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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.08.030.

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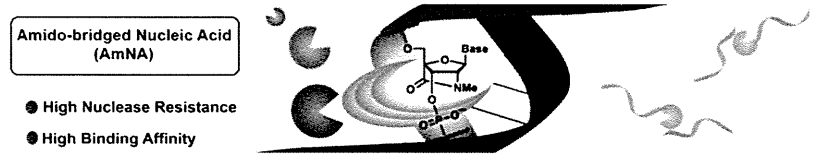

Towards the next generation: New LNA analogues based on a cyclic amide structure, termed amido-bridged nucleic acids (AmNAs), have been synthesized. Oligonucleotides modified with these residues showed high nuclease resistance along with high binding affinities towards complementary strands.

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Amido-Bridged Nucleic Acids (AmNAs): Synthesis, Duplex Stability, Nuclease Resistance, and in Vitro Antisense Potency

Aiko Yahara, Ajaya Ram Shrestha, Tsuyoshi Yamamoto, Yoshiyuki Hari, Takashi Osawa, Masaki Yamaguchi, Masaru Nishida, Tetsuya Kodama, and Satoshi Obika*[a]

Since their discovery, 2′,4′-bridged nucleic acids (2′,4′-BNAs)[1] [or locked nucleic acids (LNAs)[2]] have been considered promising candidates for antisense technology because of their unprecedented high binding affinities toward complementary strands,[1b,2] sequence selectivities,[3] and potential for in vivo applications.[4,5] Our group[6] and others[7–11] are continuing efforts to develop other bridged nucleic acids with improved properties based on the LNA structural concept. Recently, our group has focused on developing new bridged nucleic acids with carbonyl functionality in the bridge. The introduction of a carbonyl group into the bridge would be expected, as a consequence of its trigonal planarity, to restrict the flexibility of the sugar moiety. We have so far reported two analogues of bridged nucleic acids with carbonyl functionality in their bridges; both exhibited interesting properties. In one report we described a seven-membered bridged nucleic acid with a cyclic urea structure,[6c] which showed high nuclease resistance along with high RNA selectivity. We recently introduced another interesting class of LNA analogues: hydroxamate-bridged nucleic acids (HxNAs),[6d] which possessed unique nuclease resistance.

Among the challenges to the development of a potent antisense oligonucleotide, in vivo enzymatic digestion of the oligonucleotide remains an important factor. Previous studies have shown that the nuclease resistance of a bridged nucleic acid can be enhanced by increasing the size of the bridge, but this reduces binding affinity.[6,7] Optimization of the bridged nucleic acids to improve nuclease resistance without losing binding affinity is therefore needed. To accomplish this, the relationship between the size of the bridge and the properties of the bridged nucleic acid were investigated. Here we report the synthesis and properties of a new set of analogues of LNAs with cyclic amide structures, named amido-bridged nucleic acid (AmNAs). This is the first report of an amide linkage in a bridged nucleic acid, although amide functionality has found other uses in nucleic acid chemistry, for example, in modification of the phosphate linkage[12,13] and in peptide nucleic acids (PNAs).[14] Structurally, an AmNA has a five-membered bridged structure similar to those of an LNA or an 2′-amino-LNA.[15] Previous reports on 2′-amino-LNAs demonstrated that their binding affinities toward complementary strands are comparable to those of LNAs.[15b] Introduction of a carbonyl group into the LNA structure, as in an AmNA, should enhance the rigidity and polarity of the structure. These structural characteristics of an AmNA would be expected to impart properties appropriate for antisense oligonucleotides.

Compound **1** (Scheme 1), synthesized by known procedures,[6d] was used as the starting material for the synthesis of AmNAs. Triflylation of **1** followed by an S_N2 reaction with NaN_3 yielded **3** in good yield. The silyl protecting group of **3** was removed (tributylammonium fluoride, TBAF) to produce **4** with a free hydroxy moiety that was oxidized with pyridinium dichromate (PDC) in DMF to afford the carboxylic acid **5**. By means of a Staudinger reaction (i.e., treatment of **5** with PBu_3 in THF), the azido group at the 2′-position was converted into an amino group to yield **6**. The carboxylic acid function at the 4′-position and the amino function at the 2′-position of **6** were linked together by a condensation reaction, activated by *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDCI) to afford the desired cyclic product **7**. Debenzylation of **7** by catalytic hydrogenolysis yielded monomer **8** in excellent yield.

The desired monomer containing a carbonyl function in a five-membered bridge had thus been successfully synthesized. The functionalizable nitrogen at the 2′-position allowed the synthesis of various *N*-substituted derivatives of the monomer. The first attempt to methylate this functionalizable nitrogen also caused *N*-methylation at the thymine moiety, so the thymine moiety was first protected with a benzyloxymethyl (BOM) protecting group, followed by *N*-methylation of the bridge nitrogen and then by debenzylation by catalytic hydrogenolysis to afford the desired *N*-methylated monomer **10**.

To incorporate the synthesized monomers **8** and **10** into oligonucleotides, the primary hydroxy groups of **8** and **10** were selectively tritylated with 4,4′-dimethoxytrityl chloride (DMTrCl), followed by phosphitylation of secondary hydroxy groups with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite to afford the desired thymine phosphoramidites **9** and **12.** For interconversion of the nucleobase (i.e., thymine into cytosine), the secondary hydroxy group of tritylated nucleoside **11** was protected with a triethylsilyl (TES) group, and subsequent treatment with 2,4,6-triisopropylbenzenesulfonyl chloride[16] (iPr_3ArSO_2Cl) in the presence of 4-dimethylaminopyridine (DMAP) and triethylamine (TEA) sulfonated the 4-oxo group of the thymine moiety. The sulfonylated nucleoside was subjected to substitution with ammonia to afford a 5-methylcytidine derivative. The exocyclic amino moiety of the 5-methylcytidine was protected with an acetyl group, followed by desilylation and phosphitylation of the free secondary hydroxy group by 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite to afford the desired N^4-acetyl-protected 5-methylcytidine phosphordiamidite **13** (Scheme 1).

[a] *A. Yahara, Dr. A. R. Shrestha, Dr. T. Yamamoto, Dr. Y. Hari, T. Osawa, M. Yamaguchi, Dr. M. Nishida, Dr. T. Kodama, Prof. Dr. S. Obika*
Graduate School of Pharmaceutical Sciences, Osaka University
1-6 Yamadaoka, Suita, Osaka 565-0871 (Japan)
E-mail: obika@phs.osaka-u.ac.jp

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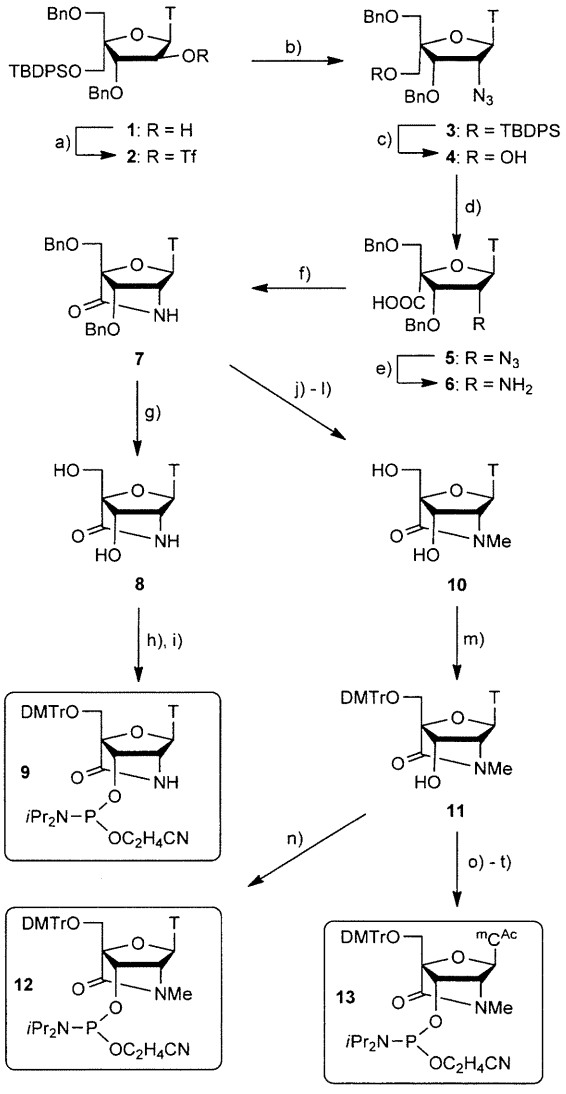

Scheme 1. Synthesis of AmNA monomers **8** and **10** and of phosphoramidites **9**, **12**, and **13**. Reagents and conditions: a) Tf_2O, pyridine, CH_2Cl_2, 0 °C; b) NaN_3, DMF, RT, 66% (two steps), c) TBAF/THF (1 N), THF, RT, 92%; d) PDC, MS (4 Å), DMF, RT, quant.; e) PBu_3, THF/H_2O, RT; f) EDCI, DMF, RT, 54% (two steps); g) 20% $Pd(OH)_2/C$, H_2, THF, RT, 93%; h) DMTrCl, pyridine, RT, 85%; i) $(iPr_2N)_2POCH_2CH_2CN$, *N,N*-diisopropylammonium tetrazolide, MeCN/THF (3:1), RT, 27%; j) BOMCl, 1,8-diazabicyclo[5.4.0]undec-7-ene, DMF, 0 °C→RT, 82%; k) NaH, MeI, DMF, 0 °C→RT, 86%; l) 20% $Pd(OH)_2/C$, H_2, THF, RT, 95%; m) DMTrCl, pyridine, RT, 90%; n) $(iPr_2N)_2POCH_2CH_2CN$, *N,N*-diisopropylammonium tetrazolide, MeCN/THF (3/1), RT, 97%; o) TESOTf, TEA, CH_2Cl_2, RT. 92%; p) iPr_3ArSO_2Cl, DMAP, TEA, MeCN, 0 °C; q) aq. NH_3, MeCN, RT; r) Ac_2O, pyridine, RT, 61% (three steps); s) TBAF/THF (1 N), CH_2Cl_2, RT, 86%; t) $(iPr_2N)_2POCH_2CH_2CN$, *N,N*-diisopropylammonium tetrazolide, MeCN/THF (3:1), RT, 86%.

The structures of AmNA monomers **8** and **10** were confirmed by ^1H NMR, which in each case showed the appearance of a singlet proton at C1′, due to the absence of coupling between the hydrogen atoms at C1′ and C2′, as previously observed in LNAs and other bridged nucleic acids, which strongly supports the N-type sugar conformation. The conformation of the sugar in the AmNA was further supported by X-ray crystallographic examination of **10**[17] (Figure 1). The pseudorotation

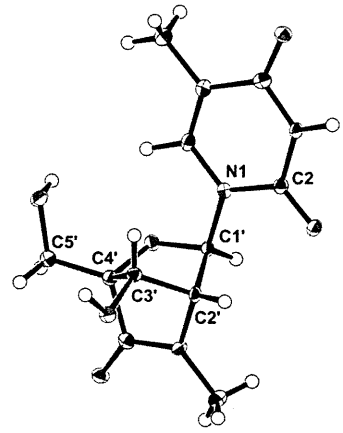

Figure 1. X-ray structure of **10** at the 50% probability level.

phase angle (*P*) in the crystal structure of **10** was 11.6°, which is similar to that in an LNA ($P = 17.4°$); this indicates that the sugar puckering of AmNA corresponds to the C3′-*endo*-N conformation. The torsion angle (δ) and maximum torsion angle (ν_{max}) of the AmNA ($\delta = 68.6°$, $\nu_{max} = 56.9°$) were comparable to those of the corresponding LNA ($\delta = 66.2°$, $\nu_{max} = 56.6°$). Furthermore, the bond length of the bridge (i.e., the distance between the carbonyl carbon atom of the bridge and the nitrogen atom at the 2′-position, C–N = 1.35 Å) is shorter than that in the corresponding LNA (C–O = 1.47 Å). These results indicate that the amide linkage could be a plausible alternative for efficiently bridging the 2′- and 4′-positions of the furanose ring for further improvement of the properties of bridged nucleic acids.

To study the properties of AmNA-modified oligonucleotides, the synthesized AmNA monomers were incorporated into oligonucleotides by a standard phosphoramidite protocol in an automated DNA synthesizer.[18] As a preliminary study, a set of T-decamer oligonucleotides modified with AmNA[NH] and AmNA[NMe] at various positions (**ON-3**, **ON-4**, **ON-6**, and **ON-7**) were synthesized and characterized by melting temperature (T_m). The study revealed that both the AmNA[NH]- and AmNA[NMe]-modified oligonucleotides exhibited excellent binding affinities toward complementary strands, with T_m values comparable to those of LNA-modified oligonucleotides (Table 1).

For a study of nuclease resistance, two T-decamer oligonucleotides, modified at the second position from their 3′-ends either with AmNA[NH] (**ON-11**) or with AmNA[NMe] (**ON-12**), were synthesized. The nuclease resistance of AmNA was appreciably better than those of the natural (**ON-1**) and LNA-modified (**ON-10**) oligonucleotides (Figure 2). Note that the nuclease resistance of AmNA was improved when the nitrogen of the amide bridge was substituted. The improved nuclease resistance of AmNA[NMe] can be attributed to the steric and electrostatic effects of the polar carbonyl function in the bridge and the *N*-methyl substituent. These properties of AmNA are comparable with those of recently reported 2′,4′-constrained 2′-*O*-methoxyethyl and ethyl bicyclic nucleic acids (cMOE BNAs and cEt BNAs, respectively)[19] which also exhibited high nuclease resistance along with high binding affinities.

Table 1. T_m [°C] values of duplexes formed by AmNA[NMe]-modified oligonucleotides with complementary ssRNAs and ssDNAs.

Oligonucleotides	RNA complement		DNA complement	
	T_m	ΔT_m per modification	T_m	ΔT_m per modification
5′-(TTTTTTTTTT)-3′ (ON-**1**)	19	–	22	–
5′-(TTTTT*T*TTTT)-3′ (ON-**2**)	26	+7	24	+2
5′-(TTTTTtTTTT)-3′ (ON-**3**)	25	+6	22	0
5′-(TTTTTITTTT)-3′ (ON-**4**)	24	+5	21	−1
5′-(TTT*T*T*T*TTT)-3′ (ON-**5**)	42	+8	37	+5
5′-(TTTtTtTtTT)-3′ (ON-**6**)	42	+8	32	+3
5′-(TTTITITITT)-3′ (ON-**7**)	40	+7	26	+2

Conditions: phosphate buffer (10 mM, pH 7.2), NaCl (100 mM), 4 μM each oligonucleotide, 0.5 °C min^{-1}. Target strand: 3′-r(AAAAAAAAAA)-5′ (**ON-8**), 3′-d(AAAAAAAAAA)-5′ (**ON-9**). T: AmNA[NMe]. t: AmNA[NH]. *T*: LNA.

Table 2. T_m values (°C) of duplexes formed by AmNA[NMe]-modified oligonucleotides in ApoB sequence with complementary ssRNA.

Oligonucleotides	RNA complement	
	T_m	ΔT_m per modification
5′-(TTCAGCATTGGTATTC)-3′ (ON-**13**)	51	–
5′-(*TT*CAGCATTGG*TATT*C)-3′ (ON-**14**)	66	+3
5′-(TTCAGCATTGGTATTC)-3′ (ON-**15**)	68	+3
5′-(*TT*CAGCATTGG*TATT*C)-3′ (ON-**16**)	72	+4
5′-(TTCAGCATTGGTATTC)-3′ (ON-**17**)	73	+4
5′-(TCAGCATTGGTATT)-3′ (ON-**18**)	48	–
5′-(*T*CAGCATTGG*TATT*)-3′ (ON-**19**)	64	+4
5′-(TCAGCATTGGTATT)-3′ (ON-**20**)	65	+4

Conditions: phosphate buffer (pH 7.2, 10 mM), NaCl (100 mM), 4 μM each oligonucleotide, 0.5 °C min^{-1}, 260 nm. Target strand: 3′-(AAGUCGUAACCAUAAG)-5′ (**ON-21**). T = AmNA[NMe] thymine. *C* = AmNA[NMe] 5-methylcytosine. *T* = LNA thymine. *C* = LNA 5-methylcytosine.

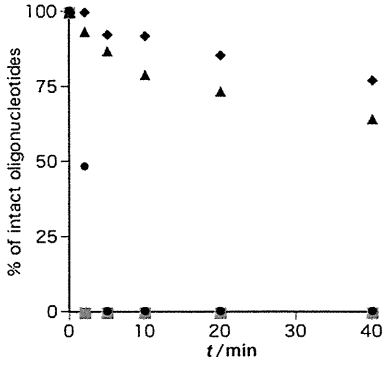

Figure 2. Nuclease resistance of 5′-d(TTTTTTTTTT)-3′ against *Crotalus admanteus* venom phosphodiesterase (CAVP). T = natural (■; ON-**1**), LNA (●; ON-**10**), AmNA[NH] (▲; ON-**11**), AmNA[NMe] (◆; ON-**12**). Experiments were performed at 37 °C in 100 μL of buffer containing 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 0.175 mg mL^{-1} CAVP, and 7.5 μM of oligonucleotide.

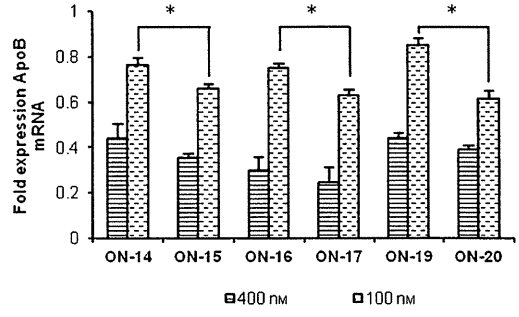

Figure 3. Comparative effects of AmNA[NMe]-modified antisense oligonucleotides targeting human ApoB mRNA versus LNA counterparts. Huh-7 cells were treated with 100 nM and 400 nM antisense oligonucleotides shown in Table 2 in complexation with Lipofectamine 2000. Data are means ± SDs ($n = 3$). *$P < 0.01$.

This showed that modification at the 4′-moiety of an LNA could impart improved properties.

In addition, a study of the serum stabilities of AmNAs with fetal bovine serum (FBS) indicated excellent biostabilities: the AmNA[NMe]-modified oligonucleotide remained almost undigested during the experimental period, whereas the natural and LNA oligonucleotides were gradually digested (see the Supporting Information). Furthermore, a comparative study of the silencing potency of AmNA[NMe]-modified and LNA-modified antisense oligonucleotides (ON-**14** to ON-**17**, ON-**19**, and ON-**20**), targeting an apolipoprotein B (ApoB) mRNA, was conducted (Table 2). We modified the 5′- and 3′-ends of the oligonucleotides with AmNAs, because both end-modified LNA oligonucleotides are reported to exhibit high stability even in the absence of PS linkages.[20] We left one nucleotide at the 3′-end unmodified because the previous experiment on exonuclease has shown that LNAs and their analogues inhibit the digestion of the 3′-end phosphodiester linkage, probably due to steric hindrance. All of the AmNA[NMe]-modified antisense oligonucleotides (ON-**15**, **-17**, **-20**) exhibited more efficient ApoB silencing than their LNA counterparts (ON-**14**, **-16**, **-19**, Figure 3), probably due to the high T_m values of the AmNAs, which are comparable with those of LNAs, and the greater exonuclease resistance of AmNA-based antisense oligonucleotides. The potency of an AmNA-based antisense oligonucleotide is up to 28% greater than that of the corresponding LNA congener. The prominent silencing potencies of AmNA[NMe]-modified oligonucleotides were observed both at the lower concentration of 100 nM and at the higher concentration of 400 nM. In particular, statistical analysis found significant superiorities of AmNA-based antisense molecules at 100 nM ($P < 0.01$).

In conclusion, new analogues of bridged nucleic acids, termed amido-bridged nucleic acids (AmNAs), were synthesized and exhibited high exonuclease resistance while maintaining binding affinities toward complementary strands. The hybridization affinities of AmNAs were comparable to those of LNAs. This study on AmNAs has thus demonstrated that the amide linkage could be an appropriate modification for improving the properties of LNAs. The in vitro antisense potencies of AmNAs were tested by synthesizing antisense oligonucleotides modified with AmNAs with the sequence of the *apoB* gene. The test also revealed improved potencies of AmNAs relative to LNAs. These results indicate that AmNAs are good candidates for further improvement of the potencies of antisense oligonucleotides based on LNAs.

Experimental Section

General methods, procedures for the synthesis and biological studies of AmNAs, and spectral data for corresponding products are provided in the Supporting Information.

Acknowledgements

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Keywords: bridged nucleic acids · nuclease resistance · oligonucleotides · synthesis design · thermal stability

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[18] Although most antisense agents are modified with PS linkages to enhance their stabilities against nucleases, we used PS-free oligonucleotides in order to compare the natures of the compounds with those of LNAs.

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Research Article

Superior Silencing by 2′,4′-BNA^{NC}-Based Short Antisense Oligonucleotides Compared to 2′,4′-BNA/LNA-Based Apolipoprotein B Antisense Inhibitors

Tsuyoshi Yamamoto,[1,2] Hidenori Yasuhara,[1,2] Fumito Wada,[1,2] Mariko Harada-Shiba,[2] Takeshi Imanishi,[3] and Satoshi Obika[1]

[1] *Applied Biopharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan*
[2] *Department of Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*
[3] *BNA Inc, 7-7-20 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan*

Correspondence should be addressed to Satoshi Obika, obika@phs.osaka-u.ac.jp

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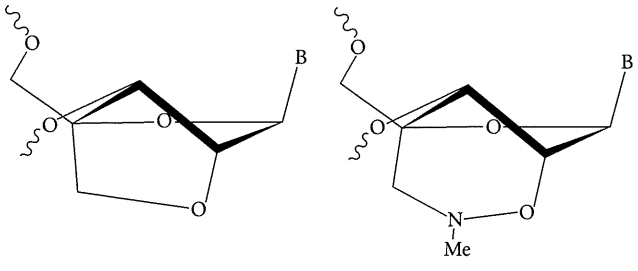
The duplex stability with target mRNA and the gene silencing potential of a novel bridged nucleic acid analogue are described. The analogue, 2′,4′-BNA^{NC} antisense oligonucleotides (AONs) ranging from 10- to 20-nt-long, targeted apolipoprotein B. 2′,4′-BNA^{NC} was directly compared to its conventional bridged (or locked) nucleic acid (2′,4′-BNA/LNA)-based counterparts. Melting temperatures of duplexes formed between 2′,4′-BNA^{NC}-based antisense oligonucleotides and the target mRNA surpassed those of 2′,4′-BNA/LNA-based counterparts at all lengths. An *in vitro* transfection study revealed that when compared to the identical length 2′,4′-BNA/LNA-based counterpart, the corresponding 2′,4′-BNA^{NC}-based antisense oligonucleotide showed significantly stronger inhibitory activity. This inhibitory activity was more pronounced in shorter (13-, 14-, and 16-mer) oligonucleotides. On the other hand, the 2′,4′-BNA^{NC}-based 20-mer AON exhibited the highest affinity but the worst IC_{50} value, indicating that very high affinity may undermine antisense potency. These results suggest that the potency of AONs requires a balance between reward term and penalty term. Balance of these two parameters would depend on affinity, length, and the specific chemistry of the AON, and fine-tuning of this balance could lead to improved potency. We demonstrate that 2′,4′-BNA^{NC} may be a better alternative to conventional 2′,4′-BNA/LNA, even for "short" antisense oligonucleotides, which are attractive in terms of drug-likeness and cost-effective bulk production.

1. Introduction

Recently designed and synthesized high-performance modified-nucleic-acids (HiPerNAs) such as 2′-*O*-methyl RNA (2′-OMe), 2′-*O*-methoxyethyl RNA (MOE), and 2′,4′-bridged nucleic acid/locked nucleic acid (2′,4′-BNA/LNA) have improved performance compared to phosphorothioate antisense oligonucleotides (AONs). HiPerNAs overcome the systemic antisense effects of these earlier antisense oligonucleotides and show promise as antisense therapeutics for the treatment of a variety of diseases [1–5]. However, more potent and less toxic AONs are required, since several clinical trials of AON drugs carrying HiPerNAs have been recently terminated due to the lack of efficacy or because of safety concerns. In addition, toxicity and delivery problems remain [6–8].

We previously described a unique modified nucleic acid, 2′,4′-bridged nucleic acid (2′,4′-BNA; also known as LNA) [9, 10]. Its high therapeutic efficacy is based on the extraordinarily high target binding of the original 2′,4′-BNA/LNA-based AON. 2′,4′-BNA/LNA-based AON is widely accepted as one of the most promising antisense drugs, so fine-tuning the structure of BNA is the key for further improving the therapeutic potency and toxicological properties of



2′,4′-BNA/LNA 2′,4′-BNA^{NC}

FIGURE 1: Structures of the BNAs. The chemical structure on the left is the original BNA, 2′,4′-BNA/LNA, and the structure on the right is 2′,4′-BNA^{NC}.

AON. In this context, Seth and coworkers developed BNA analogues and elucidated their potency and safety *in vivo*. They reported BNA or LNA analogues with 2′,4′-BNA/LNA-like binding affinities and biological activities with increased nuclease resistance and reduced toxicity [11–14]. We have also reported the synthesis and physicochemical properties of several novel BNAs, including 2′,4′-BNA^{COC} and 2,′4′-BNA^{NC} [15–17], and recently demonstrated the biological activity of a 2′,4′-BNA^{NC} -based AON. Our results indicate that 2′,4′-BNA^{NC} may be a better candidate as an antisense therapeutic [7]. 2′,4′-BNA^{NC} is a six-membered bridged structure containing a hydrophilic aminooxy moiety. A wide range of functional groups can be easily introduced, and the introduction of nitrogen atoms would improve the stability of the duplex by reducing repulsions between phosphates in the backbone [18, 19] (Figure 1). 2′,4′-BNA^{NC}-modified oligonucleotides have very high RNA affinity, similar to or even higher than their 2′,4′-BNA/LNA counterparts. Moreover, 2′,4′-BNA^{NC}-modified oligonucleotides are more resistant to endonucleolytic cleavage by nucleases than their 2′,4′-BNA/LNA counterparts [17]. Although there is limited information regarding the biological activity or therapeutic potency of 2′,4′-BNA^{NC}-based AONs, we have demonstrated the high systemic effects and safety of 2′,4′-BNA/LNA- and 2′,4′-BNA^{NC}-based 20-nucleotide-long (20-nt-long) AONs that target PCSK9 mRNA [7]. Additionally, Prakashet al. independently showed the high potency and the nontoxicity of 2′,4′-BNA^{NC}-based AONs [11].

Straarup et al. recently shortened the length of 2′,4′-BNA/LNA-based phosphorothioate AONs to eliminate the latent potency of 2′,4′-BNA/LNA drugs [20]. These short phosphorothioate AONs contain central 6- to 10-nt-long DNA regions flanked by terminal 2- to 4-nt-long 2′,4′-BNA/LNA segments. These short (12- to 14-nt) AONs would be beneficial in terms of target specificity. The introduction of only small numbers of modifications into short 2′,4′-BNA/LNA-based AONs can greatly increase target affinity. Thus, short 2′,4′-BNA/LNA phosphorothioate AONs can minimize length-dependent disadvantages such as phosphorothioate-related protein binding and RNase H inactivation [21–23] while maintaining satisfactory affinity and specificity. Additionally, short AONs are easier to produce on a bulk scale and could exhibit more drug-like characteristics. Based on the assumption that the strand-shortening strategy is also applicable to 2′,4′-BNA^{NC}-based AONs, we shortened 2′,4′-BNA^{NC}-based phosphorothioate AONs and directly compared their silencing activities against the corresponding 2′,4′-BNA/LNA-based apolipoprotein B (apoB)-targeting AONs.

2. Materials and Methods

2.1. Oligonucleotides. A series of 2′,4′-BNA/LNA-based antisense 10- to 20-nucleotide-long phosphorothioate gapmers reported previously by Straarup et al. [20] were prepared and used in this study. These AONs were designed with complementary target sites for both cynomolgus monkey and human apoB mRNA sequences. The 10- to 16-nt-long AONs can also target murine apoB mRNA (GenBank accession number NM_000384 and NM_009693 for human and mouse apoB mRNA, resp.). Additionally, we prepared 2′,4′-BNA^{NC}-based counterparts in which all the 2′,4′-BNA/LNAs were substituted by 2′,4′-BNA^{NC}. The synthesis of 2′,4′-BNA^{NC} with pyrimidine bases was previously reported [16, 17]; the synthesis of 2′,4′-BNA^{NC} with purine bases is currently being optimized and will be reported elsewhere. All the modified oligonucleotides were synthesized by Gene Design, Inc. (Ibaraki, Osaka, Japan) using standard phosphoramidite procedures and purified using HPLC.

2.2. Thermal Melting Study of Duplexes. UV melting experiments were carried out using a SHIMAZU UV-1650 spectrometer equipped with a T_m analysis accessory. Equimolar amounts of two single-stranded oligonucleotides were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to give a final strand concentration of 4.0 μM. The mixture was annealed by heating at 90°C followed by slow cooling to room temperature. The melting profile was recorded at 260 nm in the forward and reverse direction from 5 to 90°C at a scan rate of 0.5°C/min.

2.3. In Vitro Transfection Procedures. For AON transfection experiments, Huh-7 cells were seeded at 15×10^4 cells per well in 12-well plates. AONs were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's procedures. After a 4-hour transfection, cells were washed with PBS, fresh medium was added, and the cells were incubated for an additional 20 hours at 37°C. After incubation, cells were collected and subjected to analyses.

2.4. mRNA Quantification Procedures. Total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's procedure. Gene expression was evaluated by a two-step quantitative reverse transcription-PCR method. Reverse transcription of RNA samples was performed by using a High Capacity cDNA Reverse-Transcription Kit (Applied Biosystems, Foster City, CA), and quantitative PCR was performed using a Fast TaqMan Gene Expression Assay (Applied Biosystems). The mRNA levels of target genes were normalized to the GAPDH

Table 1: Oligonucleotides used in this study.

ID	Sequence	T_m (°C)	IC_{50} (nM)
ApoB-LNA-20	5′-TTCAGcattggtattCAGTG-3′	75 ± 0.8	7.9 ± 1.7
ApoB-LNA-16	5′-CAGcattggtatTCAG-3′	64 ± 0.8	2.9 ± 0.4[a]
ApoB-LNA-14	5′-AGCattggtatTCA-3′	61 ± 0.2	1.4 ± 0.5[b]
ApoB-LNA-13	5′-GCattggtatTCA-3′	58 ± 0.2	2.8 ± 0.8[a]
ApoB-LNA-10	5′-CattggtatT-3′	25 ± 1.4	N.D.
ApoB-NC-20	5′-*TTCAG*cattggtatt*CAGTG*-3′	79 ± 1.0	11.4 ± 3.0
ApoB-NC-16	5′-*CAG*cattggtat*TCAG*-3′	69 ± 0.8	1.3 ± 0.3[b]
ApoB-NC-14	5′-*AG*Cattggtat*TCA*-3′	64 ± 0.3	0.6 ± 0.1
ApoB-NC-13	5′-*G*Cattggtat*TCA*-3′	61 ± 0.1	0.9 ± 0.1
ApoB-NC-10	5′-Cattggtat*T*-3′	32 ± 0.4	N.D.

2′,4′-BNA/LNA was shown in uppercase and 2′,4′-BNA^{NC} was in italic. Natural DNA was shown in lowercase. All the linkages are phosphorothioated. We measured T_m and IC_{50} values of all entries. T_m values were determined in three independent experiments (±SD). Nondetectable IC_{50} values, due to low potency, were marked ND. [a,b]Pairs of two IC_{50} values with superscript letters are NOT statistically significant.

mRNA level. The following primer sets were used for quantitative PCR. For human apoB and GAPDH, assay IDs of Hs01071209_m1 and Hs02758991_g1 were used, respectively.

2.5. Western Blotting. Two days after transfection, the cultures were subjected to centrifugation at 4°C, 10,000 rpm for 15 min. Each supernatant was collected into an Amicon Ultra-4 Centrifugal Filter Ultracel PL-10k (Millipore) and centrifuged at 4°C at 3,000 rpm for 1 h, and then each supernatant was added to individual Vivaspin 500 units (Sartorius Stedim Biotech) and centrifuged at 4°C at 3,000 rpm for 0.5 h. Each sample (9 μL) was added to 9 μL of Novex Tris-Glycine SDS Sample Buffer (2x) (Invitrogen) and applied to a 3–8% NuPAGE Tris-Acetate Gel (Invitrogen). Electrophoresis was performed at 180 V for 130 min. The separated proteins were transferred to a PVDF membrane (Millipore) at 220 mA for 120 min. Membranes were then incubated with 10 mL of blocking buffer (Blocking One; Nacalai Tesque) for 12 h at 4°C. Membranes were successively incubated with primary antibody of anti-human ApoB antibody (R&D Systems) for 80 min at room temperature. Then, each membrane was washed with PBS containing 0.1% tween (PBST) 4 times. Membranes were incubated with goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology) for 80 min at room temperature. Chemiluminescent detection was performed using an ECL Advance Western Blot Detection Kit (Amersham Biosciences) according to the manufacture's procedure. Bands were visualized using an LAS-4000 mini (Fujifilm).

2.6. Statistics. Application of linear regression techniques to plots of the expression of apoB mRNA levels versus the logarithm of transfection concentration allows estimation of the coefficient (slope) and intercept values using the following equation: apoB mRNA = Coefficient∗log (Concentration) + Intercept. These estimates can be useful guides for the efficacies of the AONs. Linear regression analysis was applied to 500 bootstrap sample sets obtained from three independent cellular assays. The coefficient and intercept of each regression line were compared using Tukey's test or one-sample t-test.

3. Results and Discussion

To better understand the effect of strand shortening on 2′,4′-BNA^{NC}-based AONs, a series of 2′,4′-BNA^{NC}-based antisense oligonucleotides of 10- to 20-nt-long phosphorothioate gapmers and 2′,4′-BNA/LNA-counterparts were synthesized (Table 1). Formation of a stable duplex with the target mRNA is a minimum essential step of the onset of an antisense effect. We first evaluated the thermal stability of the duplex formed between each modified AON and the single-stranded 20-nt-long oligoribonucleotide complementary to ApoB-LNA-20 and ApoB-NC-20. As expected, the longer the strand or the greater the number of modifications, the higher the T_m value. Moreover, the T_m values of 2′,4′-BNA^{NC}-based AONs surpassed those of their 2′,4′-BNA/LNA-based counterparts for any given length (Table 1), in good agreement with previous reports [11, 17]. Note that the exact T_m values of LNAs in Table 1 are different from those given by Straarup et al., due to differences in composition of the measurement buffer solutions and in the length of the complementary RNAs between the two studies.

We next used *in vitro* mRNA silencing assays to estimate the potency of 2′,4′-BNA^{NC}-based AONs and to compare their potency directly to the corresponding 2′,4′-BNA/LNA-based AONs. We used the Huh-7 human hepatoma cell line, which expresses high levels of apoB mRNA in cells and secrets its protein into the medium. Each AON was introduced using standard lipofection procedures. All the AONs, except the 10-mers, ApoB-LNA-10, and ApoB-NC-10, reduced apoB mRNA and protein expression (and hence secreted protein) levels in a dose-dependent manner in the cells and culture medium, respectively, (Figures 2(a) and 2(b)). ApoB-LNA-10 did not reduce apoB mRNA levels even at concentrations above 64 nM. This may be because ApoB-LNA-10 did not bind target mRNA at 37°C due to

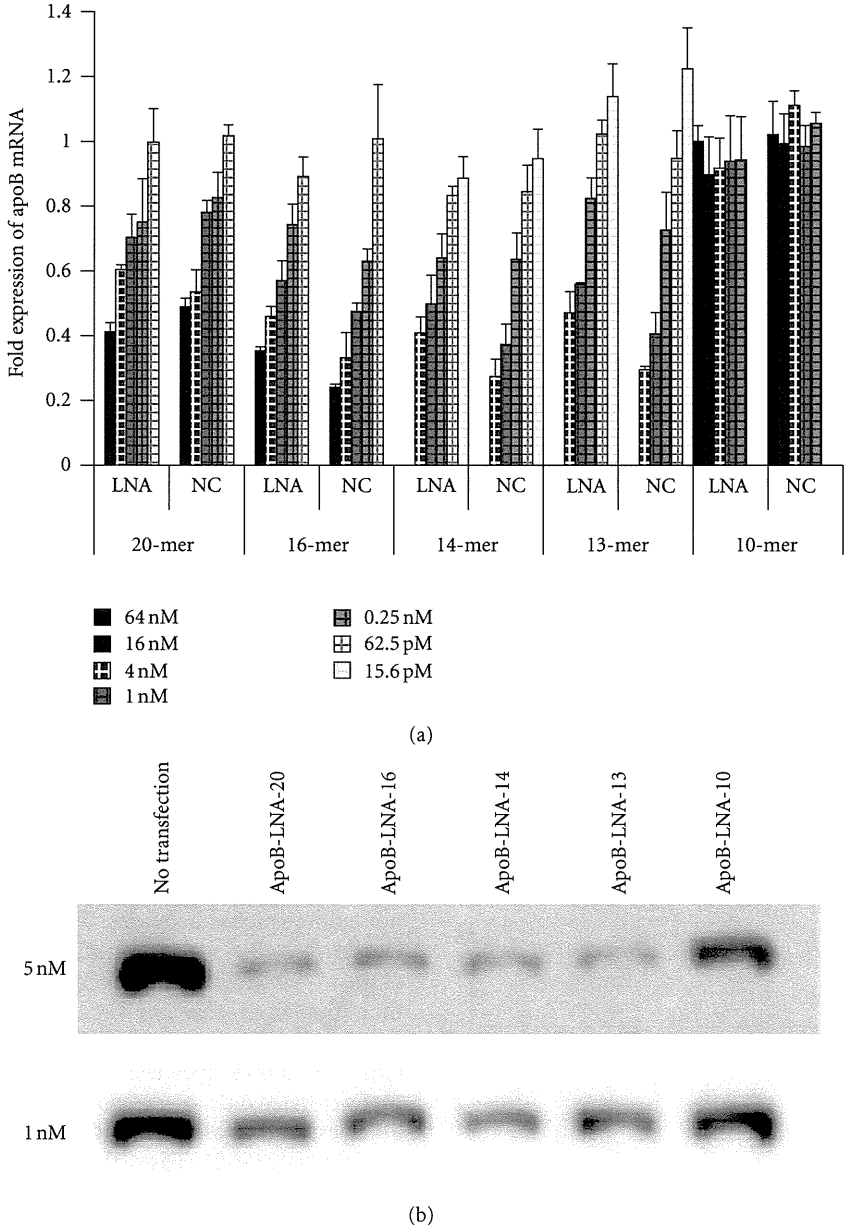


FIGURE 2: *In vitro* silencing properties of BNA-based AONs. (a) Various concentrations (15.6 pM–64 nM) of AONs were introduced into Huh-7 cells using Lipofectamine 2000. After 24-h incubation, the cells were collected, and the expression levels of apoB mRNA were determined. Data represent means ± SD. (b) Reduction of Apo B protein levels in the culture medium following transfection was confirmed by western blotting.

lack of affinity. ApoB-NC-10 also did not reduce apoB mRNA expression, despite its higher T_m value compared to that of ApoB-LNA-10. Application of linear regression techniques to plots of the expression of apoB mRNA levels versus the logarithm of transfection concentration allowed estimation of the coefficient (slope) and intercept values. Statistical comparison of these parameters between arms with the identical length would provide useful guides for the efficacies of the AONs. One-sample *t*-tests revealed that the coefficients (slopes) of the 10-mer AONs had statistically insignificant downward slopes, suggesting that these 10-mers have little or no silencing effect (Figure 3).

A length-dependent decrease in potency in 14- to 20-nt-long 2′,4′-BNA/LNA and 2′,4′-BNA^{NC}-based AONs was observed (Table 1 and Figure 1). In each series of AONs, the ApoB-LNA-14 and ApoB-NC-14 were the most potent, whereas the ApoB-LNA-13 was the most potent AON reported by Straarup et al. [20]. IC_{50} values of the 15-, 14-, 13-, and 12-mer AONs were so close to each other (≈0.5 nM) that Straarup et al. confirmed the order by

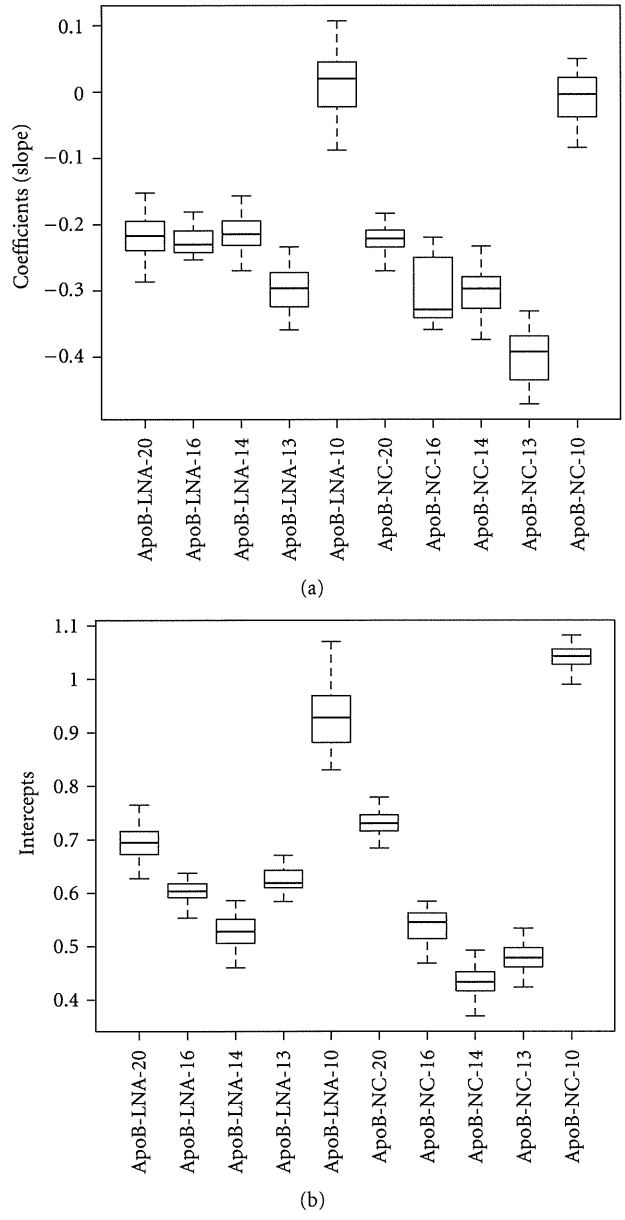


FIGURE 3: Boxplots of coefficients and intercepts of regression lines obtained from cellular assay data. Bold lines in the box indicate medians. Top and bottom lines of the box indicate upper and lower quartile points, respectively. Top and bottom lines in contact with a dotted line indicate the highest and lowest data point, respectively. Open circles indicate outliers.

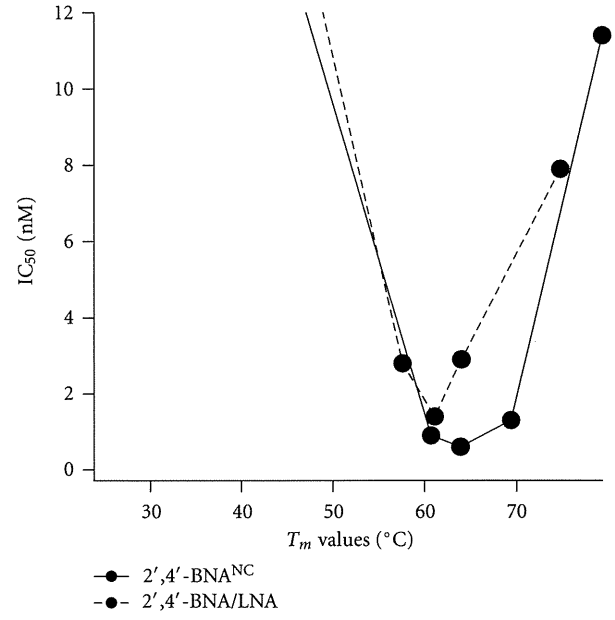


FIGURE 4: Relationship between target affinity and antisense potency. IC_{50} as a function of T_m values of 2′,4′-BNA^{NC}-based AONs (solid line) and 2′,4′-BNA/LNA-based AONs (dotted line).

conducting an *in vivo* silencing study and showed that 12- and 13-mer AONs are the most potent. In contrast, we confirmed the order of *in vitro* silencing effects of all the entries in Table 1 by using larger 12-well culture dishes. These differences in experimental conditions (*in vivo* versus *in vitro*) may explain the differences in the IC_{50} values of 2′,4′-BNA/LNA-based AONs and their order of potency. An *in vivo* study might be necessary to estimate the true order. Nevertheless, we observed in both chemistries that shorter AONs (16-, 14-, and 13-mer) are statistically significantly more potent than 20-mer AONs. Because the 10-mers did not show any activity, activity is positively correlated with binding affinity and indicates the presence of a "threshold affinity." However, longer AONs with higher affinities did not exhibit higher activities. This suggests the presence of a "length penalty" [20, 22] and the presence of an "optimal affinity," which might be a more appropriate description than threshold affinity. The variables that independently govern "length penalty" and "optimal affinity" remain largely unknown. However, plasma and intracellular proteins preferentially bind to the phosphorothioate internucleotide linkages, and these linkages are known to control RNase H activity [23–26]. Thus, the number of phosphorothioate linkages is partly related to the "length penalty."

Surprisingly, when compared to the identical length 2′,4′-BNA/LNA-based counterpart, the corresponding 2′,4′-BNA^{NC}-based AON showed stronger inhibitory activity. The differential in inhibitory activity is more pronounced in shorter (13-, 14-, and 16-mers) AONs than in the 20-mer (Table 1 and Figure 4). Indeed, as shown in Figure 3, statistical comparison using the Tukey test of the coefficients and intercepts of the regression lines, which indicate how efficiently and strongly AONs reduce apoB mRNA, revealed that 2′,4′-BNA^{NC}-based 13-, 14-, and 16-mers exhibit significantly stronger inhibitory activity than their 2′,4′-BNA/LNA counterparts. These potency differences could not be explained simply by the higher T_m of the 2′,4′-BNA^{NC}-based AONs compared to their 2′,4′-BNA/LNA-based counterpart. 2′,4′-BNA^{NC}-based AONs might exhibit less "length penalty" than their 2′,4′-BNA/LNA-based counterpart. A weaker affinity for protein binding or lower RNase H inhibitory activity of 2′,4′-BNA^{NC}-based AONs could cause this potency difference. On the other hand,

Figure 4 shows a plot of IC_{50} versus T_m, in which ApoB-NC-20, which has the highest affinity, exhibited worse IC_{50}value than ApoB-LNA-20. These findings suggest that very high affinity possibly undermines antisense potency, although binding affinity to the target generally correlates positively with potency in the case of traditional small molecule drugs. The precise onset mechanism giving rise to this phenomenon remains unclear due to the lack of experimental data. However, the U- or V-shaped curves in Figure 4 clearly indicate that the potency of AONs is the result of a delicate balance of reward term and penalty term. Thus, fine adjustment to the optimal affinity and elimination of the penalties such as excess protein binding and RNase H inhibitory activity could result in superior efficacy, represented as the lowest point on the U- or V-shaped curve.

In conclusion, we have shown that 2′,4′-BNA^{NC}-based AONs targeting apoB mRNA have higher binding affinities to the target RNA than do 2′,4′-BNA/LNA-based AONs. Additionally, *in vitro* transfection studies revealed the superior silencing effect of short 2′,4′-BNA^{NC}-based AONs (<20-nt-long), indicating that 2′,4′-BNA^{NC} may have advantageous properties as short antisense drugs. We are currently investigating the potential and safety of 2′,4′-BNA^{NC}-based AONs as therapeutic drugs.

Acknowledgments

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Development of a 2′,4′-BNA/LNA-based siRNA for Dyslipidemia and Assessment of the Effects of Its Chemical Modifications *In Vivo*

Shunsuke Wada[1,2], Satoshi Obika[3], Masa-Aki Shibata[4], Tsuyoshi Yamamoto[1,3], Moeka Nakatani[1,3], Tetsuji Yamaoka[5], Hidetaka Torigoe[2] and Mariko Harada-Shiba[1]

Recent advances in RNA interference (RNAi)-based drug development have partially allowed systemic administration of these agents *in vivo* with promising therapeutic effects. However, before chemically modified small-interfering RNAs (siRNAs) can be applied clinically, their *in vivo* effects should be thoroughly assessed. And while many studies have assessed the effects of chemically modified siRNAs *in vitro*, there has been no comprehensive assessment of their effects *in vivo*. Here, we aimed to elucidate the effects of administering chemically modified siRNAs *in vivo* and to propose a 2′,4′-bridged nucleic acid (BNA)/locked nucleic acid (LNA)-based siRNA candidate for dyslipidemia. A potentially therapeutic siRNA, siL2PT-1M, was modified with phosphorothioate (PS) and 2′,4′-BNA/LNA in its sense strand and with 2′-methoxy (2′-OMe) nucleotides in its immunostimulatory motif; administration of siL2PT-1M resulted in sustained reductions in serum total cholesterol (TC) (24 days) and a concomitant apolipoprotein B (apoB) mRNA reduction in liver without adverse effects. The 2′,4′-BNA/LNA modification in the sense strand was greatly augmented the duration of the RNAi effect, whereas cholesterol conjugation shortened the duration. Cholesterol-conjugated immunostimulatory siRNA (isRNA) induced higher serum interferon-α (IFN-α) levels than did nonmodified isRNA, indicating that the immune reaction was facilitated by cholesterol conjugation. Our results indicated that modification of the adenosine residues complementary to the immunostimulatory motif and of central 5′-UG-3′ in the sense strand would ameliorate the negative immune response.

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Subject Category: Nucleic Acid chemistries

Introduction

RNA interference (RNAi) is an emerging therapeutic strategy for chronic or genetic disorders.[1,2] However, RNAi agents such as small-interfering RNA (siRNA) have some unfavorable properties including low stability toward nucleases, inadequate biodistribution and nonspecific off-target effects (OTEs).[3–5] Many attempts have been made to develop siRNA delivery vehicles and artificial nucleic acids to achieve RNAi effects for *in vivo*. Indeed, a number of studies have succeeded in delivering siRNA to specific organs–including the liver, kidney, lung, or brain–or to several types of tumors.[6–8] Notably, stable nucleic acid lipid particle formulations[9] and lipidoids[10] are representative delivery vehicles that efficiently deliver the siRNA to the liver.

In nucleic acid chemistry, many different artificial nucleic acids have been developed to alter the behavior of siRNAs under physiological conditions. In particular, phosphorothioate (PS), 2′-methoxy (2′-OMe), and 2′-fluoro nucleic acid have often been used to modify the siRNA.[4,11,12] Our group has developed a novel class of conformationally restricted artificial nucleic acids [bridged nucleic acids (BNAs)] and characterized their basic properties.[13] The bridged structure between 2′-*O* and 4′-*C* fixes the furanose ring to the *N*-type and 2′,4′-BNA gives its nucleotide analog high nuclease resistance and affinity to complementary RNA.[14,15] The concept of introducing a bulky group at the 2′-position of the furanose ring to achieve high nuclease resistance has little impact on sugar fluctuation, whereas in the BNAs, the bicyclic structure provides steric hindrance at the phosphodiester backbone and the reduction of entropic loss by the sugar fluctuation, and thus both high nuclease resistance and stabilization of the RNA duplex are realized. Many studies related to usage of 2′,4′-BNA/locked nucleic acid (LNA) modification in siRNAs have been conducted *in vitro*.[15–19] Elmén *et al.* showed the RNAi effect by kind of 2′,4′-BNA/LNA-modified siRNAs targeting luciferase *in vitro* and elucidated the alteration of strand bias, elongation of half-life of siRNA and influences of modification site by 2′,4′-BNA/LNA modification in canonical siRNA.[15] Kjems *et al.* suggested a novel structural siRNA variant, the small internally segmented interfering RNA, and showed that the 2′,4′-BNA/

[1]Department of Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan; [2]Graduate School of Chemical Sciences and Technology, Tokyo University of Science, Shinjuku-ku, Tokyo, Japan; [3]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [4]Faculty of Health Science, Osaka Health Science University, Osaka, Japan; [5]Department of Biomedical Engineering, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan. Correspondence: Mariko Harada-Shiba, Department of Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail: mariko.shiaba.ri@mail.ncvc.go.jp (or) Satoshi Obika, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: obika@phs.osaka-u.ac.jp

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LNA-modified small internally segmented interfering RNA improved the RNAi effect and abolished unintended mRNA degradation by RNA-induced silencing complex (RISC) containing the sense strand.[16] They also demonstrated combination effect on RNAi effect *in vitro* by using 2′,4′-BNA/LNA, unlocked nucleic acid or other artificial nucleic acids in canonical siRNA targeting enhanced green fluorescent protein.[18] However, to the best of our knowledge, no study has attempted to comprehensively assess the effects caused by systemic administration of 2′,4′-BNA/LNA-modified siRNAs combined with other artificial nucleic acids *in vivo*. Many unclear functions remain on the 2′,4′-BNA/LNA with or without other artificial nucleic acids in siRNA *in vivo*. Here, we assessed the effects caused by 2′,4′-BNA/LNA modification in combination with other modifications (PS, 2′-OMe, and 3′-cholesterol conjugation) in siRNA using an *in vivo* model of dyslipidemia (**Figure 1a**).

Most siRNAs designed for a given target with phosphorodiester backbone can stimulate cytokine production, because most siRNAs contain immunostimulatory motifs such as GU-rich, AU-rich, 5′-UCA-3′.[20] Because chemical modification of an antisense strand with artificial nucleic acids is generally not tolerable in the RNAi machinery, except in the case of 2′-fluoro nucleotide,[21] efforts to reduce OTEs on immune responses have focused on chemical modification of the sense strand of siRNA, if possible, when the antisense strand of a potential siRNA contains an immunostimulatory motif. However, there are no universal rules regarding modification of siRNAs with the intent to abrogate the innate immune responses while maintaining their RNAi activity, and thus is necessary to identify the relevant properties of each modification empirically.[22] In the present work, therefore, we attempted to elucidate the impacts of four types of chemical modification on several important biological parameters–pharmacological effects, innate immune response and other toxicities–in chronological order for *in vivo*.

Familial hypercholesterolemia is one of the best-known of the genetic and metabolic disorders, and is caused by mutations on genes related to the low-density lipoprotein (LDL) receptor pathway. In particular, in homozygous familial hypercholesterolemia patients, LDL cholesterol levels do not respond to pharmaceutical treatment including statins, ezetimibe, and resin enough to reduce coronary risk.[23,24] There is thus an enormous need to develop novel drugs for the efficient and continuous reduction of LDL cholesterol levels in these patients. Several studies have attempted to use siRNAs that target apolipoprotein B (apoB) as a means of lowering LDL cholesterol, and one of the reported potential sequences has an immunostimulatory motif in the antisense strand.[25] Here, we have used this siRNA sequence to generate chemically modified variants (*e.g.*, PS, 2′-OMe, 2′,4′-BNA/LNA, and cholesteryl-triethyleneglycol-conjugated variants), and these siRNAs were administered to C57BL/6J mice that were fed an atherogenic diet to assess the effects of each or combined modification on pharmacological parameters, innate immune response, and toxicities and to identify the candidate modification for development of a therapeutic siRNA-targeting apoB mRNA.

Results

2′,4′-BNA/LNA-modified siRNAs showed similar levels of RNAi efficacy as well as siApoB-1

We designed two types of 2′,4′-BNA/LNA-modified siRNA, called siLNA-1 and siLNA-2, according to our previous study in addition to nonmodified siApoB-1 (**Table 1**).[19] There are five consecutive 2′,4′-BNA/LNA modifications at the 5′-end of the sense strand of siLNA-1 that were designed to prevent the OTE related to incorporation of an unexpected strand into RISC.[15,26,27] In contrast, siLNA-2 has three 2′,4′-BNA/LNA modifications around the 5′-end of the sense strand and two near the 3′-end of the sense strand. The 3′-end modifications were designed to enhance the nuclease resistance, because

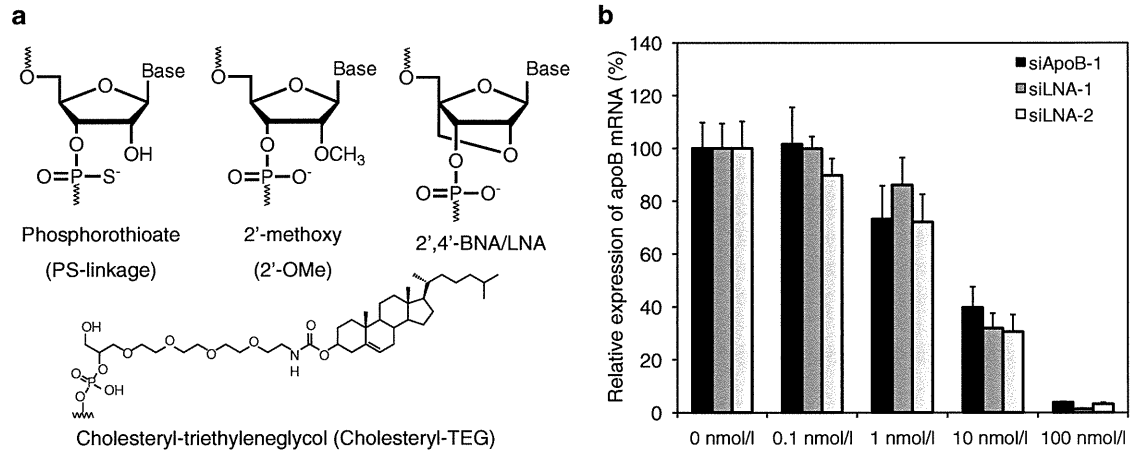

Figure 1 Structures of chemical modifications used in the study and concentration-dependent RNAi effect of nonmodified or 2′,4′-BNA/LNA modified siRNAs. (**a**) Structures of the artificial nucleic acid and cholesterol conjugates used in this study. (**b**) RNAi effect of 2′,4′-BNA/LNA modified siRNAs on apolipoprotein B (apoB) mRNA in NMuLi, mouse hepatic cells. ApoB mRNA expression was normalized to GAPDH expression. Error bar indicates SD values in this experiment.

Table 1 Sequence and T_m value (°C) of modified and nonmodified siRNAs used in this study

siRNA	*Strand*	*Sequence*	*Tm (°C)*
siApoB-1	Sense	5′-gucaucacacugaauaccaauu(dT)(dT)-3′	70.6
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siLNA-1	Sense	5′-GTCATcacacugaauaccaauu(dT)(dT)-3′	80.8
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siLNA-2	Sense	5′-GTcaTcacacugaaTacCaauu(dT)(dT)-3′	83.3
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PT-1	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)-3′	82.9
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PTC-1	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)_Chol-3′	83.0
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PTC-1M	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)_Chol-3′	84.9
	Antisense	3′-(dT)(dT)caguagu(M)gu(M)gacuuaugguuaa-5′	
siL2PTC-1L	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)_Chol-3′	85.8
	Antisense	3′-(dT)(dT)caguagTgTgacuuaugguuaa-5′	
siApoB-1C	Sense	5′-gucaucacacugaauaccaauu(dT)(dT)_Chol-3′	70.0
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PT-1M	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)-3′	85.6
	Antisense	3′-(dT)(dT)caguagu(M)gu(M)gacuuaugguuaa-5′	
siNTCtrl	Sense	5′-guaucucuucauagccuua(dT)(dT)-3′	N.D.
	Antisense	3′-(dT)(dT)cauagagaaguaucggaau-5′	

Abbreviations: apoB, apolipoprotein B; BNA, bridged nucleic acid; siRNA, small-interfering RNA; LNA, locked nucleic acid.
The following characters were used in this table to express each modification, upper cases: 2′,4′-BNA/LNA; lower cases: natural RNA; small "s": phosphorothioate modification; "Chol": cholesteryl-TEG conjugation; "(M)": 2′-Methoxy modified nucleotide; and "dT": thymine deoxyribonucleotide which was introduced at two overhang nucleotides. As the duplex stability, T_m values were analyzed with a UV experiment in a subset of siRNAs. The T_m value of siNTCtrl was not determined, and marked ND.

siRNAs are mainly degraded by 3′-exonuclease.[12,28,29] siLNA-1 and -2 have the same number of 2′,4′-BNA/LNA modifications, and each has a higher T_m value than does siApoB-1 (**Table 1**).

Using mouse hepatic cells (NMuLi), the RNAi effect of each siLNA was measured and compared to that of siApoB-1. Treatment with either siLNA-1 or -2 resulted in a concentration-dependent reduction in apoB mRNA expression, and the maximum reduction by siLNA-1 or -2 was by up to >95% at 100 nmol/l concentration of siRNA as did siApoB-1 (**Figure 1b**). The IC_{50} values of siApoB-1, siLNA-1, and siLNA-2 were 4.4, 4.4, and 2.9 nmol/l, respectively. There was no loss of RNAi effect by 2′,4′-BNA/LNA modification in these siLNAs.

2′,4′-BNA/LNA modification extends the half-life of siRNA

Previously, the bicyclic structure of nucleotides was shown to substantially enhance the nuclease resistance of siRNA.[15,19,30] We assessed the effects of 2′,4′-BNA/LNA modifications on half-life of siLNAs compared with that of nonmodified siApoB-1. Each siRNA (siApoB-1, siLNA-1, and siLNA-2) was incubated in mouse serum at 37 °C, and samples of the siRNA-serum mixtures were taken at several time points and analyzed using polyacrylamide gel electrophoresis. Nonmodified siApoB-1 was rapidly degraded within 2 hours, whereas each siLNA showed significant elongation of the stability against enzymatic degradation (**Figure 2a**). Notably, the intact duplex band of siLNA-2 was still detectable until 48 hours of incubation (**Figure 2a**). The half-life of siApoB-1 was ~138 minutes, whereas those of siLNA-1 and siLNA-2 were 268 minutes and 1,106 minutes, respectively (**Figure 2b**). Chemical modification of the sense strand alone resulted in a substantial increase in the half-life of the siRNAs. The half-life of siLNA-2 was longer than that of either of the other siRNAs (siApoB-1 or siLNA-1), and it was approximately eightfold longer than that of siApoB-1. The extremely long half-life of siLNA-2 was attributed to 2′,4′-BNA/LNA-mediated protection of the region around the 3′- and 5′-ends of the sense strand, which appeared to change the environment around 3′-end of both the sense and antisense strands of siRNA.

Design of further modified siRNAs based on siLNA-2 and their RNAi effect *in vitro*

Because siLNA-2 had a substantial RNAi effect and was highly resistant to nuclease, four other siRNAs with additional chemical modifications were designed based on siLNA-2 (**Table 1**). PS modifications were added to the 5′- and 3′-ends of the sense strand of siLNA-2 to generate siL2PT-1. These

additional modifications were designed to achieve further enhanced stability toward nucleases and to assess the alterations on the immune response by partially overlapping the complementary motif of the immunostimulatory motif (the underlined motif in **Table 1**). Based on the work reported by Davies *et al*, 3′-end conjugation on a canonical siRNA might disturb the interaction between 3′-conjugated siRNA and Toll-like receptor 3 (TLR3) by its steric hindrance.[31] We hypothesized that conjugation of a cholesterol moiety to the sense strand of siRNA would affect its immunostimulatory properties and, therefore, that the cholesterol-conjugated siRNA would induce a smaller immune response than its nonmodified counterparts, or possibly no immune response at all. Thus, siL2PTC-1 was designed to have an additional cholesterol conjugation at the 3′-end of the sense strand of siL2PT-1. Previously, the 2′-OMe[32] and 2′,4′-BNA/LNA[33] modifications were demonstrated to eliminate the immune response of the siRNA if these modifications were in the immunostimulatory motif. Therefore, siL2PTC-1M and siL2PTC-1L, which were designed based on siL2PTC-1, have two additional 2′-OMe or 2′,4′-BNA/LNA modifications, respectively, in immunostimulatory motif of the antisense strand. These additional modifications were intended to completely abrogate the innate immune response caused by the siRNA. First, we measured the T_m value of each siRNA, and despite the PS, 2′-OMe modifications and cholesterol conjugation, each siRNA had a T_m value comparable to that of siLNA-2 (**Table 1**).

We evaluated the RNAi effect of each siRNA in the siLNA-2 series using mouse hepatic cells (NMuLi), and two siRNA concentrations, 10 or 100 nmol/l, were used in this assay. Regardless of the concentrations (10 or 100 nmol/l), each of the newly designed siRNAs was less potent as an RNAi agent than siLNA-2 or siApoB-1. At concentrations of 100 nmol/l, siL2PT-1, siL2PTC-1 and siL2PTC-1M showed reduction of apoB mRNA by approximately up to 80%, whereas siL2PTC-1L reduced apoB mRNA by just 60% (**Figure 3a**). Although

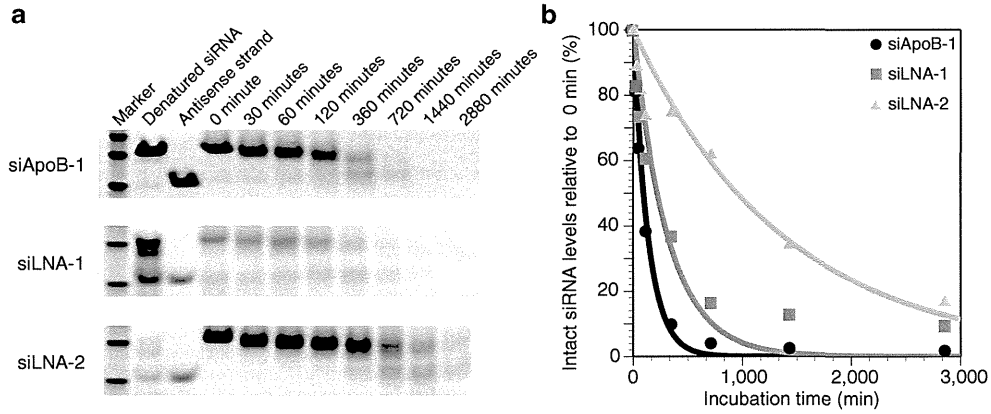

Figure 2 2′ A′-BNA/ LNA modification contributed to the nuclease resistance of the siRNAs. Serum stability of siLNAs and nonmodified siApoB-1 in mouse serum was analyzed using incubation times of 30, 60, 120, 360, 720, 1,440, and 2,880 minutes. (**a**) Samples taken at each time were subjected to native polyacrylamide gel electrophoresis in a 20% TBE gel. Images of polyacrylamide gels stained with SYBR gold are shown. (**b**) The density of bands representing duplex siRNAs was quantified using Image J software, which is freely available on the Internet. Band intensities were normalized using the band intensity of the first "0 minute" time point.

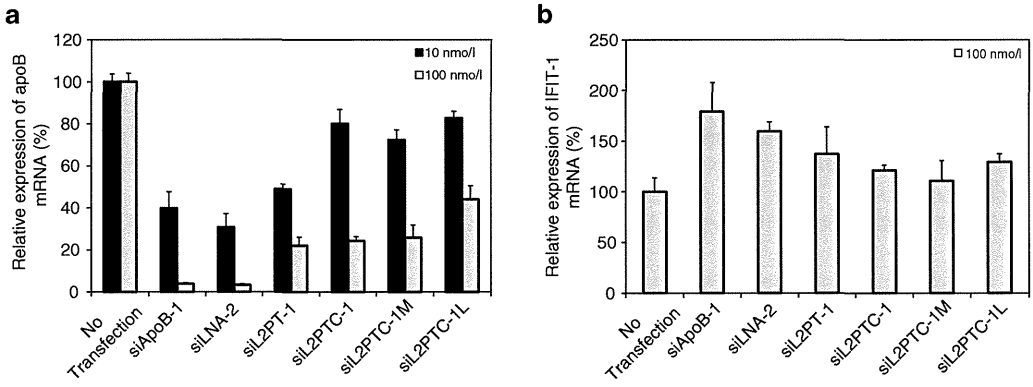

Figure 3 RNAi effects on apoB mRNA of modified siRNAs based on siLNA-2 and the downstream effects on IFIT-1 mRNA levels. (**a**) All siRNAs were transfected into NMuLi cells at concentrations of 10 or 100 nmol/l using Lipofectamine RNAiMAX. ApoB mRNA expression was normalized to GAPDH expression, and relative values were calculated using the no-transfection group. (**b**) Expression of interferon-induced tetratricopeptide repeats 1 (IFIT-1) mRNA was measured as the inflammatory response of the cell; IFIT-1 expression was normalized to GAPDH expression. Error bars indicate SD.

siL2PTC-1M and siL2PTC-1L had modifications at the same positions in their antisense strand, siL2PTC-1L showed a less effective RNAi effect than siL2PTC-1M, indicating that the 2′,4′-BNA/LNA markedly affected the RNAi machinery. At concentrations of 10 nmol/l, each cholesterol-conjugated siRNA had a less pronounced RNAi effect than did the nonconjugated siRNAs (**Figure 3a**). We also evaluated the expression of IFIT-1 (interferon-induced protein with tetratricopeptide repeats 1) mRNA, which was used as an indicator of immune responses and was related to the production of interferon-α (IFN-α).[34] At concentrations of 100 nmol/l, IFIT-1 mRNA expression was significantly higher (1.8-fold higher; $P < 0.01$) in siApoB-1-treated cells than in phosphate-buffered saline (PBS)-treated cells (**Figure 3b**). On the other hand, the elevation of IFIT-1 mRNA was gradually inhibited by each additional chemical modification (**Figure 3b**).

Therapeutic experiment with siLNA-2 series and their effects *in vivo*

To evaluate the pharmacological and toxic effects of each siRNA in this collection, we used atherogenic diet-fed C57BL/6J mice as a mouse model of dyslipidemia. We used Invivofectamice 2.0 reagent (Invitrogen, Carlsbad, CA) as a delivery vehicle of the siRNA. Mice were treated with a single tail vein injection of 5 mg/kg of one siRNA formulated with Invivofectamine 2.0 and adjusted to a final volume of 200 µl with PBS. Mice in the control PBS-treated group were injected with 200 µl of PBS via the tail vein.

Mice receiving siApoB-1 exhibited overt body weight loss, shedding up to 9% of their initial body weight by day 1 ($P < 0.01$) (**Figure 4a**). Among chemically modified siRNAs, siLNA-2, and siL2PTC-1, in particular, seemed to cause overt body weight loss of up to >6% of their initial body weight by day 1. Previous studies have indicated that overt body weight loss is often caused by a strong innate immune response to the siRNA.[32] On the other hand, the average body weight change in each of the other siRNA-treated groups was <3%.

Serum total cholesterol (TC) levels were measured at several time points (day 2, 7, 14, and 24). All siRNAs achieved a similar serum TC reduction of ~55–60% at 2 days after injection (**Figure 4b**). The cholesterol-conjugated siRNAs (siL2PTC-1, siL2PTC-1M, and siL2PTC-1L) showed similar or shorter duration of serum TC reduction than those

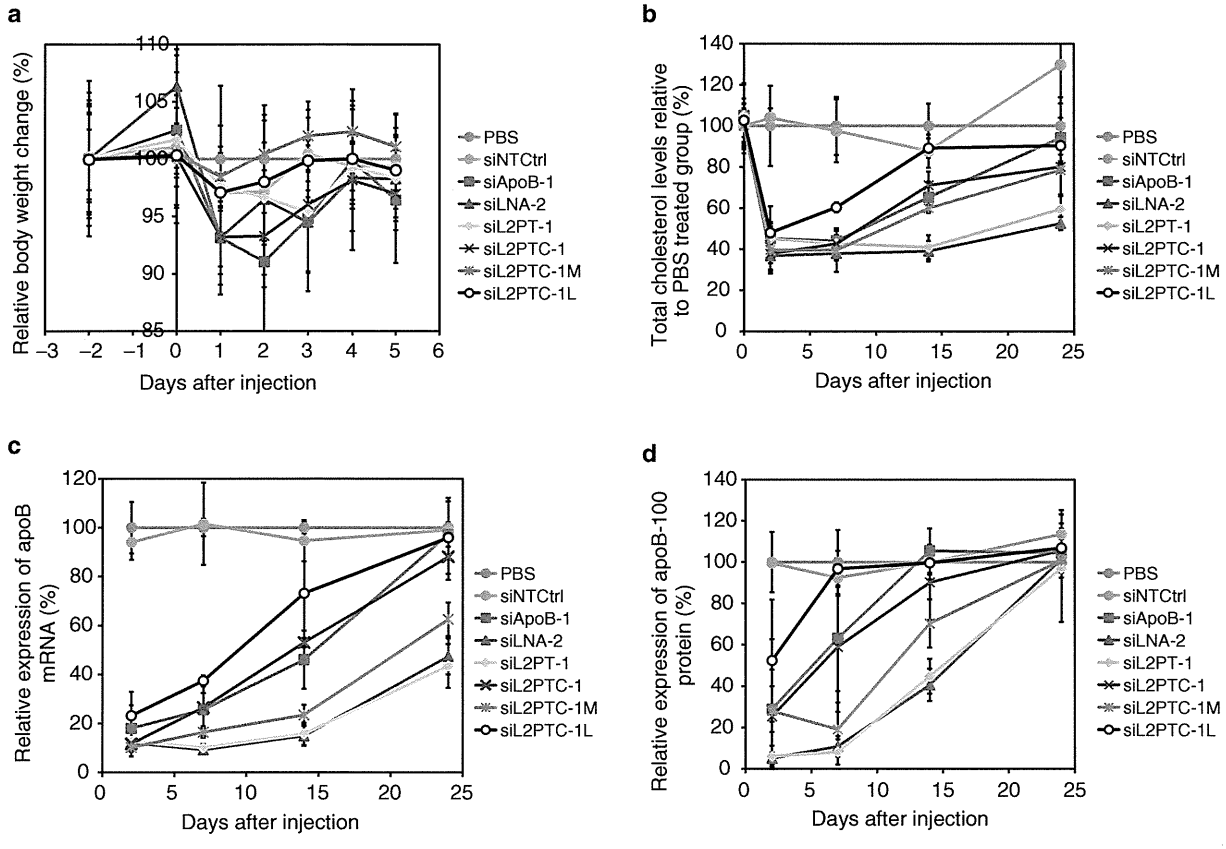

Figure 4 Therapeutic effects and body weight changes in mice receiving an siRNAs. (**a**) Body appearance, including body weight, of each mouse was observed for a week prior to the 2 days before the injection. Body weight changes in mice treated with an siRNA were indicated as ratio relative to that in the PBS-treated group. (**b**) Serum total cholesterol levels were indicated as ratio relative to that in the PBS-treated group. (**c**) ApoB mRNA expression in the liver tissues was normalized to GAPDH expression and was indicated as ratio relative to that in the PBS-treated group. (**d**) ApoB-100 protein levels in liver tissues were quantified by determining the intensity of the band on a western blot membrane, and these intensities are presented as a ratio relative to the band intensities of samples from the PBS-treated group. All values are the means ±SD of three to five animals.

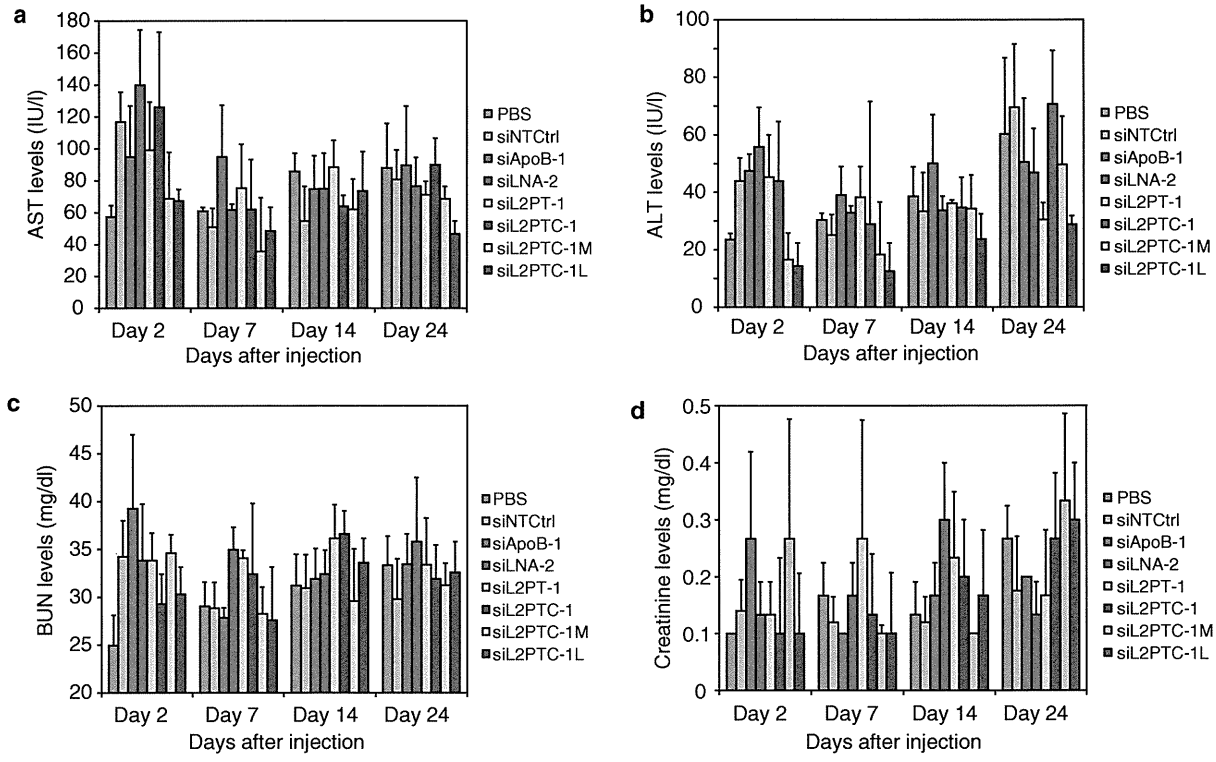

Figure 5 Changes in serum chemistry after the injection of an siRNAs. Aspartate aminotransaminase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine levels were measured at each time point as indicators of hepatotoxicity and nephrotoxicity. Raw data on (**a**) AST, (**b**) ALT, (**c**) BUN, and (**d**) Creatinine levels are indicated. All values are the means ±SD of values from three to five animals.

of siApoB-1, whereas siLNA-2 and siL2PT-1 showed significantly longer duration of lipid-lowering effect than that of siApoB-1. In mice treated with siLNA-2 or siL2PT-1, reductions in serum TC of up to >50% lasted until approximately day 24. Serum TC levels of siLNA-2- or siL2PT-1-treated mice did not recover to the baseline levels by day 40 (**Supplementary Figure S1**). Injection of a Nontargeting control siRNA, siNTCtrl, did not cause any reduction in serum TC levels compared with the PBS-treated group (**Figure 4b**).

ApoB mRNA levels in the liver samples were also measured at the same time points. All siRNAs achieved significant inhibitions in the apoB mRNA levels of up to 90% compared to the PBS-treated group on day 2 (**Figure 4c**). A similar tendency in recovery curve was observed between apoB mRNA and serum TC levels. In contrast, siNTCtrl did not cause a significant reduction in apoB mRNA expression. In mice receiving siL2PTC-1L, a slight loss of RNAi effect was observed and the recovery of apoB mRNA expression was fastest. Significant RNAi effects (>50% inhibition of apoB mRNA) were sustained for 24 days after injection of siLNA-2 or siL2PT-1. In contrast, the RNAi effects were no longer significant at 24 days after injection of any other siRNAs (**Figure 4c**). The time required for apoB mRNA expression to recover by 50% (hereafter referred to as RT_{50}) in the siLNA-2 and siL2PT-1 treatment groups was 25 and 26 days, respectively. The RT_{50} values for the siL2PTC-1M, siL2PTC-1, siApoB-1, and siL2PTC-1L treatment groups were 22, 16, 15, and 12 days, respectively.

For each group, relative apoB-100 protein levels in the liver samples were measured by western blotting. While the recovery curves for apoB mRNA expression and serum TC levels were similar, levels of apoB-100 protein recovered faster than did apoB mRNA levels in all siRNA-treated groups (**Figure 4d**). In the siLNA-2- or siL2PT-1-treated group, up to >50% inhibition of apoB-100 was still observed on day 14, whereas, in mice receiving any other siRNAs, full expression of apoB-100 protein levels was observed on day 14. In contrast, siL2PTC-1L resulted in the least inhibition of apoB-100 production (up to 50% on day 2), and apoB-100 levels in these mice had recovered by day 7. In all pharmacological parameters (TC levels, mRNA and protein expression), the cholesterol-conjugated siRNAs (siL2PTC-1, siL2PTC-1M and siL2PTC-1L) shortened the duration, even though two of these siRNAs (siL2PTC-1 and siL2PTC-1M) showed a level of RNAi effects comparable to that of siL2PT-1 *in vitro* at 100 nmol/l. No remarkable reductions of apoB-100 protein level were observed in the siNTCtrl-treated group.

In translation, there was a high correlation between apoB mRNA recovery and apoB-100 protein recovery ($R^2 = 0.929$) (**Supplementary Figure S2**). When apoB mRNA expression recovered to ~40% of the initial levels, apoB-100 protein expression had already recovered to the initial level, indicating that target knockdown by siRNA facilitates the translation of target mRNA to protein. Despite the high correlation between apoB mRNA and apoB-100 protein, the correlation

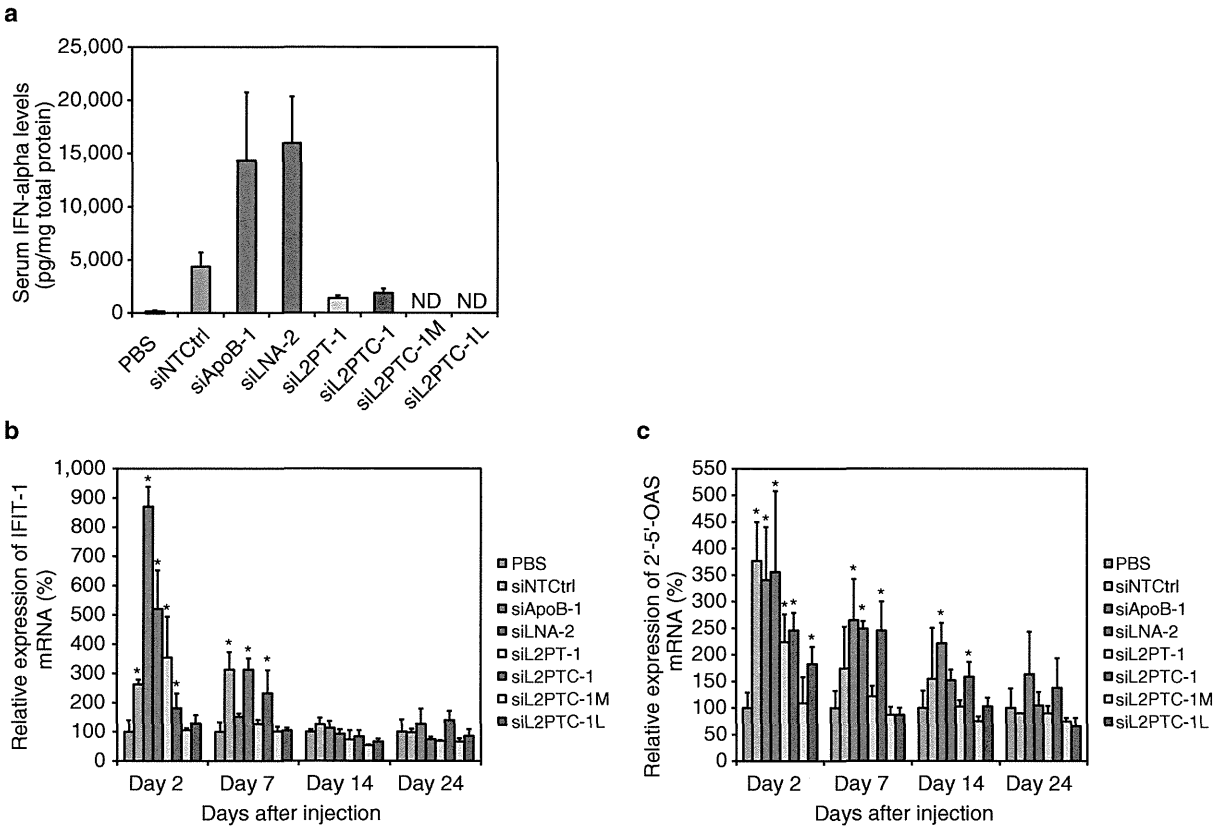

Figure 6 Serum interferon-α (IFN-α) levels and IFN-α-related gene expression. (**a**) Serum IFN-α levels were measured 6 hours after the injection of each siRNAs using enzyme-linked immunosorbent assay. (**b**) Interferon-induced tetratricopeptide repeats 1 (IFIT-1) and (**c**) 2′-5′-oligoadenylate synthetase 1 (OAS-1) mRNA expression in the liver was measured at each time point. IFIT-1 and OAS-1 mRNA expression was normalized to GAPDH expression. All values are the means ±SD of four to five animals.

between serum TC levels and apoB-100 protein levels was less pronounced (R^2 = 0.710).

Hepatotoxicity and nephrotoxicity in each siRNA-treated group

Serum levels of liver aminotransferase [aspartate aminotransaminase (AST) and alanine aminotransferase (ALT)] activities were measured as indicators of hepatotoxicity, and blood urea nitrogen (BUN) and creatinine levels were measured as indicators of nephrotoxicity. Although, on day 2, the mice injected with siRNA that had no modifications to the immunostimulatory motif had higher AST activities, ALT activities and BUN levels than did PBS-treated mice, the elevations of ALT activities and of BUN levels were within the normal range (**Figure 5a–c**). Gradual increases in ALT levels were observed in all groups throughout the experiment, indicating the influence of the atherogenic diet. No notable changes in creatinine levels were observed in any of the siRNA-treated groups (**Figure 5d**).

IFN-α concentrations in serum and IFN-α-related gene expression in liver

IFN-α levels in serum collected 6 hours after injection were measured using enzyme-linked immunosorbent assay. Overt increases in IFN-α levels were observed in mice receiving siApoB-1 or siLNA-2, which showed overt body weight loss on day 1, whereas the elevation of IFN-α levels was significantly attenuated in mice receiving siL2PT-1, which contained additional PS modification (**Figure 6a**). However, additional inhibition of the overt increases in IFN-α levels was not observed in mice receiving siL2PTC-1. The IFN-α concentrations were especially similar in mice receiving siL2PTC-1 or siL2PT-1. In contrast, no IFN-α production was observed in mice receiving siL2PTC-1M or siL2PTC-1L, the immunostimulatory motifs of which were modified with 2′-OMe or 2′,4′-BNA/LNA, respectively. The nontargeting control siRNA, siNTCtrl, also had a different immunostimulatory RNA motif in its sense strand. Mice receiving siNTCtrl had much higher serum IFN-α levels than did the PBS-treated group, but significantly lower serum IFN-α levels than those mice receiving siLNA-2 or siApoB-1 ($P < 0.05$), indicating that the different immunostimulatory motifs induced different levels of innate immune response (**Figure 6a**).

We also confirmed the changes in the expression of two IFN-α–related gene, IFIT-1 and 2′-5′-oligoadenylate synthetase 1 (OAS-1), using quantitative PCR. OAS-1 is induced by IFN-α and synthesizes 2′,5′-oligoadenylates that activate the RNase L. On day 2, mice receiving siApoB-1 exhibited approximately tenfold higher expression of IFIT-1 mRNA than did PBS-treated mice, and the overexpression diminished gradually with each additional chemical modification (**Figure 6b**). On day 2, mice receiving siL2PTC-1 exhibited