Letters

Short Antisense Oligonucleotides with Novel 2′−4′ Conformationaly Restricted Nucleoside Analogues Show Improved Potency without Increased Toxicity in Animals

Punit P. Seth,* Andrew Siwkowski, Charles R. Allerson, Guillermo Vasquez, Sam Lee, Thazha P. Prakash, Edward V. Wancewicz, Donna Witchell, and Eric E. Swayze

Isis Pharmaceuticals, 1891 Rutherford Road, Carlsbad, California 92008

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Abstract: The potency of second generation antisense oligonucleotides (ASOs) in animals was increased 3- to 5 -fold ($ED_{50} \approx 2-5$ mg/kg) without producing hepatotoxicity, by reducing ASO length (20-mer to 14-mer) and by employing novel nucleoside modifications that combine structural elements of 2′-*O*-methoxyethyl residues and locked nucleic acid. The ability to achieve this level of potency without any formulation agents is remarkable and likely to have a significant impact on the future design of ASOs as therapeutic agents.

Antisense technology is a powerful method to modulate gene expression in animals and represents a novel therapeutic platform.[1] The most advanced second generation antisense oligonucleotides (ASOs) are chimeric phosphorothioate[2,3] (PS) modified oligonucleotides, which have a central DNA region of 8−16 nucleotides, flanked on the 5′ and 3′ ends with five to two 2′-*O*-methoxyethyl (MOE) residues.[4] This "gapmer" design supports RNase H mediated degradation of target mRNA due to the central DNA region. The flanking MOE residues increase hybridization to complementary mRNA and further stabilize the oligonucleotide toward enzymatic degradation. The PS backbone not only provides stabilization to nucleases but also confers a substantial pharmacokinetic benefit by increasing the binding to plasma proteins. This prevents rapid renal excretion of the ASO and facilitates binding to other acceptor sites which promote uptake to tissues.[5,6]

There are currently multiple second generation ASOs in development for a variety of disease indications including hypercholesteremia, diabetes, and cancer, among others. One particular compound, mipomersen (ISIS 301012), targeting apolipoprotein B (ApoB),[7] reduced LDL cholesterol by 6−41% in normal volunteers at doses ranging from 50 to 400 (mg/kg)/week in a phase I clinical trial.[8] Ongoing phase II clinical trials with mipomersen have further substantiated clinical efficacy and demonstrated an attractive safety profile for this drug.[9]

The improved performance of second generation ASOs in animals can be attributed in part to the higher affinity of MOE (**B**, Figure 1) residues ($\Delta T_m \approx 1.5$ °C/incorporation),[10] which typically translates to increased binding affinity for the biological receptor (complementary mRNA). To probe if further increases in affinity could enhance the potency of gapmer ASOs, we replaced MOE residues in the wings with bicyclic nucleic acids

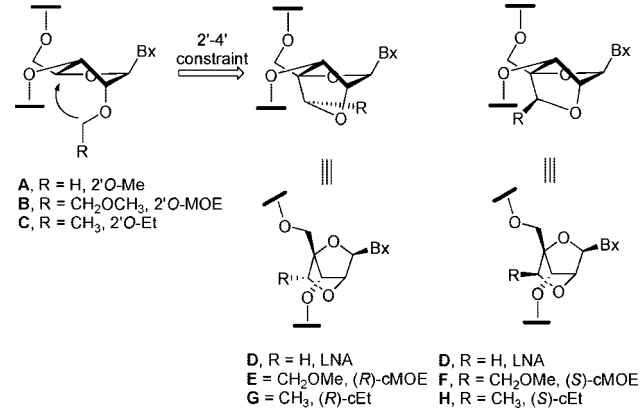

Figure 1. Design of constrained MOE (cMOE) and constrained ethyl (cEt) nucleoside analogues.

such as 2′,4′-methylene bridged nucleic acid (BNA)[11] commonly called LNA (**D**, locked nucleic acids, Figure 1).[12] While this substitution resulted in improved potency of some ASOs in animals, it was accompanied by a significant increase in the risk for hepatotoxicity.[13] In contrast, the MOE modification employed in second generation ASOs is well tolerated in a variety of animal models and has also demonstrated an excellent safety profile in human clinical trials.[14]

Our previous study with LNA gapmers had indicated that the motifs that provided the greatest increase in potency of LNA containing ASOs were gapmer designs with a central gap region of 12−16 DNA residues flanked on either end by 2−3 LNA residues.[13] In the same set of experiments, it was observed that a 16-mer LNA gapmer maintained potency (relative to MOE) while showing lower hepatotoxicity than the longer LNA ASOs. To follow up on this observation, we prepared and evaluated additional 16-, 15-, and 14-mer, LNA gapmers that were shortened gap sequence matched versions of a previously identified active MOE ASO **3a** (ISIS 116847)[15] targeting mouse PTEN (*Mus. musculus* phosphatase and tensin homologue).

A surprising discovery made in this study was that the 14-mer ASO exhibited similar potency in vivo as the 15-mer, 16-mer, and 20-mer ASOs (Supporting Information S1, S2, and S3). This observation was especially intriguing considering that the ASO concentration in liver tissue for the 14-mer ASO, a property typically modulated by overall PS content,[16] was expected to be significantly lower than the 16-mer or 20-mer ASOs. Further screening experiments (Supporting Information S1, S2, and S3) with LNA gapmers containing 2 LNA monomers at each end of a 10 base deoxyribose nucleoside gap (2−10−2 design) led to the identification of a number of sequences with different potency and hepatotoxicity profiles. For this study, we chose two sequences that were at either extremes of the potency and hepatotoxicity spectrum. LNA ASO **1a** (Table 1) was moderately potent but severely hepatotoxic after a single administration, while LNA ASO **2a** was potent but toxic at doses 5−10 times the ED_{50}.

The above screening exercise demonstrated that it was possible to reduce the hepatotoxicity of LNA ASOs by reducing the length of the ASO. However, as shown by ASO **1a**, this alone was not sufficient to eliminate the risk of hepatotoxicity

* To whom correspondence should be addressed. Phone: 760-603-2587. Fax: 760-603-3891. E-mail: pseth@isisph.com.

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Table 1. Sequence and Composition of ASOs Used for Current Study[a]

ASO	modification	ASO sequence (5′ to 3′)
1a	LNA	T^{m}CatggctgcagmCT
1b	*R*-cMOE	UCatggctgcagCU
1c	*S*-cMOE	UCatggctgcagCU
1d	*R*-cEt	UCatggctgcagCU
1e	*S*-cEt	UCatggctgcagCU
1f	MOE	T^{m}Catggctgcag^{m}CT
2a	LNA	^{m}CTtagcactggc^{m}CT
2b	*R*-cMOE	CUtagcactggcCU
2c	*S*-cMOE	CUtagcactggcCU
2d	*R*-cEt	CUtagcactggcCU
2e	*S*-cEt	CUtagcactggcCU
2f	MOE	CUtagcactggcCU
3a	MOE	^{m}CTG^{m}CTag^{m}c^{m}ct^{m}ctggaTTTGA

[a] Base code is as follows: a = adenine, t = thymine, g = guanine, c = cytosine, u = uracil, ^{m}c = 5-methylcytosine. **Upper case letters indicate BNA or MOE monomers in "wings" flanking a central DNA "gap" region (lower case letters). All internucleosidic linkages are phosphorothioate. m denotes 5-methyl substitution on cytosine nucleobase. LNA nucleosides are only available commercially with 5-Me substitution on the pyrimidine nucleobases.

completely. We hypothesized that it might be possible to further reduce the risk of liver toxicity by replacing LNA with novel nucleoside modifications that combine the structural elements of MOE and LNA.

LNA (or 2′−4′ BNA) can essentially be considered a 2′-*O*-Me nucleoside (**A**, Figure 1) where the methyl group is constrained back to the 4′- position of the furanose ring system. The 2′−4′ constraint enforces an N-type sugar pucker of the furanose ring, which in turn leads to improved hybridization with complementary RNA. By use of a similar strategy, constraining the ethyl chain in the MOE residue back to the 4′-position of the furanose ring system provided nucleosides **E** (*R*-constrained MOE or *R*-cMOE,[a] Figure 1) and **F** (*S*-cMOE).[17] As such, we expected the methoxymethyl groups in the cMOE nucleosides to mimic the steric and hydration attributes of MOE nucleosides[18] and thereby improve the safety profile of ASOs containing these modifications.

To determine if ASOs containing the cMOE nucleosides produce less hepatoxicity compared to LNA, mice were injected intraperitonealy (ip) with a single dose of ASOs **1a**, **1b**, and **1c** containing LNA (**A**), *R*-cMOE (**C**), and *S*-cMOE (**D**) nucleosides, respectively, and the transaminase (ALT) levels were determined 72 h after ASO administration. In this study, the *R*-cMOE and *S*-cMOE containing ASOs **1b** and **1c** did not result in elevated ALT levels while the LNA ASO **1a** showed a highly significant ALT elevation, indicative of hepatotoxicity (Figure 2). After validation of our premise that replacing LNA with novel nucleoside monomers that combine the structural elements of MOE and LNA could mitigate the hepatotoxicity caused by LNA, the cMOE nucleosides were evaluated for their effects on ASO potency. LNA ASO **2a** and its sequence matched *R*- and *S*-cMOE and MOE ASOs **2b**, **2c**, and **2f**, respectively, were evaluated in thermal stability and cell culture experiments. The cMOE ASOs **2b** and **2c** exhibited similar binding affinity (as measured by T_m) to the LNA ASO **2a** (Table 2). Consistent with the T_m data, ASOs **2b** and **2c** demonstrated similar IC_{50} values (compared to the LNA ASO **2a**) for PTEN reduction when transfected into mouse brain endothelial (bEND) cells using lipofectin reagent (see Supporting Information S4 for dose response curves). The previously described 5−10−5 MOE ASO **3a** (ISIS 116847), included as a positive control for the study,

[a] Abbreviations: cMOE, constrained 2′*O*-methoxyethyl; cEt, 2′*O*-constrained ethyl.

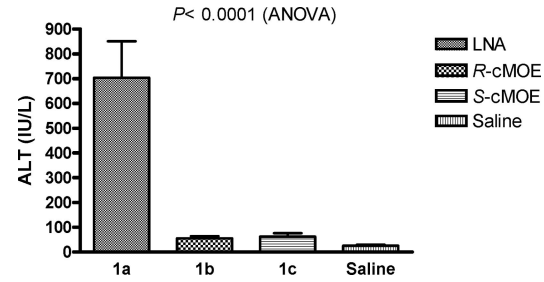

Figure 2. Substituted BNA and MOE nucleosides can mitigate the hepatotoxicity produced by LNA ASO **1a**. Mice (6-week-old male Balb/c, n = 4/dose group) were injected (ip) with 35 mg/kg ASOs **1a**, **1b**, and **1c**. The animals were sacrificed 72 h after dosing and the ALT levels recorded. The increase in ALT levels for LNA ASO **1a** treated animals was found to be significant ($P < 0.0001$, ANOVA) versus saline and ASOs **1b** and **1c** ($P < 0.001$, Tukey's post hoc test).

Table 2. Biophysical and Biological Evaluation of LNA, cMOE, cEt, and MOE Gapmer ASOs

ASO	modification	T_m[a] (° C)	IC_{50}[b] (nM)	ED_{50}[c] (mg/kg)
2a	LNA	60.6	4.9	6
2b	*R*-cMOE	56.9	6.0	29
2c	*S*-cMOE	57.6	5.0	25
2d	*R*-cEt	58.9	10.3	13
2e	*S*-cEt	59.1	5.8	9
2f	MOE	50.8	>20	>45
3a	MOE	67.9	6.2	36

[a] Melting temperature of ASO-RNA duplex. [b] IC_{50} for PTEN mRNA reduction after transfection with lipofectin in bEND cells. [c] ED_{50} for PTEN mRNA reduction in liver. Mice (6-week-old male Balb/c, n = 4/dose group) were injected (ip) with 4.5, 9, 18, and 36 mg/kg ASOs **2a**, **2b**, **2c**, **2d**, **2e**, **2f** and with 1.6, 10, 33, and 100 mg/kg MOE ASO **3a**. The animals were sacrificed 72 h after administration of ASO, and liver PTEN RNA was quantified. All errors are in ±sd.

was equipotent to ASOs **2a**−**c**, while the 2−10−2 MOE ASO **2f** demonstrated lower binding affinity and potency in cell culture.

To evaluate the effect of the cMOE nucleosides on ASO potency in animals, ASOs **2a**, **2b**, **2c**, **2f**, and **3a** were injected (ip) into mice and PTEN expression in liver was determined (Table 2). In a dose response study, the *S*- and *R*-cMOE BNA ASOs **2b** and **2c** (ED_{50} of 25 and 29 mg/kg, respectively) were found to be significantly less potent compared to the LNA ASO **2a** (ED_{50} = 6 mg/kg; see Supporting Information S5 for dose response curves). The loss in potency (as measured by ED_{50}) for the cMOE ASOs **2b** and **2c** was surprising considering that these ASOs demonstrated very similar binding affinity (T_m) and potency for target reduction in cell culture compared to the LNA ASO **2a**.

Since the data clearly suggested that introduction of steric bulk (methoxymethyl groups) on the BNA scaffold had a positive impact on reducing the hepatotoxicity of LNA ASOs, we further hypothesized that reducing the size and hydrophilicity of the substituent group might restore potency while maintaining the beneficial effects on hepatotoxicity. To test this hypothesis, we prepared nucleoside analogues *R*-cEt (**G**) and *S*-cEt (**H**, see Supporting Information S6 for NMR spectra of *R*-cEt and *S*-cEt uridine nucleosides) which now had methyl groups (instead of methoxymethyl) on the BNA scaffold (Figure 1).

ASOs **1d** and **1e** (Table 1) containing nucleosides *R*-cEt (**E**) and *S*-cEt (**F**) were evaluated for their ability to mitigate the hepatotoxicity observed with LNA ASO **1a**. As seen with the cMOE nucleosides, no significant ALT elevations were observed for ASOs containing the novel nucleoside monomers while the LNA ASO **1a** showed significant ALT increases indicative of hepatotoxicity (Supporting Information S7).

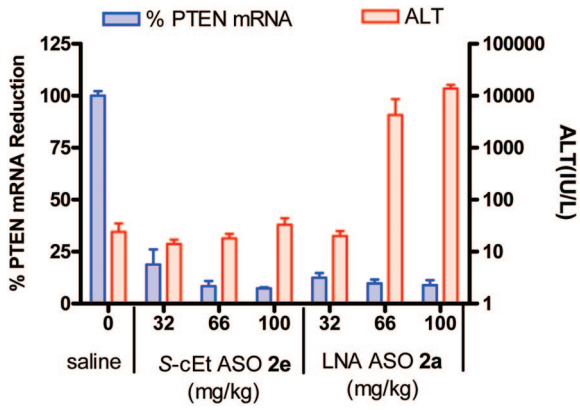

Figure 3. PTEN mRNA reduction in liver and ALT levels after single high dose administration of ASOs **2a** and **2e**. Mice (6-week-old Balb/c, n = 4/dose group) were injected (ip) with 32, 66, and 100 mg/kg ASOs **2a** and **2e**. The animals were sacrificed 72 h after administration of ASO, and ALT levels and liver mRNA were quantified after sacrifice. All errors in ±sd.

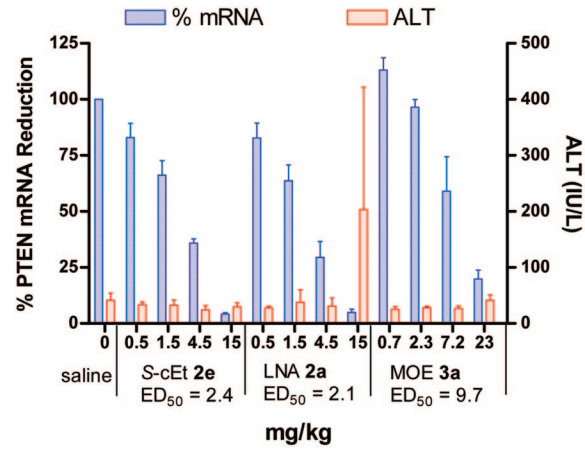

Figure 4. PTEN mRNA reduction in liver and ALT levels after a 3-week multiple administration study. Mice (6-week-old male Balb/c) were injected (ip) twice a week for 3 weeks with 0.5, 1.5, 4.7, and 15 mg/kg ASOs **2a** and **2e** and with 0.7, 2.3, 7.2, and 23 mg/kg ASO **3a**. The animals were sacrificed 72 h after administration of the last dose, and ALT levels and liver mRNA were quantified after sacrifice. All errors are in ±sd.

ASOs **2d** and **2e** (Table 1) containing the nucleoside analogues *R*-cEt and *S*-cEt, respectively, were prepared and evaluated for binding affinity and potency in cell culture experiments (Table 2). As seen previously with the cMOE ASOs **2b** and **2c** and consistent with the T_m data, ASOs **2d** and **2e** demonstrated similar IC_{50} values (compared to the LNA ASO **2a**) for PTEN reduction when transfected into bEND cells using lipofectin (see Supporting Information S8 for in vitro dose response curves).

To evaluate the effect of the *R*-cEt and *S*-cEt nucleosides on ASO potency in animals, ASOs **2d** and **2e** were injected (ip) into mice and PTEN expression in liver was determined (Table 2). In this study, *S*-cEt BNA containing ASO **2e** showed good potency (ED_{50} = 9 mg/kg) while there was a slight shift to the right in the dose response curves for ASO **2d** containing the *R*-cEt modification (ED_{50} = 13 mg/kg) (see Supporting Information S9 for dose response curves). The concomitant reduction of PTEN protein levels in liver tissue was further confirmed by Western blot analysis (see Supporting Information S10). To ascertain if the differences in potency between ASOs **2a**, **2d**, and **2e** were due to differences in gross tissue levels, the amounts of ASO in liver tissue from the two high dose groups of the study were quantified. In all cases we found similar tissue levels of intact drug (25−30 μg/g tissue at the 18 mg/kg dose), thereby ruling out any gross changes in metabolic stability or pharmacokinetic behavior between the LNA, *S*-cEt, and *R*-cEt modifications (Supporting Information S11).

We also examined if the reduced hepatotoxicity observed by substituting LNA (**A**) with the *S*-cEt (**F**) modification in sequence **1a** translated to the more potent LNA ASO **2a**. Mice were injected with increasing doses of LNA ASO **2a** and *S*-cEt ASO **2e**, and PTEN expression in liver and serum ALT levels were determined (Figure 3). At all doses (32, 66, and 100 mg/kg) evaluated for both ASOs, PTEN mRNA was lowered to near maximal levels. Consistent with a previous study, at the 32 mg/kg dose LNA ASO **2a** was not hepatotoxic, but at the 66 and 100 mg/kg dosage levels, severe (>100-fold) elevations in ALT were observed. In contrast, the *S*-cEt ASO **2e** showed no elevation in transaminases even at the 100 mg/kg dose, despite reducing PTEN mRNA levels to >90%.

To further characterize the in vivo behavior of the modified BNA containing ASOs and to better understand the toxicological impact of dosing over an extended time period, ASOs **2a** (LNA), **2e** (*S*-cEt), and **3a** (MOE positive control) were evaluated in a multiple administration (3 week) dose response study (Figure 4). In this study, the LNA and *S*-cEt ASOs **2a** (ED_{50} = 2.1 mg/kg) and **2e** (ED_{50} = 2.4 mg/kg), respectively, demonstrated similar potency and maximal target reduction. Consistent with our earlier studies, the MOE ASO **3a** (ED_{50} = 9.5 mg/kg) demonstrated reduced potency relative to the *S*-cEt modification. Elevations in liver transaminase levels (ALT), increased liver and spleen weights, and reduced body weight gain were observed in animals dosed with the LNA ASO **2a**, while these parameters were normal for MOE and *S*-cEt ASOs **3a** and **2e**, respectively (Supporting Information S12). Thus, results from the longer dosing schedule further support our hypothesis that introducing steric bulk on the BNA scaffold can provide potent, high affinity BNA containing ASOs with an attractive safety profile.

The amount of the *S*-cEt ASO **2e** in liver tissue from the 3-week study was quantified to better understand the pharmacokinetic/pharmacodynamic relationships for shorter ASOs compared to their 20-mer counterparts. The tissue levels observed for the *S*-cEt ASO **2e** ($EC_{50} \approx 6$ μg/g; see Supporting Information S13) were substantially less than that observed for a typical 20-mer 5−10−5 MOE ASO ($EC_{50} \approx 80$ μg/g).[6] Thus, shorter BNA-containing ASOs appear to be at least 3- to 5-fold more potent than a 5−10−5 MOE ASO on an ED_{50} basis and to be 10- to 15-fold more potent on an EC_{50} basis (drug concentration in liver tissue).

In this study we show that the potency of second generation ASOs in animals can be increased 3- to 5-fold without producing hepatotoxicity, by reducing ASO length (20-mer to 14-mer) and by employing novel nucleoside modifications that combine structural elements of MOE and LNA nucleosides. While shortening ASO length raises concerns regarding the theoretical specificity of gene inhibition, these questions have been addressed to some extent previously.[19] However, a thorough understanding of specificity will have to involve a detailed microarray analysis of shorter ASOs versus their longer gap sequence matched counterparts in multiple targets and sequences. These studies are currently underway and will be reported in due course. The precise mechanisms for reducing hepatotoxicity by introduction of steric bulk along the 2′−4′ bridging substituent are as yet unclear. Preliminary investigations regarding toxicity of LNA ASOs had implicated apoptosis

pathways involved in sensing foreign RNA,[13] which could conceivably be activated differentially by LNA but not by appropriately substituted BNAs.

In conclusion, a short BNA gapmer ASO showed an unexpected improvement in potency relative to a second generation ASO such as the 5−10−5 MOE gapmer **3a** and an improved safety profile relative to a short LNA ASO. We are currently evaluating short ASOs with novel BNA modifications in numerous preclinical animal models at Isis. Preliminary data have suggested that the results obtained with PTEN are generally translatable to other liver targets and also to down-regulating target mRNA in other tissues and species. The ability to achieve this level of potency in animals without any formulation or delivery agents is remarkable and likely to have a significant impact on the future design of ASOs as therapeutic agents.

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Supporting Information Available: PTEN mRNA and ALT levels for 14-mer in vivo screen, proton NMR spectra for *R*-cEt and *S*-cEt uridine nucleosides, in vitro and in vivo dose response curves for all ASOs tested, Western blot for PTEN protein reduction, ALT levels after dosing ASOs **1a**−**f**, ASO concentration in liver tissue for modified BNA ASOs, organ weight data from 3-week dosing study. This material is available free of charge via the Internet at http://pubs.acs.org.

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