OL Organic Letters

pubs.acs.org/OrgLett

Letter

Improved *In Vivo* Metabolic Stability and Silencing Efficacy of siRNAs with Phosphorothioate Linkage-Free, GalNAc-Conjugated Sense Strands Containing Morpholino-LNA Modifications

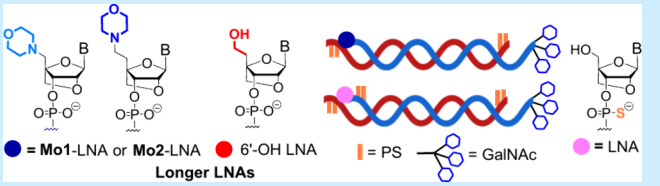
Dhrubajyoti Datta, Pawan Kumar, Jayanta Kundu, June Qin, Jason A. Gilbert, Sally Schofield, Daniel P. Donnelly, Ju Liu, Rohan Degaonkar, Martin Egli, and Muthiah Manoharan*

Cite This: *Org. Lett.* 2024, 26, 10061−10065

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: To ensure specificity, loading of the sense strand of small interfering RNAs (siRNAs) into RISC must be inhibited. We show here that siRNAs with 5′- and 6′-morpholino LNA residues or 6′-OH-LNA at the 5′ terminus of a fully phosphodiester sense strand resulted in metabolically stable siRNAs with a potency and a duration of action in mice that were greater than those of an siRNA in which the 5′ terminus of the sense strand has two terminal phosphorothioate linkages and regular LNA.



Synthetic short interfering RNAs (siRNAs) act through the RNA interference (RNAi) pathway, an endogenous mechanism for the control of gene expression. Synthetic siRNAs must be chemically modified to ensure metabolic stability, efficacy, and cell permeation and to overcome off-target related toxicities.[1−4] All clinically approved siRNAs have 2′-modified nucleosides (2′-OMe and 2′-F) and some phosphorothioate (PS) linkages to provide metabolic stability. The 3′ ends of sense strands of most clinically approved siRNAs are conjugated to trivalent *N*-acetylgalactosamine (GalNAc), a ligand that mediates efficient uptake into liver hepatocytes and provides metabolic stability (Figure S1).

After an siRNA interacts with RISC, only the antisense strand is retained. The antisense strand then specifically interacts through base pairing with the targeted mRNA to downregulate gene expression. Incorporation of the sense strand into RISC can result in the undesired silencing of unrelated mRNAs. We previously reported that the presence of 5′- or 6′-morpholino at the 5′ end of the sense strand improves activity by blocking loading of the sense strand into RISC.[5−7] Alternate backbones such as amide linkages have been shown to achieve the same goal.[4]

Locked nucleic acid (LNA) oligomers bind strongly to target RNA and are resistant to degradation by nucleases.[8−10] In addition, the LNA modification is a poor substrate for polymerases and kinases.[11] We reasoned that combining LNA with morpholino at the 5′ end of the sense strand would strongly disfavor its loading into RISC and would further improve the metabolic stability. Here, we tested this hypothesis by incorporating novel 5′-morpholino (Mo1) LNA, 6′-morpholino (Mo2) LNA, and 6′-OH-LNA (Figure 1) into GalNAc-conjugated sense strands with or without 5′-terminal PS linkages. PS linkages have chirality-dependent metabolic

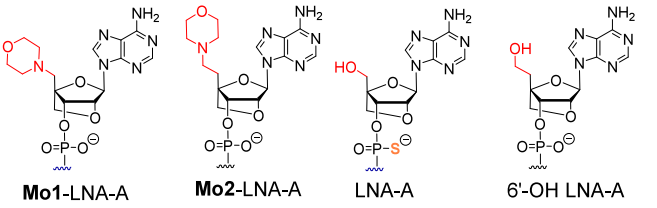
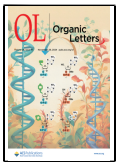


Figure 1. Structures of 5′- and 6′-morpholino LNAs, LNA, and 6′-OH-LNA adenosine building blocks.

stability and RNAi activity[12] and as diastereomeric mixtures complicate purification (Figure S2). We reasoned that the enhanced stability provided by morpholino and LNA might enable the removal of PS linkages without compromising siRNA activity.

To understand the effect of LNA combined with Mo1 on the 5′ end of the sense strand, a building block with Mo1 and LNA (**5**) was synthesized (Scheme 1). In brief, the 5′-OH group of a reported nucleoside **1**[13] was tosylated and treated with morpholine to afford **3** in moderate yield. The *tert*-butyldimethylsilyl (TBS) group of **3** was then removed to afford compound **4**, which was phosphitylated to afford **5** in good yield. Phosphoramidite **5** was then used in the synthesis of 5′-modified sense strands [**ON1**−**ON4** (Table S1)]. These modified sense strands were used to prepare **si-3**−**si-6** (Table

Received: August 7, 2024
Revised: September 10, 2024
Accepted: September 19, 2024
Published: November 11, 2024



Scheme 1. Synthesis of 5′-Morpholino LNA-A Phosphoramidite 5

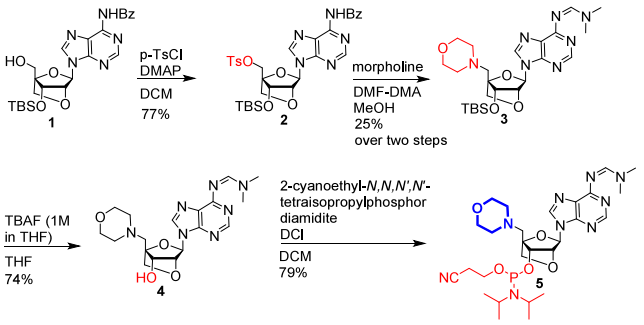
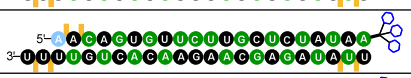
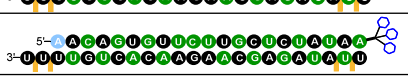


Table 1. Sense and Antisense Strand Sequences and Chemistries of *Ttr*-Targeted siRNAs Used in This Study

siRNA ID	siRNA sense (top) and antisense (bottom) strands [a]
si-1	
si-2	
si-3	
si-4	
si-5	
si-6	
si-7	
si-8	
si-9	
si-10	
si-11	
si-12	
si-13	

[a]Black and green circles indicate 2′-OMe and 2′-F sugar modifications, respectively. Orange lines indicate PS. Light blue, pink, dark blue, and red colored circles represent Mo1-LNA-A (**5**), LNA-A, Mo2-LNA-A (**14**), and 6′-OH-LNA-A (**18**) modifications, respectively. The trivalent GalNAc ligand at the 3′ terminus of the sense strand is indicated as hexagonal rosettes.

1). We also prepared **si-2**, in which the 5′ end of the sense strand is modified with LNA with a 3′-phosphodiester linkage. All siRNAs were fully modified with 2′-F and 2′-OMe, and all sense strands were conjugated to trivalent GalNAc and differed in the number and position of PS linkages. These siRNAs were evaluated in mice for their ability to reduce the circulating levels of TTR. The control in this experiment was **si-1**, an siRNA that has been previously reported to effectively silence expression of *Ttr*.[14]

Mo1-LNA-modified siRNAs **si-3**−**si-6** were more potent at the nadir on day 7 and had longer durations of action than did **si-1** and **si-2** (Figure 2A). Surprisingly, **si-6**, which has no PS

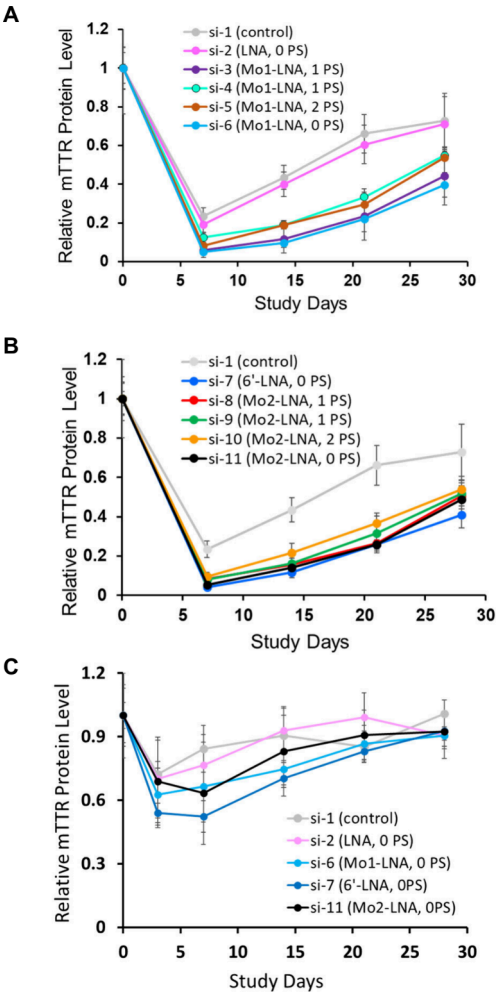


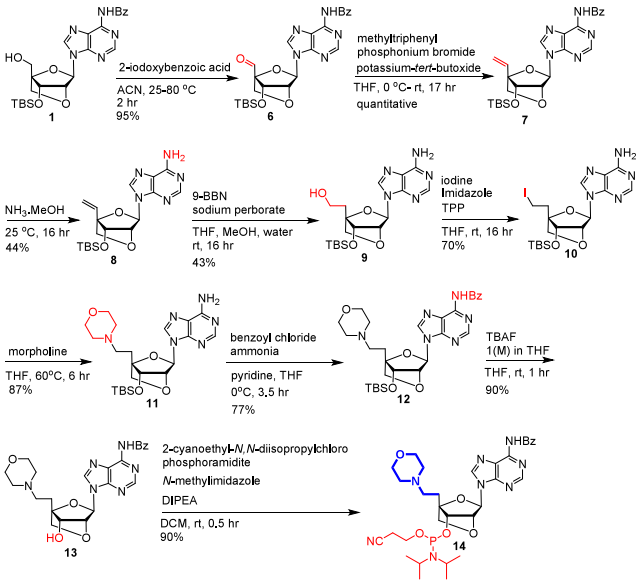
Figure 2. PS linkages are unnecessary in the sense strand when the sense strand is modified with Mo1-LNA, Mo2-LNA, or 6′-OH-LNA. Plotted are relative levels of TTR protein in the serum of mice treated with one subcutaneous dose of (A) siRNAs with LNA or the LNA-Mo1 modification at 1.0 mg/kg, (B) siRNAs with 6′-LNA or the Mo2 modification at 1.0 mg/kg, and (C) the indicated siRNAs at 0.3 mg/kg. TTR was evaluated by an enzyme-linked immunosorbent assay at the indicated times after dosing. Each data point is the average ± standard deviation normalized to predose levels in individual animals (n = 3 per group). Data in panels A and B were acquired in a single experiment, and the combined data are shown in Figure S3A.

linkages in the sense strand, was more efficacious than **si-5**, which has two PS linkages at the 5′ end (Figure 2A). This indicates that PS linkages are unnecessary when the Mo1-LNA modification is incorporated at the 5′ end of the sense strand.

Our previous work showed that Mo2 is a better RISC antagonist than Mo1 in the context of 2′-OMe.[7] To test Mo2 in the context of LNA, we synthesized building block **14** (Scheme 2). The 5′-hydroxy group of nucleoside **1** was oxidized with 2-iodoxybenzoic acid to obtain aldehyde **6**, which was used in a Wittig reaction to afford **7** in quantitative yield. The hydroboration−oxidation reaction of **7** produced a complex reaction mixture. Therefore, the N^6-benzoyl was removed from compound **7** to afford compound **8**, which was then treated

https://doi.org/10.1021/acs.orglett.4c02903
Org. Lett. 2024, 26, 10061−10065

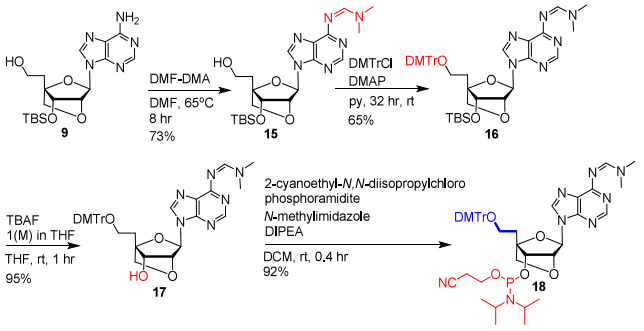
Scheme 2. Synthesis of 6′-Morpholino LNA-A Phosphoramidite 14



with 9-borabicyclo(3.3.1)nonane to afford 6′-OH-containing compound **9** in moderate yield. Compound **9** was then iodinated using triphenyl phosphine and iodine to afford 6′-iodo compound **10**, which was converted into 6′-morpholino LNA nucleoside **11** in good yield. The exocyclic amine group of **11** was benzoyl protected to afford **12**. The TBS group of **12** was desilylated to afford **13**, which was phosphitylated to afford **14** in very good yield.

We also synthesized the 6′-OH-LNA building block to evaluate a longer homologue without morpholino using the chemistry we published.[7] The exocyclic N^6-amino group of compound **9** was protected with *N,N*-dimethylformamide dimethyl acetal to afford **15**. Dimethoxytrityl protection of the 6′-OH afforded **16** in moderate yield, and subsequent removal of TBS afforded **17** in very good yield. The phosphitylation of **17** produced **18** (Scheme 3). Building blocks Mo2-LNA-A (**14**)

Scheme 3. Synthesis of 6′-OH-LNA-A Phosphoramidite 18



and 6′-OH-LNA-A (**18**) were used in syntheses of sense strands (Table S1) that were annealed with the complementary antisense strand to form **si-7**–**si-11** (Table 1).

As was observed for siRNAs with sense strands modified with Mo1-LNA, irrespective of the number or placement of PS linkages, Mo2-LNA-modified sense strands resulted in siRNAs that were more active than control **si-1** (Figure 2B). The 6′-OH-LNA modification (**si-7**) also provided an advantage compared to the control (Figure 2B). At a dose of 1 mg/kg, it was not possible to differentiate the efficacies of **si-6**, **si-7**, and **si-11**, which are modified with Mo1-LNA, 6′-OH-LNA-A, and Mo2-LNA, respectively. At the lower dose of 0.3 mg/kg, **si-7** appeared to be slightly more active than **si-6** and **si-11** (Figure 2C).

To understand the advantages of LNA linked through a phosphodiester over the standard 2′-OMe linked through a PS, we synthesized building blocks for incorporation of Mo2-2′-OMe-A and 6′-OH-2′-OMe-A (Schemes S1 and S2). We also used the reported Mo1-2′-OMe-A[5] building block for this comparative study. These building blocks were incorporated at the 5′ ends of the sense strands through phosphodiester linkages. The sense strands were annealed with antisense strands to afford siRNAs **si-15**–**si-17** (Table S3). When tested in mice at a dose of 0.3 mg/kg, the siRNAs with morpholino modifications in the context of 2′-OMe (**si-15** and **si-17**) had activity that was higher than that of the siRNA with a sense strand modified with 6′-OH-2′-OMe (**si-16**) (Figure S3B). **si-16** was also less active than **si-7**, which has a sense strand modified with LNA (Figure S4A). The efficacies of **si-15** (modified with Mo2-2′-OMe) and **si-17** (modified with Mo1-2′-OMe) were similar to those of **si-11** and **si-6**, which combine morpholino analogues with LNA (Figure S4B). Furthermore, consistent with our previous observations,[4] the Mo2-LNA and 6′-OH-LNA modifications on the 5′ end of the antisense strand result in the complete loss of *Ttr* silencing as these modifications block loading of the antisense strand into the RISC (Figure S5).

To assess how well LNA and morpholino protect sense strands from nucleases present in plasma, two additional siRNA duplexes with sense strands modified with LNA were prepared. These siRNAs had a 5′ LNA modification with PS linkages at position 1 or 2 [**si-12** or **si-13**, respectively (Table 1)]. These siRNAs as well as siRNAs with sense strands modified with LNA (**si-2**), Mo1-LNA (**si-6**), 6′-OH-LNA (**si-7**), and Mo2-LNA (**si-11**) without PS linkages were incubated in rat plasma for 24 h. No 5′ sense strand metabolism was observed, and the extent of removal of the 3′-terminal residue of the antisense strand was similar for all siRNAs (Figure S6).

To evaluate the 5′ exonuclease and overall metabolic stability of these duplexes in the liver, mice were treated with 10 mg of siRNA/kg, and livers were collected at 24 h. For **si-12**, with an LNA and a single terminal PS, no metabolism of the sense strand was detected. Metabolism of the sense strand modified with LNA with a penultimate PS linkage (**si-13**) was detected, but no 5′ sense strand metabolism was observed for any other siRNA (Figure S6). For **si-13**, ∼29% of the sense strand signal resulted from the sense strand lacking the LNA residue, which has the penultimate PS. No metabolism from the 5′ terminus was detected for **si-2**, which also has an LNA terminus but is not stabilized by a PS linkage. Close examination of the LCMS data for **si-2** revealed a significant loss of the total sense strand signal (Figure S6), suggesting that after removal of the terminal residue metabolism of the entire strand occurred. In rat plasma, removal of the 3′-terminal residue of the antisense strands was similar for all siRNAs, with 40–50% of the signal due to a strand that is shorter by a single residue. These data indicate that Mo1-LNA, Mo2-LNA, and 6′-OH-LNA protect the sense strand of an siRNA to a roughly equivalent extent from degradation by nucleases present in plasma.

We used computational modeling to visualize the interactions of 6′-P-LNA and Mo2-LNA at the active site of a 5′-exonuclease to gain insight into the origins of the increased metabolic stability afforded by these modifications (Figure 3). Human

https://doi.org/10.1021/acs.orglett.4c02903
Org. Lett. 2024, 26, 10061−10065

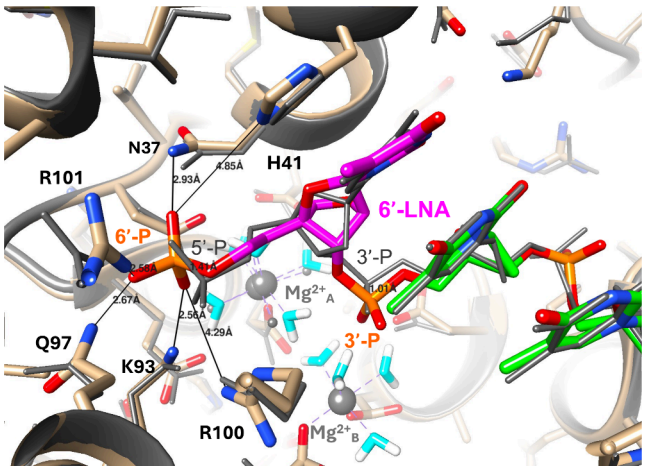


Figure 3. Model of Xrn1 bound to an oligonucleotide with a phosphorylated 6′-OH-LNA residue at the 5′ end. The crystal structure (Protein Data Bank 2y35) is shown as thin gray lines, revealing deviations between the LNA 6′-phosphate and the parent 5′-phosphate as well as between the 3′-phosphates. The guanidino moiety of R100 that forms salt bridges to OP1 and OP2 of the 5′-phosphate in the crystal structure does not contact the 5′-terminal phosphate in the 6′-OH-LNA model.

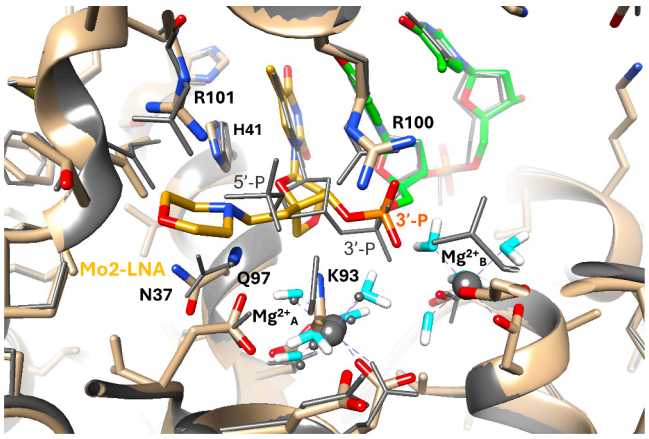


Figure 4. Model of Xrn1 bound to an oligonucleotide with a 5′ Mo2-LNA residue. The crystal structure (Protein Data Bank entry 2y35) is shown with thin gray lines, illustrating the electrostatic incompatibility of the Mo2-LNA modification with surrounding basic and/or polar side chains K93, R100, R101, N37, and H41.

phosphodiesterase II is a 5′-exonuclease that is often used for stability assays conducted *in vitro*; however, no three-dimensional structural information is available for this nuclease. Therefore, we modeled the oligonucleotides in the active site of a eukaryotic 5′-exonuclease, Xrn1, as we did previously.[15] Xrn1 is an exonuclease conserved from yeast to mammals.[16]

At the Xrn1 active site with two Mg^{2+} ions (Figure S7), we changed the 5′ residue to phosphorylated 6′-OH-LNA using UCSF Chimera,[17] followed by energy minimization with Amber 14[18] until conversion. The resulting model demonstrates that the 6′-phosphate of the 6′-OH-LNA is inserted more deeply into the Xrn1 binding pocket than the 5′-phosphate of the oligonucleotide in the crystal structure. In addition, R100, which is in direct contact with the 3′-phosphate group of the 5′-terminal residue in the crystal structure, does not contact the LNA phosphate group [4.29 Å (Figure 3)]. The terminal phosphate of the LNA-containing oligonucleotide is shifted by 1.4 Å, and the 3′-phosphate is shifted by 1 Å relative to the positions in the crystal structure. The LNA C4″−O2′ bridge sits as close as 3.3 Å to a water coordinated to one of the active site Mg^{2+} ions (Mg^{2+}_A) and as close as 3.6 Å to a main chain atom of Xrn1 residue N37. The misaligned phosphates in the model with 6′-OH-LNA are consistent with the enhanced metabolic stability of this modification. Next, we used molecular mechanics to evaluate the Mo2-LNA modification bound at the active site of the dual-metal ion model of Xrn1. The energy-minimized model of the complex shows shifts of key residues at the active site away from the morpholino moiety (Figure 4). These include N37, H41, K93, R100, and R101 that directly contact or are in the proximity of the 5′ phosphate in the parent structure and are important for activity. The scissile 3′ phosphate also undergoes a shift (Figure S8). The inability of nucleases to establish interactions with the hydrophobic morpholino moiety likely precludes the degradation of sense strands modified with morpholino.

Similar to the electrostatic and steric incompatibility of Mo2 at the Xrn1 active site, this modification is not tolerated at the Ago2 MID domain that harbors the binding site for the 5′-terminal residue of the guide strand. This was illustrated in previously published modeling studies.[5,7] 5′-OH-LNA and extended 6′-OH-LNA exhibit poor affinity for MID, which recognizes the terminal phosphate of the guide strand through interactions with Lys and Arg side chains. Even LNA with a phosphate or (*E*)-vinyl phosphonate is not a good fit as the bicyclic LNA sugar cannot adopt the DNA-like C2′-endo conformation imposed by the Ago2 on the ribose of the 5′-terminal nucleotide.[2] The LNA sugar is restricted to the RNA-like C3′-endo conformation. Moreover, LNA is a poor substrate for kinases,[11] as rationalized on the basis of a model of LNA at the active site of eukaryotic polynucleotide kinase Clp1.[19] Accordingly, the C4″−O2′ bridge clashes with Clp1 side chains (not shown).

In conclusion, combinations of Mo1 or Mo2 with LNA linked through a phosphodiester provided as much or even more metabolic stability than an LNA linked through a PS linkage at the 5′ end of the sense strand of an siRNA. In addition, siRNAs with sense strands modified at the 5′ ends with Mo1-LNA, Mo2-LNA, or 6′-OH-LNA without PS linkages had efficacy, potency, and duration of silencing that were greater than those with sense strands with 2′-OMe linked through PS. siRNA strands with PS linkages have chirality-dependent properties and are diastereomeric mixtures that cause difficulties in purification and cause analytical challenges. Thus, these metabolically stable, off-target-mitigating morpholino LNA modifications warrant further investigation in the context of RNAi therapeutics. Yamada et al. recently confirmed the numerous applications possible for such "extended" backbone linkages[20] and corroborated the findings reported here and previously.[7,21−23]

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.4c02903.

Experimental section and compound characterization (PDF)

https://doi.org/10.1021/acs.orglett.4c02903
Org. Lett. 2024, 26, 10061−10065

AUTHOR INFORMATION

Corresponding Author

Muthiah Manoharan − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States;* orcid.org/0000-0002-7931-1172; Email: mmanoharan@alnylam.com

Authors

Dhrubajyoti Datta − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

Pawan Kumar − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

Jayanta Kundu − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

June Qin − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

Jason A. Gilbert − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

Sally Schofield − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

Daniel P. Donnelly − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

Ju Liu − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States;* orcid.org/0000-0003-1030-9266

Rohan Degaonkar − *Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States*

Martin Egli − *Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232, United States;* orcid.org/0000-0003-4145-356X

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.orglett.4c02903

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Tang, Q.; Khvorova, A. RNAi-based drug design: considerations and future directions. *Nat. Rev. Drug Discovery* **2024**, *23*, 341.

(2) Egli, M.; Manoharan, M. Re-Engineering RNA Molecules into Therapeutic Agents. *Acc. Chem. Res.* **2019**, *52*, 1036.

(3) Egli, M.; Manoharan, M. Chemistry, structure and function of approved oligonucleotide therapeutics. *Nucleic Acids Res.* **2023**, *51*, 2529.

(4) Kotikam, V.; Rozners, E. Amide-Modified RNA: Using Protein Backbone to Modulate Function of Short Interfering RNAs. *Acc. Chem. Res.* **2020**, *53*, 1782.

(5) Kumar, P.; Parmar, R. G.; Brown, C. R.; Willoughby, J. L. S.; Foster, D. J.; Babu, I. R.; Schofield, S.; Jadhav, V.; Charisse, K.; Nair, J. K.; et al. 5′-Morpholino modification of the sense strand of an siRNA makes it a more effective passenger. *Chem. Commun.* **2019**, *55*, 5139.

(6) Janas, M. M.; Schlegel, M. K.; Harbison, C. E.; Yilmaz, V. O.; Jiang, Y.; Parmar, R.; Zlatev, I.; Castoreno, A.; Xu, H.; Shulga-Morskaya, S.; et al. Selection of GalNAc-conjugated siRNAs with limited off-target-driven rat hepatotoxicity. *Nat. Commun.* **2018**, *9*, 723.

(7) Datta, D.; Theile, C. S.; Wassarman, K.; Qin, J.; Racie, T.; Schmidt, K.; Jiang, Y.; Sigel, R.; Janas, M. M.; Egli, M.; et al. Rational optimization of siRNA to ensure strand bias in the interaction with the RNA-induced silencing complex. *Chem. Commun.* **2023**, *59*, 6347.

(8) Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.-i.; In, Y.; Ishida, T.; Imanishi, T. Synthesis of 2′-O,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3, -endo sugar puckering. *Tetrahedron Lett.* **1997**, *38*, 8735.

(9) Singh, S. K.; Koshkin, A. A.; Wengel, J.; Nielsen, P. LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. *Chem. Commun.* **1998**, 455.

(10) Hagedorn, P. H.; Persson, R.; Funder, E. D.; Albæk, N.; Diemer, S. L.; Hansen, D. J.; Møller, M. R.; Papargyri, N.; Christiansen, H.; Hansen, B. R.; et al. Locked nucleic acid: modality, diversity, and drug discovery. *Drug Discovery Today* **2018**, *23*, 101.

(11) Lundin, K. E.; Højland, T.; Hansen, B. R.; Persson, R.; Bramsen, J. B.; Kjems, J.; Koch, T.; Wengel, J.; Smith, C. I. Biological activity and biotechnological aspects of locked nucleic acids. *Adv. Genet.* **2013**, *82*, 47.

(12) Jahns, H.; Taneja, N.; Willoughby, J. L. S.; Akabane-Nakata, M.; Brown, C. R.; Nguyen, T.; Bisbe, A.; Matsuda, S.; Hettinger, M.; Manoharan, R. M.; et al. Chirality matters: stereo-defined phosphorothioate linkages at the termini of small interfering RNAs improve pharmacology in vivo. *Nucleic Acids Res.* **2022**, *50*, 1221.

(13) Manoharan, M.; Das, R. S.; Rajeev, K. G.; Datta, D.; Theile, C. Modified oligonucleotides and double-stranded RNAs. WO2024073709, 2024.

(14) Nair, J. K.; Willoughby, J. L. S.; Chan, A.; Charisse, K.; Alam, M. R.; Wang, Q.; Hoekstra, M.; Kandasamy, P.; Kel'in, A. V.; Milstein, S.; et al. Multivalent N-Acetylgalactosamine-Conjugated siRNA Localizes in Hepatocytes and Elicits Robust RNAi-Mediated Gene Silencing. *J. Am. Chem. Soc.* **2014**, *136*, 16958.

(15) Mikami, A.; Erande, N.; Matsuda, S.; Kel'in, A.; Woods, L. B.; Chickering, T.; Pallan, P. S.; Schlegel, M. K.; Zlatev, I.; Egli, M.; et al. Synthesis, chirality-dependent conformational and biological properties of siRNAs containing 5′-(R)- and 5′-(S)-C-methyl-guanosine. *Nucleic Acids Res.* **2020**, *48*, 10101.

(16) Jinek, M.; Coyle, S. M.; Doudna, J. A. Coupled 5′ Nucleotide Recognition and Processivity in Xrn1-Mediated mRNA Decay. *Mol. Cell* **2011**, *41*, 600.

(17) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605.

(18) Case, D. A.; Cheatham, T. E., III; Darden, T.; Gohlke, H.; Luo, R.; Merz, K. M., Jr; Onufriev, A.; Simmerling, C.; Wang, B.; Woods, R. J. The Amber biomolecular simulation programs. *J. Comput. Chem.* **2005**, *26*, 1668.

(19) Dikfidan, A.; Loll, B.; Zeymer, C.; Magler, I.; Clausen, T.; Meinhart, A. RNA specificity and regulation of catalysis in the eukaryotic polynucleotide kinase Clp1. *Mol. Cell* **2014**, *54*, 975.

(20) Yamada, K.; Hariharan, V. N.; Caiazzi, J.; Miller, R.; Ferguson, C. M.; Sapp, E.; Fakih, H. H.; Tang, Q.; Yamada, N.; Furgal, R. C. Enhancing siRNA efficacy in vivo with extended nucleic acid backbones. *Nat. Biotechnol.* **2024**, DOI: 10.1038/s41587-024-02336-7.

(21) Debart, F.; Vasseur, J.-J.; Sanghvi, Y. S.; Dan Cook, P. Synthesis and incorporation of methyleneoxy(methylimino) linked thymidine dimer into antisense oligonucleosides. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1479.

(22) An, H.; Wang, T.; Maier, M. A.; Manoharan, M.; Ross, B. S.; Cook, P. D. Synthesis of Novel 3′-C-Methylene Thymidine and 5-Methyluridine/Cytidine H-Phosphonates and Phosphonamidites for New Backbone Modification of Oligonucleotides. *J. Org. Chem.* **2001**, *66*, 2789.

(23) Masaki, Y.; Tabira, A.; Hattori, S.; Wakatsuki, S.; Seio, K. Insertion of a methylene group into the backbone of an antisense oligonucleotide reveals the importance of deoxyribose recognition by RNase H. *Org. Biomol. Chem.* **2022**, *20*, 8917.