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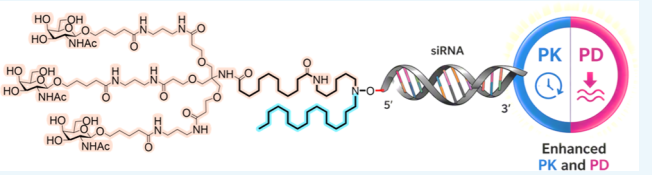
Synthesis and Characterization of Aminooxy Click Chemistry-Mediated *Bis*-Conjugates of siRNAs and Evaluation of Their RNAi Activity

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ABSTRACT: Previously, we used an aminooxy click chemistry (AOCC) strategy to introduce pairs of ligands into oligonucleotides during solid-phase synthesis by using nucleoside and non-nucleoside building blocks. However, the standard oligonucleotide synthesis conditions were incompatible with the incorporation of certain complex ligands. In this report, first, a post-oligonucleotide synthesis solution-phase AOCC approach was developed to address this issue. Different pairs of ligands were conjugated to oligonucleotides through an *O*-amino building block incorporated during the solid-phase synthesis. Second, a small interfering RNA with a sense strand conjugated using the AOCC strategy to target the *ApoB* gene using a receptor-targeting ligand (GalNAc) and a pharmacokinetic enhancer (C16 alkyl) was evaluated in mice. The *bis*-heteroconjugate had a longer duration of action and higher level of silencing than the siRNA carrying only the receptor-targeting ligand.



INTRODUCTION

Conjugation of oligonucleotides to ligands in order to alter their pharmacokinetic and pharmacodynamic properties has been critically important in the development of nucleic acid-based therapeutics, as demonstrated by the clinical successes of siRNAs conjugated to trivalent *N*-acetylgalactosamine (GalNAc).[1] Click-type chemistries, due to their efficiency and chemo-selectivity under biologically relevant conditions, have proven their utility in the synthesis of oligonucleotide bioconjugates.[2,3] Conjugation of oligonucleotides to lipophilic modifications like cholesterol[4] and fatty acid conjugates[5,6] results in their broad distribution to liver and the central nervous system,[7−11] and more specific tissue targeting has been demonstrated with folic acid,[12] which binds to the folate receptor α, resulting in targeting to cancer cells,[13] and cyclic RGD peptides (cRGD), which bind to cells that express integrins.[14] However, there are still issues related to the PK modulation and efficient tissue delivery of such novel siRNA conjugates that remain to be tackled in order to enable preclinical and clinical developments.

To facilitate conjugation to oligonucleotides, we recently introduced a "click type" aminooxy-based chemistry that we call aminooxy click chemistry (AOCC).[15] The aminooxy group has been successfully used to irreversibly or reversibly link ligands to oligonucleotides through an oxime linkage.[16−22] Unlike copper-assisted azide−alkyne cycloaddition chemistry, the limited bio-orthogonality of the oxime chemistry renders it more suitable for *in vitro* applications.[23] In our previous report, we synthesized a series of nucleoside and non-nucleoside building blocks with the highly reactive nucleophilic aminooxy (*O*-amino) functionality, appropriately protected.[15] Nucleosides modified with the reactive aminooxy group were then converted into *bis*-homo and *bis*-hetero building blocks functionalized with two identical or two different ligands respectively (Figure 1). Both *bis*-homo- and *bis*-heterotype nucleoside conjugates were successfully incorporated at the 5′ ends of oligonucleotides.

We posited that the incorporation of two different functionalities through *bis*-hetero-AOCC would enhance the therapeutic potential of oligonucleotides. For example, an agent such as GalNAc, folate, or peptide to enable tissue-type targeting could be combined with a ligand that improves endosomal release or pharmacokinetics.[24,25] However, using this building-block approach, incorporation of complex ligands such as vitamins or peptides is difficult. Complex molecules often cannot tolerate conditions required during synthesis and deprotection of oligonucleotides, resulting in the need for

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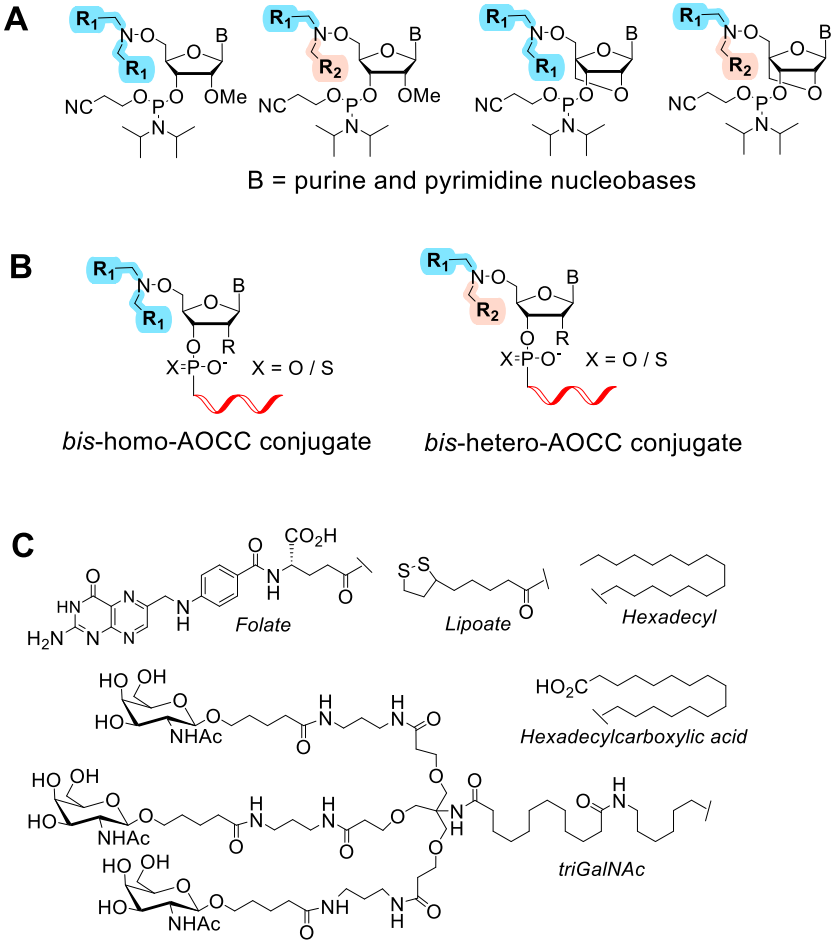


Figure 1. (A) Previously reported AOCC-mediated *bis*-homo and *bis*-hetero monomer building blocks. (B) *Bis*-homo- and *bis*-heterotype nucleoside conjugates at 5′ ends of oligonucleotides. (C) Representative targeting ligands and pharmacokinetic enhancers (represented as R_1 and R_2) conjugated to oligonucleotides in the previous study.[15]

installation of protecting groups on the ligands and subsequent deprotection. In this work, we circumvented this issue by first introducing the precursors of *O*-amino groups into the oligonucleotide strands via solid-phase synthesis. Then, after cleavage of the oligonucleotide from the solid support, we functionalized the reactive *O*-amino groups postsynthetically (i.e., after completion of solid-phase oligonucleotide synthesis). Through this approach, simultaneous access to targeting ligands and pharmacokinetic enhancers or diagnostic agents such as biotin or a fluorophore will be greatly facilitated.

RESULTS

Building Block Synthesis

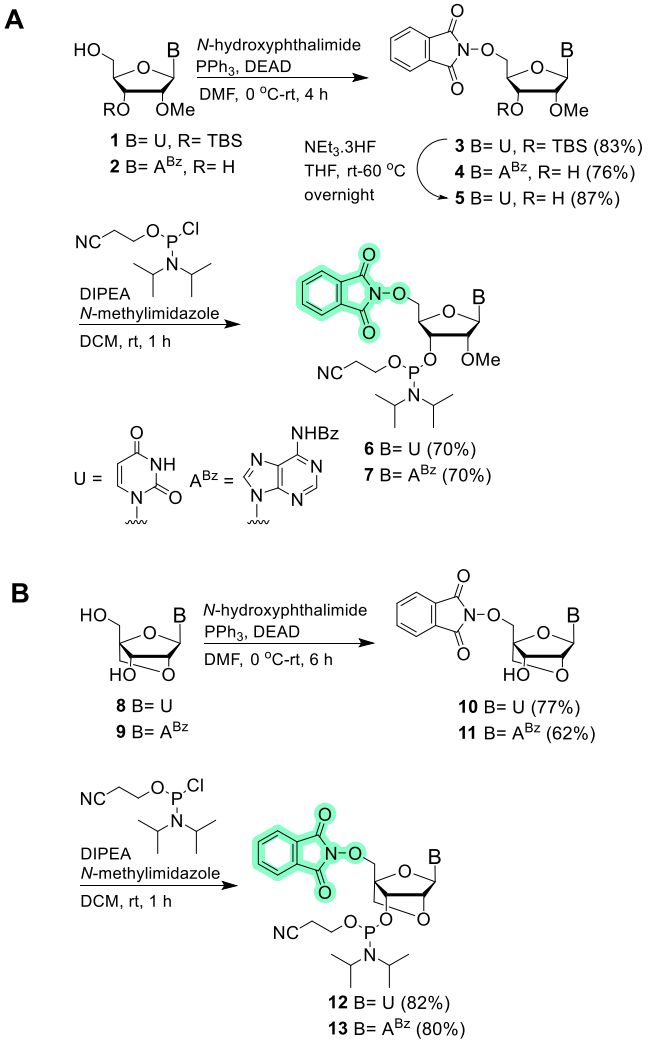
First, we synthesized *N*-hydroxyphthalimido (OPhth)-functionalized building blocks, which are the precursors of the *O*-amino group. To generate OPhth-modified nucleosides, commercially available 2′-*O*-methyl (2′-OMe) and 2′-*O*,4′-locked nucleosides with either protected or free 3′-OH were used. Mitsunobu reaction of compound **1**,[26] and **2**,[27] afforded the OPhth-containing scaffolds **3** and **4**, respectively. Deprotection of the 3′-*tert*-butyldimethylsilyl group from compound **3** yielded **5**. Phosphitylation of **4** and **5** under standard conditions afforded **6** and **7**, respectively, in good yields (Scheme 1A). The locked nucleic acid building blocks **8**, synthesized as described,[28,29] and **9**,[30,31] were also converted to the corresponding OPhth derivatives **10** and **11**, which were then converted to the respective phosphoramidites **12** and **13** (Scheme 1B). We also wanted to demonstrate the AOCC at the 2'- and 3'-positions of ribonucleosides. Therefore, we prepared *O*-amino precursor building blocks from the 2′ and 3′ ends of the sugars of pyrimidine nucleosides through a reported alkylation method.[32,33] Such modifications enable access to the *O*-amino groups at internal positions and terminal ends of oligonucleotides. The stannylated uridine **15**,[32] was treated with the OPhth-containing bromo compound **14** (see Supporting Information) (Scheme 2), resulting in a mixture of regio-isomers **16** and **17**. The isomers were separated after 5′-DMT protection, affording the 2′- and 3′-substituted isomers **18** and **19**, respectively. These regio-isomers were characterized by 2D-NMR (see Supporting Information). Phosphitylation of **18** and **19** yielded the phosphoramidite building blocks **20** and **21**, respectively, in good yields (Scheme 2).

We synthesized non-nucleoside building blocks containing OPhth groups from either known abasic *trans*-4-hydroxyprolinol amine **22**,[34] or from a similar scaffold **26**, synthesized as described[1,35] (Scheme 3). Coupling with 12-hydroxydodecanoic acid afforded **23** and **27**, respectively. Mitsunobu reactions of *N*-hydroxyphthalimide with these two compounds yielded the OPhth-functionalized compounds **24** and **28**, respectively, which upon phosphitylation reactions afforded the corresponding phosphoramidites **25** and **29**, respectively.

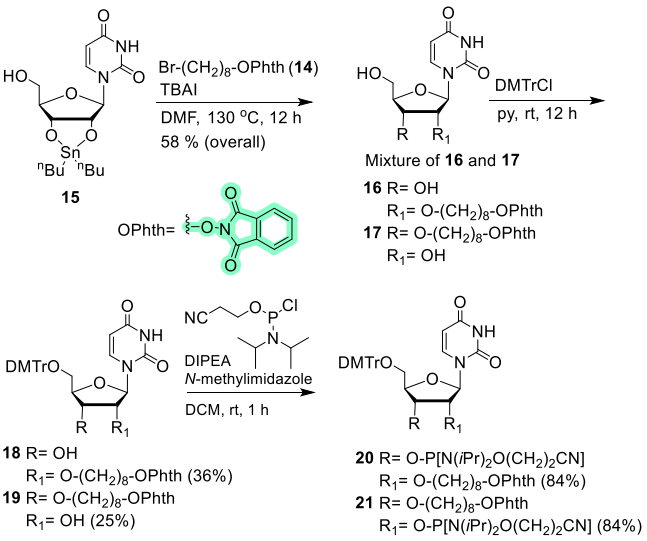
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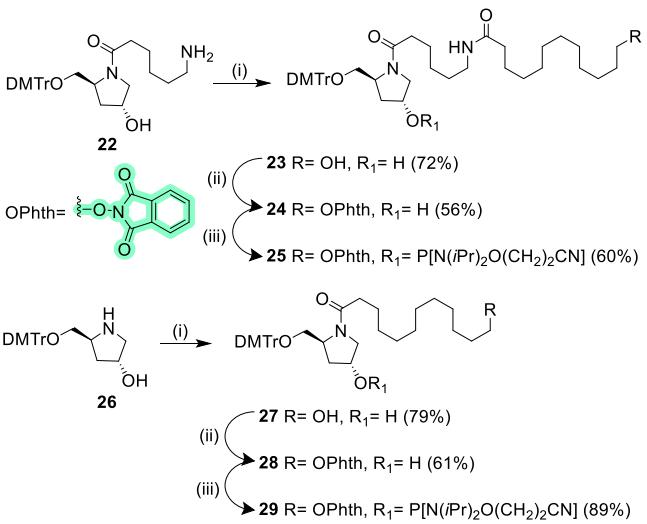
Scheme 1. Synthesis of 5′-*O*-Amino Precursor Phosphoramidite Building Blocks (A) of 2′-OMe-Uridine 6 and Adenosine 7 and (B) 2′-*O*,4′-Locked Uridine 12 and Adenosine 13



Scheme 2. Synthesis of 2′- and 3′-*O*-Amino Precursor Phosphoramidite Building Blocks of Uridine 20 and 21, Respectively



Scheme 3. Synthesis of Abasic *O*-Amino Precursor Phosphoramidite Building Blocks 25 and 29[a]



[a]Reaction conditions: (i) 12-hydroxydodecanoic acid, (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholinocarbenium hexafluorophosphate (COMU), diisopropylethylamine (DIPEA), 12 h; (ii) *N*-hydroxyphthalimide, triphenyl phosphine (TPP), diethyl azodicarboxylate (DEAD), DMF, 0 °C to room temperature, 12 h; (iii) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, *N*-methylimidazole, DIPEA, dichloromethane (DCM), room temperature, 1 h.

We also made modified succinyl controlled pore glass (CPG) building blocks from intermediates **18**, **19**, **24**, and **28**. Succinylation of 2′- and 3′-octyl-OPhth-modified uridine nucleosides under basic conditions afforded **30** and **31**, respectively, which were then coupled to the free $-NH_2$ groups of unmodified CPGs to afford *O*-amino precursor-containing CPGs **32** and **33**, respectively, with good loading capacities. Similarly, the *trans*-4-hydroxyprolinol intermediates **24** and **28** were converted to succinates **34** and **35**, respectively, which were then used to make the modified CPGs **36** and **37**, respectively, with good loading capacities (Scheme 4).

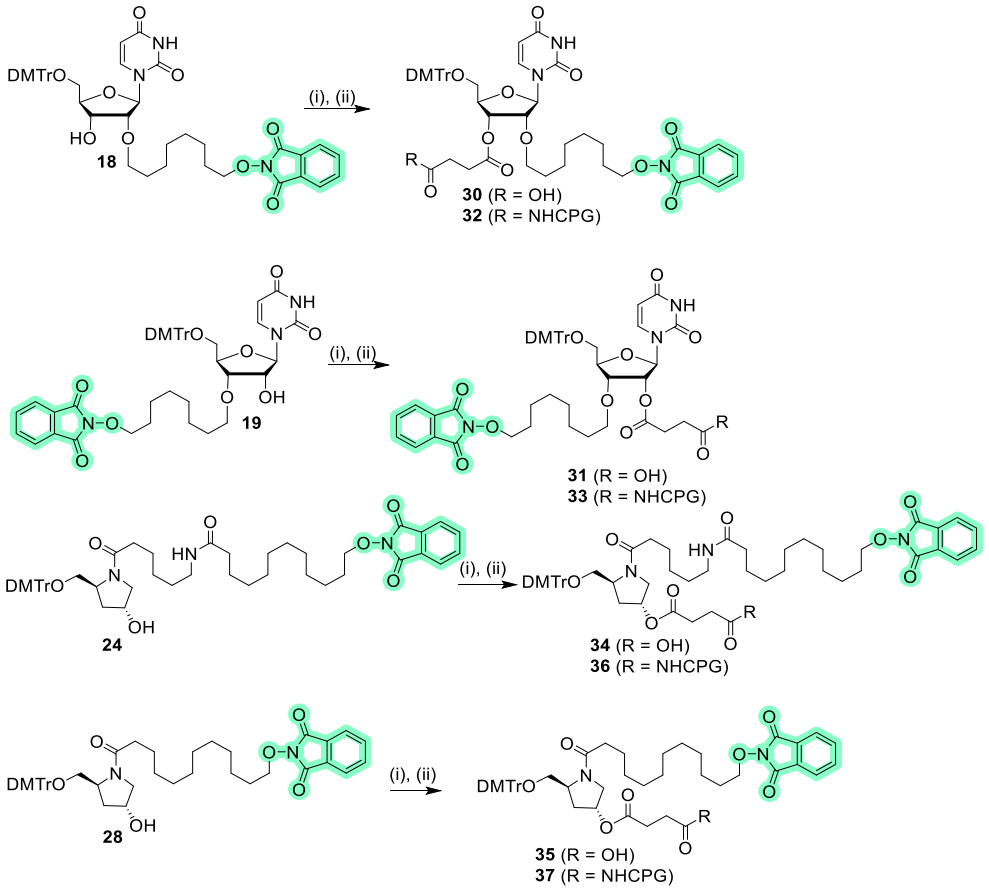
Postsynthetic AOCC *Bis*-Hetero Conjugation in Solution and on Solid Phase

The *O*-amino precursor building blocks were incorporated at the 5′ and 3′ termini and at internal positions of the sense strands of siRNAs targeting mouse *Ttr* and *Sod*-1 during solid-phase synthesis. Modified oligonucleotides were then converted into the *bis*-hetero-AOCC-functionalized oligonucleotides postsynthetically in two steps, either in solution or on support. For the solution route, first, the oxime conjugates were prepared by reacting the *O*-amino group with the first set of ligands (i.e., targeting ligands like GalNAc or cRGD) and then purified. Next, a different set of ligands (i.e., pharmacokinetic enhancers) were introduced in solution, affording the *bis*-hetero-AOCC conjugates (Scheme 5A). To deprotect the phthalimido groups cleanly, 0.5 M hydrazine acetate in pyridine was used.[19] The CPGs containing the free *O*-amino groups were then treated with aqueous ammonia to obtain the crude aminooxy oligonucleotides. These were resuspended in HEPES buffer, and aldehyde group-containing ligands, namely, GalNAc aldehyde and cRGD aldehyde, were

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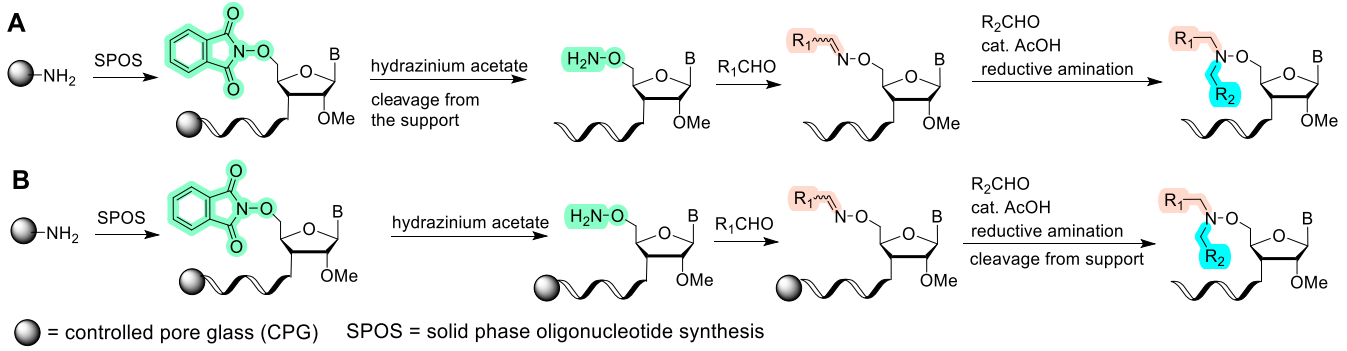
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Scheme 4. Synthesis of *O*-Amino Precursor-Modified CPGs Building Blocks 32, 33, 36, and 37[a]



[a]Reaction conditions: (i) succinic anhydride, DMAP, DCM, room temperature, 3−4 h, 66−81% yield; (ii) CPG-NH_2 (500 Å), HOBT, DIPEA, ACN, then capping reagents in THF, loading 75−93 μmol/g.

Scheme 5. Schematic Representation of AOCC-Mediated Conjugation (A) in Solution and (B) on Support



added at pH 7.0. The resulting mixture was then warmed to 37 °C for 1 h to afford the oxime conjugates, which were subsequently purified (Table 1 and Supporting Information). This afforded **ON1**, **ON2**, and **ON3** with oxime conjugates to GalNAc at the 5′ terminus, GalNAc at the 3′ terminus, and cRGD at an internal position, respectively. In the next step, these oligonucleotides were reacted with aldehyde-functionalized ligands (i.e., hexadecanal, biotin aldehyde, and ibuprofen aldehyde) under reductive amination conditions of catalytic acid and $NaBH_3CN$ to yield *bis*-heteroconjugated oligonucleotides (Table 1). **ON1**, upon treatment with hexadecanal under reductive amination conditions, afforded **ON4** conjugated to GalNAc and C16 at the 5′ terminus. **ON2** treated under identical conditions yielded **ON5** conjugated to GalNAc and C16 at the 3′ terminus. **ON3** was converted into three different AOCC conjugates functionalized with cRGD and with C16, biotin, or ibuprofen to give **ON6**, **ON7**, and **ON8**, respectively (Table 1). These crude-conjugated oligonucleotides were purified by ion exchange and reverse phase HPLC. Formation of desired conjugates was verified using analytical reverse phase HPLC chromatography (Supporting Information).

For the postsynthetic approach on support, we prepared **ON4**, but instead of cleaving the oligonucleotide from the CPG support after deprotection of the phthalimido groups with hydrazine acetate, the solid support was washed

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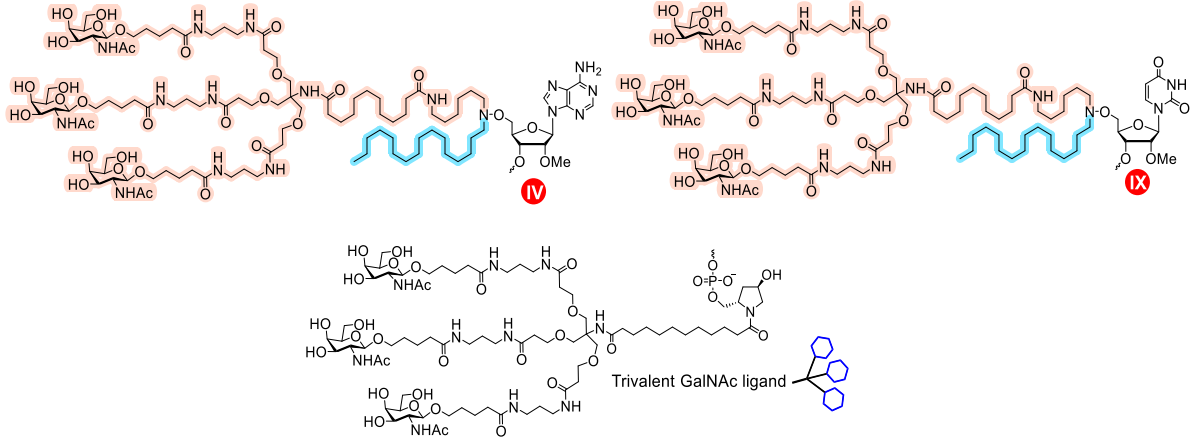
Table 1. Sense Strand Sequences with Oxime and Aminooxy Conjugates

oligo ID	Target	sequence 5′−3′[a]	aldehyde used	conjugation chemistry
ON1	*Ttr*	**I**aCaGuGu*UCU*uGcUcUaU●a●*A*	GalNAc	oxime
ON2	*Ttr*	*A*●a●CaGuGu*UCU*uGcUcUaUa**II**	GalNAc	
ON3	*Sod*-1	cauuu**III***AaUCC*ucacucuaaa	cRGD	
ON4	*Ttr*	**IV**aCaGuGu*UCU*uGcUcUaU●a●*A*	GalNAc + C16	aminooxy
ON5	*Ttr*	*A*●a●CaGuGu*UCU*uGcUcUaUa**AV**	GalNAc + C16	
ON6	*Sod*-1	cauuu**VI***AaUCC*ucacucuaaa	cRGD + C16	
ON7	*Sod*-1	cauuu**VII***AaUCC*ucacucuaaa	cRGD + biotin	
ON8	*Sod*-1	cauuu**VIII***AaUCC*ucacucuaaa	cRGD + ibuprofen	
ON9	*ApoB*	**IX**●g●*UgAcAaAUA*uGgGcAuC●a●*A*	GalNAc + C16	

[a]Uppercase italicized and lowercase letters represent 2′-fluoro and 2′-OMe nucleosides, respectively. **I** refers to GalNAc attached to 5′-modified adenosine building block **7**, **II** refers to GalNAc attached to 3′-modified *trans*-4-hydroxyprolinol building block **37**, **III** refers to cRGD attached to the 2′-modified uridine building block **20**, **IV** refers to 5′-GalNAc + C16 units derived from 5′-modified adenosine building block **7**, and **V** refers to 3′-GalNAc + C16 derived from *trans*-4-hydroxyprolinol building block **37**. **VI**, **VII**, and **VIII** refer to cRGD + C16, cRGD + biotin, and cRGD + ibuprofen, respectively, attached to the 2′-modified uridine building block **20**. **IX** refers to 5′-GalNAc + C16 derived from 5′-modified uridine reported previously.[15] Phosphorothioate linkages are indicated by the ● symbol. See Tables 2, S1, and S2 for the structures of oxime and *bis*-heteroaminooxys, **I**-**IX**. Yields and characterization of conjugates are shown in Table S2.

Table 2. Sense (Top) and Antisense (Bottom) Strand Sequences and Chemistries of *ApoB*-Targeting siRNAs Used in This Study[a]

siRNA ID	siRNA sense (top) and antisense (bottom) strands [a]	Sense strand modifications		Target
		5′ end	3′ end	
si-1		-	GalNAc	*Ttr*
si-2		GalNAc + C16	-	
si-3			GalNAc	*ApoB*
si-4		GalNAc + C16	-	



[a]Black and green circles indicate 2′-OMe and 2′-fluoro sugar modifications, respectively. Orange bars indicate phosphorothioate linkage. A circle colored in red represents 5′ GalNAc + C16. The GalNAc ligand at the 3′ terminus of the sense strand is indicated by hexagonal rosettes.

thoroughly with pyridine and DCM. The free *O*-amino group on the support was then treated with GalNAc aldehyde (Figure 1C and Supporting Information) under mild basic conditions to afford the corresponding oxime. This oxime was reduced on the support with $NaBH_3CN$ under catalytic acid conditions and immediately reacted with hexadecanal. After repeating the latter cycle twice, oligonucleotide **ON4** was cleaved from the support, purified, and characterized.

Analysis of Gene Silencing Activity of *Bis*-Heteroconjugated siRNAs in Mice

To evaluate the efficacy and duration of action of *bis*-heteroconjugated siRNA, we chose to target *apolipoprotein B* (*ApoB*) mRNA, which is expressed in hepatocytes and encodes a protein involved in cholesterol transport. *ApoB* is a validated target for agents that act through the RNA interference (RNAi) pathway to treat hypercholesterolemia and cardiovascular diseases. It is the structural component of LDL, VLDL, and Lp(a).[4] It has been established that RNAi-based silencing

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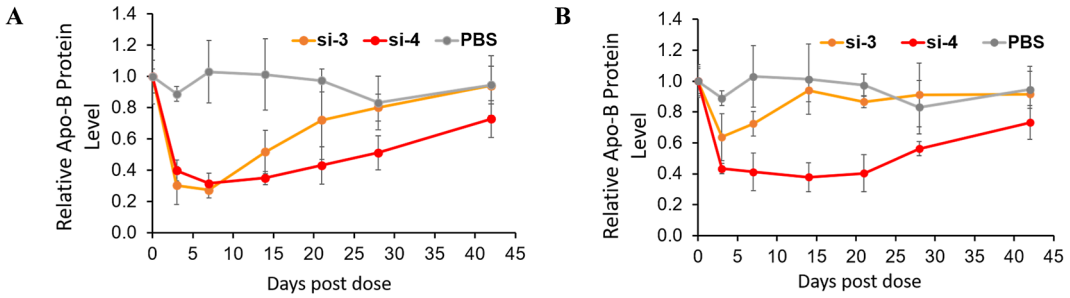


Figure 2. Modification of the sense strand of an siRNA with both GalNAc and C16 enhances efficacy and duration of silencing relative to siRNA conjugated only to GalNAc. Mice (n = 3 per group) were treated with a single (A) SC or (B) IV dose of 3 mg/kg of **si-3** or **si-4**. The levels of circulating ApoB protein were quantified by ELISA. Data are expressed relative to levels of ApoB in the PBS-treated mice.

Table 3. Percent Relative Knockdown of ApoB Protein on Days 7 and 28 after SC and IV Administrations of AOCC-Modified (si-4) and Control (si-3) Duplexes

siRNA	ApoB Sense (top) and antisense (bottom) strands	% Relative knockdown on day 7		% Relative knockdown on day 28	
		SC	IV	SC	IV
si-3		>70	~25	~20	<10
si-4		~70	~70	~50–60	>40

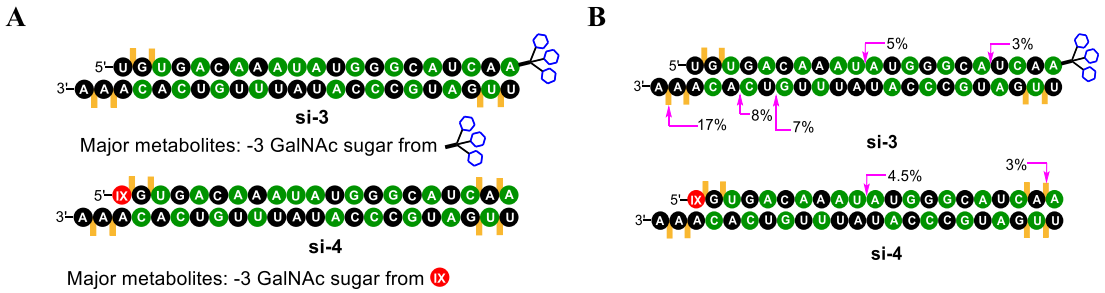


Figure 3. Identified metabolites for **si-3** and **si-4** (A) from rat liver tritosomes and (B) rat liver homogenates. Pink arrows and percentages represent location and abundance of each identified metabolite. Black and green circles indicate 2′-OMe and 2′-fluoro sugar modifications, respectively. Orange bars indicate phosphorothioate linkage. A circle colored in red represents 5′ GalNAc + C16. The GalNAc ligand at the 3′ terminus of the sense strand is indicated by hexagonal rosettes.

of *ApoB* mRNA in the liver significantly lowers LDL cholesterol by inhibiting its production. Using siRNAs, shRNAs, or artificial miRNAs, this therapeutic strategy has demonstrated successful reduction of ApoB protein by over 50% in preclinical models. ApoB-100 (in the liver)[4,36] and ApoB-48 (in the intestine) are essential for exporting cholesterol and triglycerides from hepatocytes and enterocytes.[37] Silencing these genes in the liver breaks down mRNA, inhibiting lipoprotein production and clearing cholesterol. Studies show siRNA-mediated knockdown can achieve up to 90−95% reduction in hepatic *ApoB* mRNA using lipid nanoparticles.[38] In nonhuman primates, a single dose showed reductions in cholesterol levels lasting over 11 days. While antisense oligonucleotides like mipomersen[39] exist, RNAi therapies may offer highly potent and durable knockdown of *ApoB*, with applications for homozygous familial hypercholesterolemia.

ON9, which carries GalNAc and C16 lipid attached through AOCC at the 5′ end, has the same sequence and chemical modifications as the sense strand of the *ApoB*-targeted siRNA described previously.[15] We reported the synthesis of GalNAc/C16 AOCC-conjugated oligonucleotide **ON9** previously to modify the 5′ end of the sense strand of the *ApoB*-targeting duplex using the monomer building block approach.[15] **ON9** was hybridized with an appropriately chemically modified antisense strand to yield **si-4** (Table 2). The control duplex **si-3** has a sense strand modified at the 3′ end with GalNAc (Table 2).

The *in vivo* efficacy of *bis*-heteroconjugated siRNA was evaluated in mice following subcutaneous (SC) and intravenous (IV) administration (Figure 2). Following SC dosing (3 mg/kg), both **si-3** (GalNAc only) and **si-4** (GalNAc + C16) achieved robust ApoB knockdown, with maximal reductions of ~70−80% observed at day 7. While the initial potency of the two constructs was comparable, **si-4** exhibited markedly improved durability. ApoB levels in **si-3**-treated animals returned to near baseline by day 28, whereas **si-4** maintained substantial silencing (~50−60% reduction) at the same time point (Figure 2A).

A more pronounced difference between the two constructs was observed following IV administration (Figure 2B). Consistent with prior observations for GalNAc-conjugated siRNAs by IV (due to fast renal elimination), **si-3** showed limited activity, achieving only ~30−40% knockdown at peak

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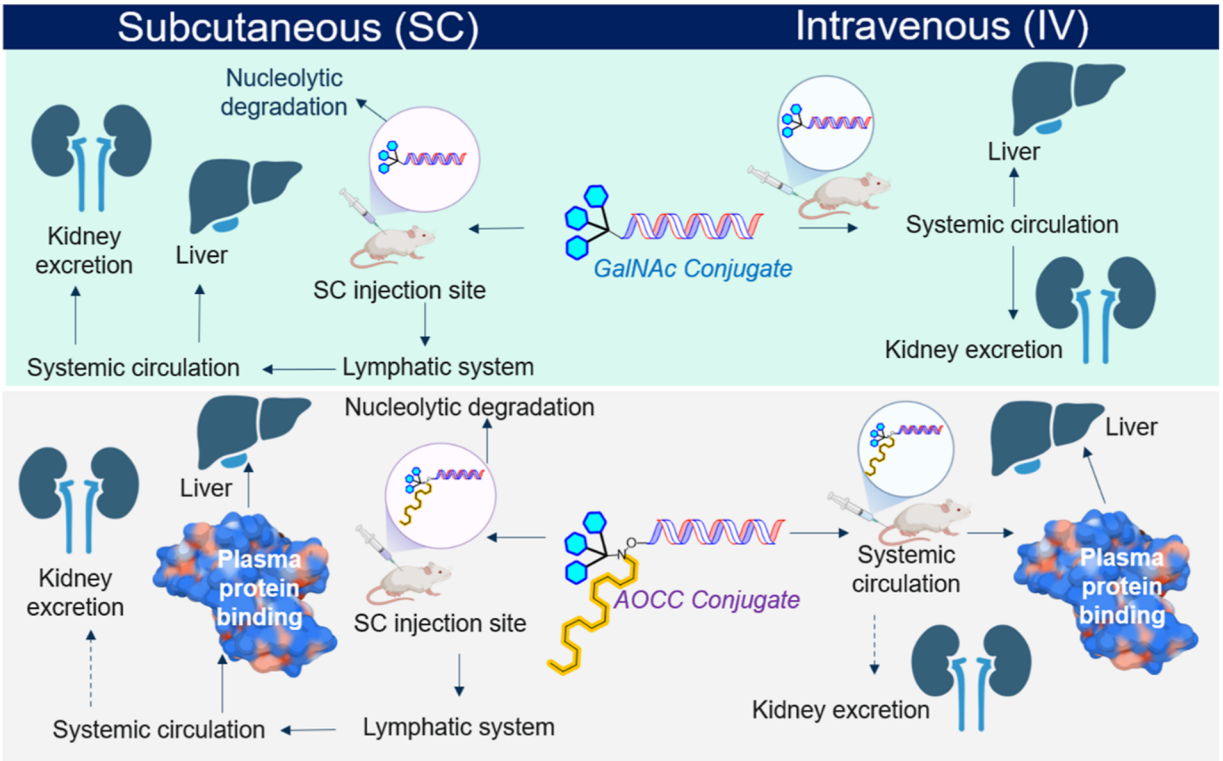


Figure 4. Pictorial representation of pathways for a conventional GalNAc conjugate and AOCC conjugates used in this study through both SC and IV routes of administration.

(3 days post dose) and rapidly returning to baseline. In contrast, **si-4** demonstrated both enhanced potency and durability under these conditions, producing ~60−70% ApoB reduction at day 7 and maintaining >40% knockdown at day 28 (Figure 2 and Table 3). These data indicate that incorporation of the C16 lipid substantially improves both the magnitude and persistence of RNAi activity, particularly under IV conditions where GalNAc-mediated uptake alone is less effective.

Metabolic Stability Improvement with AOCC Constructs

We next evaluated the metabolic stability of **si-3** and **si-4** in rat liver homogenate and rat liver tritosome. The duplexes were incubated in respective matrices for 24 h at 37 °C. Following incubation and cleanup, samples were analyzed by LC−MS for metabolite profiling. In rat liver homogenate, a significant difference in metabolic profiles between **si-3** and **si-4** was observed (Figure 3). Compared to the traditional 3′ GalNAc within **si-3,** which showed 17% 3′ n-1 antisense metabolism, no n-1 metabolite was observed for **si-4**. Only 4.5% and 3% detectable metabolites were observed for sense strand of **si-4** containing 5′ GalNAc/C16 AOCC conjugate while similar 5% and 3% metabolites were detected for sense strand of **si-3**. Also, additional endonuclease metabolites were identified in **si-3** for the antisense strand (15%, sum of 8% and 7%), which were absent for **si-4**. We hypothesize that protection of the 5′ sense strand with the GalNAc/C16 conjugate not only inhibits 5′ exonuclease activity but also hinders 3′ exonuclease activity toward the antisense strand due to enhanced steric interference in the duplex. One of the metabolites identified within **si-4** was minor exonuclease activity toward the 3′ sense strand (3%), likely resulting from the removal of the 3′-GalNAc conjugate. In the rat tritosome, the only major metabolite observed for 3′ GalNAc for **si-3** and 5′ GalNAc/C16 conjugate for **si-4** is the loss of the carbohydrate groups by glycosidase activity.[40] Results suggest that both duplexes were stable in the tritosome. These data suggest that the 5′ AOCC construct confers improved metabolic stability relative to the 3′ GalNAc control, although the relative contributions of 5′ conjugation per se and lipid-mediated pharmacokinetic enhancement cannot be fully disentangled from the present data.

DISCUSSION

In this work, we demonstrated postsynthetic, solution-phase, and solid-phase approaches for conjugation of two different ligands to the same residue of an oligonucleotide. Both postsynthetic methods proved effective for preparing bis-heteroconjugates. However, the solution-phase approach offers practical advantages for complex ligands because additional protecting group manipulations can often be avoided, thereby simplifying synthesis and purification. These methods avoid the necessity for complex building blocks and multiple protection−deprotection steps. We introduced the precursors of *O*-amino groups into the various positions of the oligonucleotide strands and performed stepwise ligand addition to prepare bis-heteroconjugates at the termini and internal positions of oligonucleotides. This postsynthetic approach broadens the scope of ligands that can be attached to oligonucleotides. Ibuprofen is one of the ligands we used that is known to bind strongly to human serum albumin (HSA) and has previously been employed as an albumin-binding moiety for oligonucleotides and other therapeutic cargos.[41,42] Such interactions can prolong systemic circulation and improve the pharmacokinetic properties through reversible albumin association. Accordingly, we included ibuprofen as a representative pharmacokinetic-enhancing ligand in the AOCC

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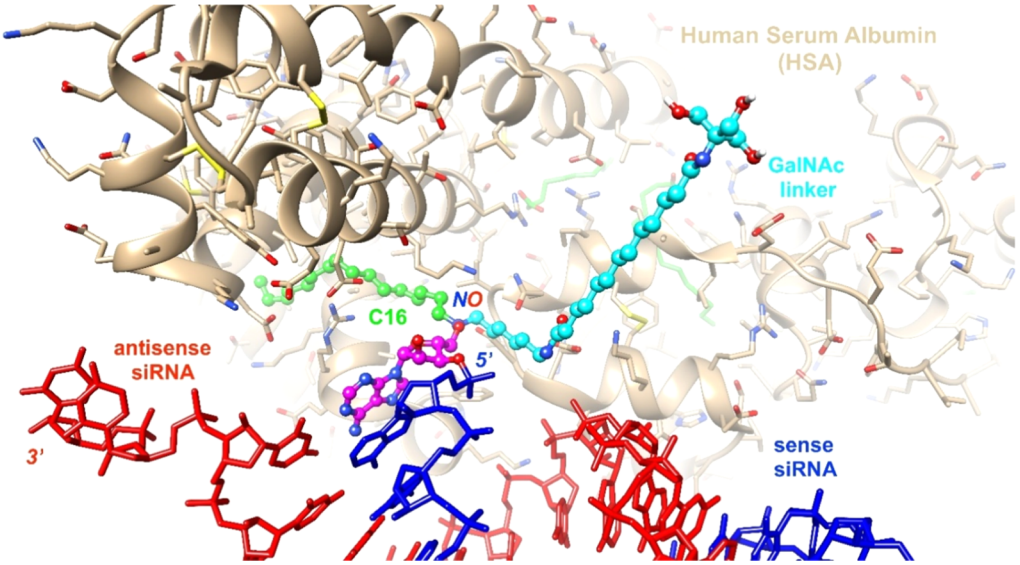


Figure 5. Computational model of an siRNA with a sense strand conjugated at the 5′ terminus to GalNAc and C16 lipid bound to HSA. The model is based on the crystal structure of HSA (shown in cartoon mode colored in tan) in complex with eight myristic acid molecules (green chains) (PDB ID 8RCP). The siRNA antisense and sense strands are colored in red and blue, respectively. To construct the model, the carboxyl group oxygen atoms of myristate #6 were removed, and the C14 chain was extended by two carbons near the surface before linking it to the *-N-O-* moiety of the 5′ terminal sense strand residue (carbon atoms colored in magenta). The C16 chain lodged in the HSA channel and the GalNAc conjugate are shown in ball-and-stick mode, with carbon atoms colored in green and cyan, respectively; only the portion up to the branch point for the triantennary GalNAc is shown for the latter. The overhanging dinucleotide at the 3′ end of the antisense strand (lower left of image) is rotated away to avoid a potential clash with the protein. The model of the complex was built using the UCSF Chimera program.[47]

platform to demonstrate the versatility of the conjugation strategy and its compatibility with diverse ligand classes.

Comparison of the silencing activities of siRNAs conjugated only to the targeting ligand GalNAc and *bis*-heteroconjugated to both GalNAc and the pharmacokinetic enhancer C16 revealed that the *bis*-heteroconjugate had improved efficacy and longer duration of silencing whether given subcutaneously or intravenously. We hypothesize that the lipid chain mediates binding to albumin, thereby creating a mini-reservoir for systemic circulation, resulting in the longer duration of action (Figure 4). In support of this model, we previously studied a spectrum of lipid conjugates and showed that lipid-mediated albumin binding improves biodistribution and, hence, the activity of siRNAs.[4,5] The enhanced duration of action of the *bis*-heteroconjugate after SC administration likely results because the lipid retains the oligonucleotide in circulation; in the absence of the lipid moiety, the hydrophilic oligonucleotides not taken up by the liver accumulate in kidneys and are excreted.

We acknowledge that the present study compares the bis-heteroconjugate (**si-4**) only to the 3′-GalNAc control (**si-3**) and does not include all possible comparator designs (e.g., 5′ lipid + 3′ GalNAc, 5′ GalNAc-only, or lipid-only constructs). The improved potency and durability observed for **si-4** may reflect the combined effects of 5′ conjugation, which protects the sense strand from exonuclease degradation, and the pharmacokinetic enhancement conferred by the C16 lipid through albumin binding.[4,5] Dissecting the individual contributions of these effects will require a dedicated cross-design pharmacokinetic study, which is beyond the scope of the present proof-of-concept work and is currently in progress.

Previous work has revealed considerable differences in how the body absorbs and processes GalNAc-conjugated siRNAs given intravenously and subcutaneously.[40,43,44] IV administration provides a higher immediate concentration in the blood and temporarily saturates liver receptors, causing poor uptake of the drug into the liver and rapid excretion through the kidneys. In contrast, SC administration results in gradual distribution into the bloodstream, resulting in lower, more stable drug levels in plasma.[44] This causes steady receptor-mediated uptake into liver hepatocytes and slow export to the kidney, and thus excretion levels are low.

The conjugation to both the hepatocyte-targeting GalNAc and a C16 lipid is advantageous after both IV and SC dosing. After IV administration, the lipid keeps the siRNA in circulation longer through its affinity for plasma proteins, slowing both liver receptor saturation and kidney-mediated elimination. Similarly, after SC administration, the lipid-binding to serum proteins results in a reservoir of the siRNA in the bloodstream, and steady release results in efficient silencing and long duration of action.

We have also observed improved metabolic stability of the AOCC constructs (Figure 3). To confirm that the *bis*-hetero conjugate we tested can bind to albumin, we constructed a computational model of an siRNA carrying a GalNAc and C16 at the 5′ end of the sense strand bound to HSA. The model is based on the previously reported complex of HSA with eight myristic acid molecules.[45,46] Binding of the lipid chain inside an HSA channel occurs without interference by the adjacent GalNAc conjugate or the 3′ dinucleotide overhang of the antisense strand (Figure 5).

■ CONCLUSIONS

In summary, we developed a postsynthetic AOCC strategy that enables efficient attachment of diverse ligands, including structurally complex molecules such as carbohydrates, peptides, lipids, and vitamins, to oligonucleotides. This approach permits the simultaneous incorporation of targeting ligands, pharmacokinetic enhancers, and diagnostic function-

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alities without the need for elaborate protecting group manipulations during oligonucleotide synthesis. *Bis*-hetero-conjugates were generated at both terminal and internal positions of oligonucleotides in high yield and purity, demonstrating the versatility of AOCC as a platform for the construction of multifunctional oligonucleotide conjugates.

Evaluation of a representative *bis*-hetero siRNA carrying both a GalNAc targeting ligand and a C16 lipid pharmacokinetic enhancer in mice following both SC and IV administration demonstrated improved *ApoB* silencing activity, enhanced durability of response, and improved metabolic stability relative to those of a GalNAc-only comparator. These findings support the concept that combining receptor-targeting and pharmacokinetic-enhancing ligands within a single conjugation site can improve the *in vivo* performance of therapeutic siRNAs.

To the best of our knowledge, this study represents the first *in vivo* demonstration of an AOCC-generated *bis*-hetero-conjugated siRNA. Together, these results establish post-synthetic AOCC as a versatile platform for the development of next-generation oligonucleotide therapeutics and provide a framework for incorporating additional functionalities designed to improve biodistribution, pharmacokinetics, endosomal escape, and extrahepatic delivery.

Recent computational and experimental studies[48] have further shown that *N,N,O*-trisubstituted hydroxylamines are thermodynamically and kinetically stable, are not unusually susceptible to oxidative metabolism relative to analogous amines and ethers, and should not be regarded as inherent structural liabilities in drug discovery. These observations provide additional support for the use of AOCC-derived chemistries in therapeutic oligonucleotide design.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.6c00339.

NMR spectra and characterization data for building blocks; HPLC chromatograms and mass spectra of oligonucleotide conjugates; detailed experimental procedures for oligonucleotide synthesis, conjugation, and purification; *in vivo* assay protocols; *in vitro* metabolism study protocols and LC−MS analyses; structures of oxime and *bis*-heteroaminooxy conjugates; and yields and characterization data for conjugates (PDF)

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Author Contributions

§D.D. and M.N.T. contributed equally.

Notes

All animal studies were conducted using protocols consistent with local, state, and federal regulations, as applicable, and were approved by the Institutional Animal Care and Use Committee (IACUC) at Alnylam Pharmaceuticals; no separate approval number was issued. Safety Statement: No unexpected or unusually high safety hazards were encountered during the course of this work.

The authors declare no competing financial interest.

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