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Multivalent *N*-Acetylgalactosamine-Conjugated siRNA Localizes in Hepatocytes and Elicits Robust RNAi-Mediated Gene Silencing

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Supporting Information

ABSTRACT: Conjugation of small interfering RNA (siRNA) to an asialoglycoprotein receptor ligand derived from *N*-acetylgalactosamine (GalNAc) facilitates targeted delivery of the siRNA to hepatocytes *in vitro* and *in vivo*. The ligands derived from GalNAc are compatible with solid-phase oligonucleotide synthesis and deprotection conditions, with synthesis yields comparable to those of standard oligonucleotides. Subcutaneous (SC) administration of siRNA−GalNAc conjugates resulted in robust RNAi-mediated gene silencing in liver. Refinement of the siRNA chemistry achieved a 5-fold improvement in efficacy over the parent design *in vivo* with a median effective dose (ED_{50}) of 1 mg/kg following a single dose. This enabled the SC administration of siRNA−GalNAc conjugates at therapeutically relevant doses and, importantly, at dose volumes of ≤1 mL. Chronic weekly dosing resulted in sustained dose-dependent gene silencing for over 9 months with no adverse effects in rodents. The optimally chemically modified siRNA−GalNAc conjugates are hepatotropic and long-acting and have the potential to treat a wide range of diseases involving liver-expressed genes.

Synthetic small interfering RNAs (siRNAs) can inhibit expression of disease-causing genes through post-transcriptional gene silencing mediated by the endogenous RNA interference (RNAi) pathway.[1,2] siRNAs have great therapeutic potential, but efficient delivery to target cells or organs remains a challenge. Some of the obstacles associated with *in vivo* delivery of siRNA have been overcome by using lipid nanoparticles (LNPs).[3] Recent clinical data showed that siRNAs formulated in LNPs inhibit expression of therapeutically relevant genes in humans.[4] Alternative approaches for *in vivo* delivery of siRNA include the use of targeted cationic cyclodextrin-containing polymer NPs[5] and charge-masked polycationic polymers.[6]

Covalent conjugation of small molecules to siRNA is an approach that may avoid side effects resulting from the use of nonviral vectors, particles, or excipient-based delivery systems. Conjugation of cholesterol and other lipophilic moieties to siRNAs results in broad biodistribution and gene silencing in multiple tissues, including liver.[7] To elicit favorable RNAi-mediated therapeutic effects, however, repeated high intravenous (IV) doses are required. For some therapeutic applications, it may not be advantageous or possible to deliver siRNAs intravenously; thus, the development of robust subcutaneously delivered siRNA conjugates is desirable. Conjugation of drugs to ligands of cell-surface receptors expressed only on certain cell types or ligands of receptors overexpressed on specific tissues as a result of certain disease conditions is a promising approach for targeted drug delivery.[8,9] Covalent conjugation of carbohydrates, peptides, and polyamines to oligonucleotides (ONs) for delivery and targeting of potential nucleic acid therapeutics has been reported.[10,11] Here we describe conjugation of siRNA to *N*-acetylgalactosamine (GalNAc), a highly efficient ligand for the asialoglycoprotein receptor (ASGPR). Upon subcutaneous (SC) administration, these siRNA−GalNAc conjugates robustly suppress gene expression of the targeted mRNA in liver.

The ASGPR, also known as the Ashwell−Morell receptor,[12] is expressed on hepatocytes and facilitates uptake and clearance of circulating glycoproteins with exposed terminal galactose and GalNAc glycans via clathrin-mediated endocytosis.[13] Multivalency and the presence of Ca^{2+} ions are prerequisites for proper recognition and binding of the ligands to the carbohydrate recognition domain of the receptor.[14,15] The binding affinity of ligands varies from micromolar to low-nanomolar and depends on the number and spatial orientation of the sugar moieties present.[16−18] Several novel ASGPR ligand mimics have also been reported recently,[19] and repression of hepatitis B virus RNA, protein, and DNA upon coinjection of cholesterol−siRNA and GalNAc−melittin-like peptide (GalNAc−MLP) conjugates in a transgenic mouse model has been demonstrated.[20] However, these two-component (cholesterol−siRNA and GalNAc−MLP) formulations were limited to IV administration.

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In the present work, we found that conjugation of optimized, chemically modified siRNAs to an engineered ASGPR ligand surprisingly resulted in conjugates with systemic stability against nucleases and improved pharmacokinetics relative to the unconjugated siRNAs. Moreover, these conjugates mediated robust and durable silencing of the targeted gene in the liver following single or multiple low-volume SC administrations.

Well-characterized bi- and triantennary GalNAc ligands[16,18,21] were reengineered to facilitate covalent conjugation to siRNAs.[22] Appropriately protected bi- and triantennary GalNAc monomers **1**−**3**, which are compatible with solid-phase ON synthesis and deprotection (SPSD) conditions, were synthesized (Figure 1). A

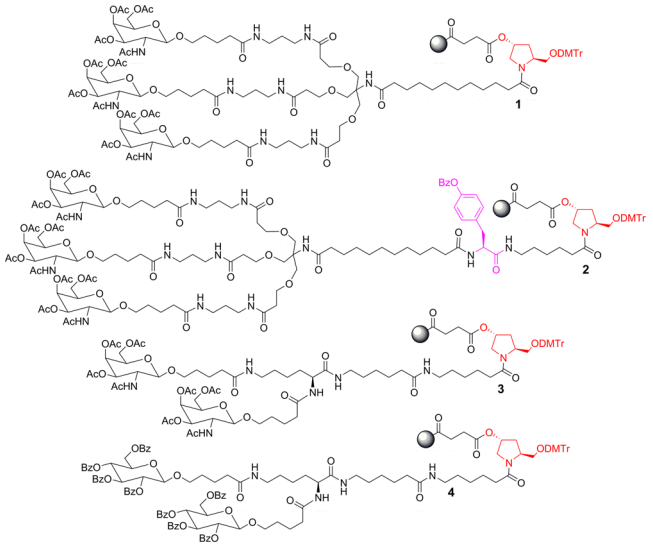

Figure 1. Triantennary (**1**, **2**) and biantennary (**3**) GalNAc and biantennary glucose (**4**) solid supports for conjugation to siRNA.

trans-4-hydroxyprolinol (*t*HP) moiety (Figure 1, red) was introduced in the design to enable site-specific conjugation of the ligand at any position of an ON during solid-phase synthesis. The triantennary GalNAc building block **1** was synthesized from D-(+)-galactosamine (**I**), 2-amino-2-(hydroxymethyl)-1,3-propanediol (**II**), *trans*-4-hydroxy-L-proline methyl ester (**III**), and a solid support **IV** (Scheme 1). The GalNAc solid support yielded the desired conjugated ONs after SPSD. In monomer **2**, a tyrosine moiety was introduced between the *t*HP linker and the triantennary ligand to enable ^{125}I radiolabeling[23] for evaluation of receptor−ligand interactions and uptake of the siRNA−GalNAc conjugate *in vivo*. The biantennary glucose monomer **4** was synthesized and conjugated to siRNA to demonstrate ligand specificity. Syntheses of **1**−**4** and their conjugation to siRNA are described in detail in the Supporting Information (SI).

siRNA **16** and ligand-conjugated siRNAs **17**−**22** (Table 1) were synthesized using standard SPSD conditions.[24] The 3′-triantennary GalNAc ($GalNAc_3$)-conjugated sense (S) strands **8**, **12**, and **13** were obtained by performing SPS on support **1** (Figure 1). The overall synthesis yield for the $GalNAc_3$ conjugate was comparable to those for standard ONs. The biantennary GalNAc ($GalNAc_2$) and glucose (Glc_2) S strands **6** and **7** were synthesized from the correponding solid supports **4** and **3**. S strand **10**, synthesized on solid support **2**, was annealed with antisense (AS) strand **11**, synthesized on solid support **49**, followed by ^{125}I radiolabeling of the resulting duplex[25] to obtain radiolabeled siRNA−GalNAc conjugate **20** (Scheme S2 in the SI). Since attempts to iodinate ONs containing phosphorothioate (PS) backbone modifications were not successful, the 3′-end of AS strand **11** devoid of PS linkages was modified with an end cap (E) to provide 3′-exonuclease protection. AS strand **9** was synthesized from solid support **44a** and Alexa Fluor 647 dye (section 3 in the SI). Annealing of equimolar amounts of S and the corresponding AS strands yielded siRNAs **16**−**22** (Table 1). The solubilities of the conjugates in water and aqueous buffers were comparable to those of the unmodified siRNAs.

Scheme 1. Synthesis and siRNA Conjugation of Monomer 1

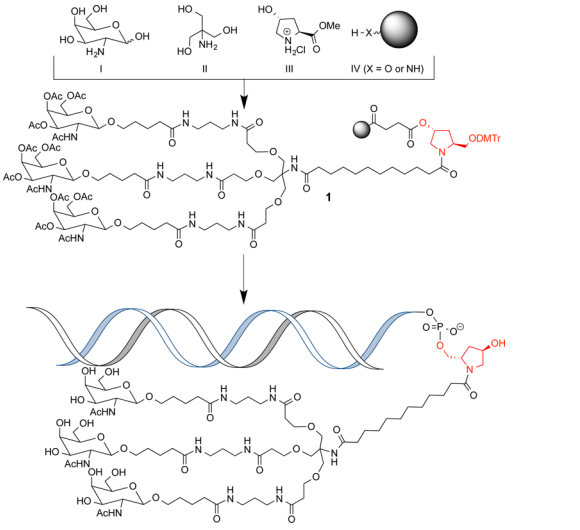

Table 1. siRNAs Used in This Study

siRNA	S/AS[a]	sequence (5′-3′)[b]	target[c]
16	**5/9**	GGAAUCuuAuAuuuGAUCcA•A/ uuGGAUcAAAuAuAAGAuUCc•c•U•F	*ApoB100*
17	**6/9**	GGAAUCuuAuAuuuGAUCcAA•(Glc_2)/ uuGGAUcAAAuAuAAGAuUCc•c•U•F	*ApoB100*
18	**7/9**	GGAAUCuuAuAuuuGAUCcAA•($GalNAc_2$)/ uuGGAUcAAAuAuAAGAuUCc•c•U•F	*ApoB100*
19	**8/9**	GGAAUCuuAuAuuuGAUCcAA•($GalNAc_3$)/ uuGGAUcAAAuAuAAGAuUCc•c•U•F	*ApoB100*
20	**10/11**	GGAAUCuuAuAuuuGAUCcAA(Tyr*-$GalNAc_3$)/ uuGGAUcAAAuAuAAGAuUCccU(E)	*ApoB100*
21	**12/14**	*AaCaGuGuUcUuGcUcUaUaA*($GalNAc_3$)/ uUaUaGaGcAaGaAcAcUgUu•U•u	*mTTR*
22	**13/15**	*A•a•CaGuGuUCUuGcUcUaUaA*($GalNAc_3$)/ u•U•aUaGaGcAagaAcAcUgUu•u•u	*mTTR*

[a]S and AS represent sense and antisense strands, respectively. [b]Uppercase, italicized upper-case, and lower-case letters indicate 2′-OH, 2′-deoxy-2′-fluoro, and 2′-*O*-methyl sugar modifications, respectively, to adenosine (A), cytidine (C), guanosine (G), and uridine (U). F, Alexa Fluor 647 fluorophore; (E), 3′-end-cap; (Glc_2), biantennary glucose; ($GalNAc_2$), biantennary GalNAc; ($GalNAc_3$), triantennary GalNAc; (Tyr*-$GalNAc_3$), ^{125}I-radiolabeled triantennary GalNAc (see Table S1 in the SI for details). • indicates a phosphorothioate (PS) linkage. [c]siRNAs target mRNAs encoding apolipoprotein B 100 (*ApoB100*)[7] and mouse transthyretin (*mTTR*).[4]

Uptake of the siRNA−GalNAc conjugates was evaluated in freshly isolated primary mouse hepatocytes (Figure 2).[26] After cells were incubated with 20 nM siRNA, robust uptake was observed with GalNAc-conjugated siRNAs **18** and **19**, in contrast to the unconjugated siRNA **16** and glucose-conjugated siRNA **17**, which showed little or no uptake. Uptake of **19** was reduced to background signal level in the presence of EGTA because of depletion of Ca^{2+}, which is crucial for binding of the ligand to the receptor.[14] Furthermore, preincubation of the receptor with

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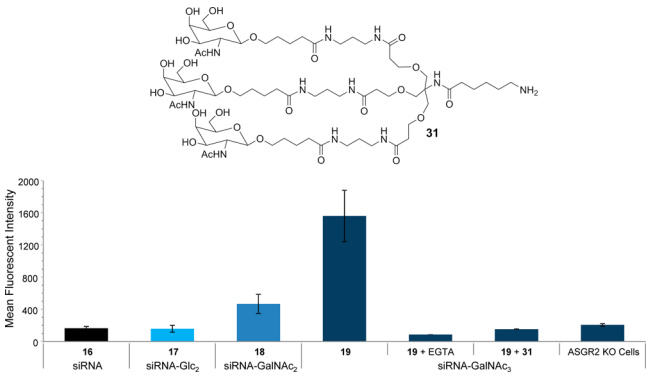

Figure 2. Uptake of conjugates into primary mouse hepatocytes. Freshly isolated hepatocytes from livers of wild-type and *ASGR2*-knockout mice were incubated with 20 nM siRNA. As controls, samples treated with **19** were also treated with EGTA or triantennary GalNAc monomer **31**. Uptake was determined by a fluorescence-based assay.[26] Error bars are standard deviations.

triantennary GalNAc ligand **31** (Figure 2 and Scheme S4) inhibited uptake of **19**, and incubation with *ASGR2*-knockout cells (which lack one of the two subunits of ASGPR) resulted in diminished uptake relative to wild-type cells. The uptake of siRNA–$GalNAc_3$ **19** was significantly higher than that of siRNA–$GalNAc_2$ **18**, indicating that at least *in vitro* the receptor binding affinity is correlated with the uptake efficiency. In addition, conjugation of the triantennary GalNAc to siRNA targeting the *ApoB100* gene[7] led to robust silencing *in vitro* in the absence of transfection agent.[26] These data show that uptake of GalNAc-conjugated siRNA into primary hepatocytes is mediated by specific binding of the GalNAc ligand to the ASGPR and depends on the receptor–ligand binding affinity.

Single and repeat IV dosing of radiolabeled siRNA–GalNAc conjugate **20** showed robust liver-specific uptake of the conjugate in mice. Predosing of animals with excess GalNAc monomer significantly inhibited uptake and resulted in higher serum levels than observed in animals that were not pretreated with GalNAc (Figure 3a,b). This is consistent with the *in vitro* uptake results

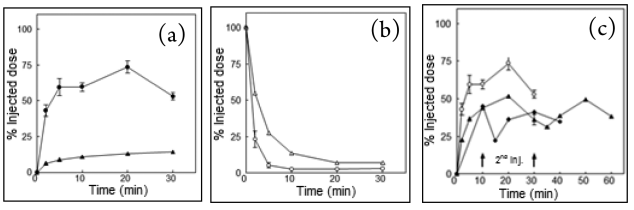

Figure 3. Uptake of ^{125}I-labeled siRNA–GalNAc conjugate **20** into livers of wild-type mice after IV administration. (a, b) % injected dose (ID) detected as a function of time after 0.5 mg/kg dose (a) in liver with (-▲-) and without (-●-) and (b) in serum with (-△-) and without (-○-) pretreatment with free ligand. (c) % ID in liver after a 0.5 mg/kg single dose (-○-) and after two consecutive 1 mg/kg doses at t = 0 and 10 min (-●-) or t = 0 and 30 min (-▲-). Error bars are standard deviations.

(Figure 2). To evaluate the ability of the receptor to engage in multiple rounds of siRNA–GalNAc uptake, mice were given two 1.0 mg/kg doses of **20** spaced either 10 or 30 min apart. Similar uptake kinetics were observed after the first and second doses of **20**, demonstrating efficient receptor recycling that enabled robust uptake of a second dose of the conjugate (Figure 3c). These findings are consistent with the short recycling time (10–15 min) reported for the ASGPR.[13]

To evaluate the impact of the mode of administration on *in vivo* uptake, radiolabeled siRNA–GalNAc conjugate **23** (SI section 6) was administered by IV and SC routes. Substantially higher levels of **23** were observed in liver with SC administration; details are summarized in SI section 7. The findings suggest that the slow release of the drug from the SC space increases plasma exposure, thereby extending the receptor–ligand interaction, which in turn enhances the uptake efficiency.

We next evaluated the ability of the siRNA–GalNAc conjugate to silence gene expression *in vivo* using conjugate **21**, designed to target the rodent *transthyretin* (*TTR*) gene. TTR is produced in liver and, when mutated, results in aberrant fibril formation and deposition in peripheral tissues, leading to neuropathy and/or cardiomyopathy.[4] Upon SC administration of a single 25 mg/kg dose to mice, >80% suppression of *TTR* mRNA in liver was observed 24 h post dose relative to animals treated with the PBS control (Figure 4a, light-blue bars). In contrast, IV administration of the same dose of the *TTR* siRNA–GalNAc conjugate resulted in only 15% inhibition of *TTR* mRNA expression.

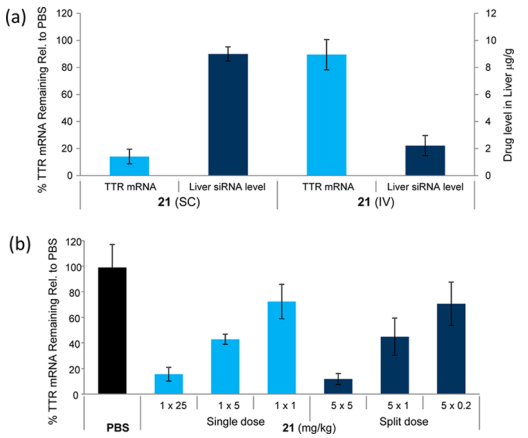

Figure 4. *TTR* gene silencing by conjugate **21** in wild-type C57BL/6 mice (n = 5). (a) % *mTTR* mRNA expression remaining (light blue, primary ordinate) and tissue levels of **21** in liver (dark blue, secondary ordinate) 24 h after a 25 mg/kg single dose (IV or SC). (b) % *TTR* mRNA expression compared with control 48 h after single doses of 25, 5, and 1 mg/kg (light blue) and after multidoses of 5 × 5, 5 × 1, and 5 × 0.2 mg/kg (dark blue) given SC once daily for 5 days. *TTR* mRNA levels in liver relative to GAPDH, depicted as percent of the PBS control, were measured 48 h after the final dose using a quantitative bDNA assay (Panomics). Error bars are standard errors for mRNA measurements and standard deviations for siRNA levels in liver.

The extent of silencing was correlated with higher siRNA levels in liver following SC compared with IV administration (Figure 4a, dark-blue bars), which is consistent with the *in vivo* uptake study using radiolabeled conjugate **20**. The level of **21** in mouse liver was quantified by a hybridization-based HPLC assay[27] using a fluorescently labeled ON probe complementary to the AS strand **14** of the siRNA.

To evaluate a potential dosing regimen that may optimally use the ASGPR for uptake, SC administration of **21** to mice in five consecutive doses of 0.2, 1, or 5 mg/kg was compared with a single cumulative dose of 1, 5, or 25 mg/kg, respectively. The two dosing regimens resulted in similar levels of mouse *TTR* gene suppression (Figure 4b), indicating that the receptor capacity was not exceeded, at least up to the highest dose tested.

With conjugate **21** as the basis, analysis of the metabolic stability and further refinement of the siRNA chemistry led to the

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design of conjugate **22** with additional PS linkages, which provide improved protection against 5′-exonucleases. Conjugate **22** elicited robust gene silencing in mice, with a single-dose median effective dose (ED_{50}) of ∼1 mg/kg, a 5-fold *in vivo* potency improvement over **21** (Figure 5a). As **21** and others in this class

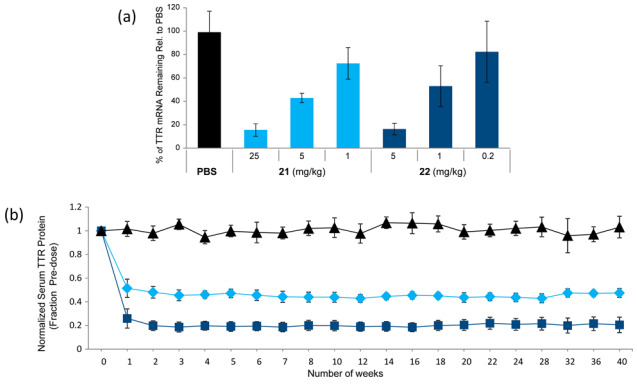

Figure 5. *mTTR* gene silencing by **21** and **22** and long-term pharmacologic effect of **22** in wild-type C57BL/6 mice. (a) SC single dose responses for **21** (light blue; 1, 5, and 25 mg/kg; *in vitro* free uptake IC_{50} = 1.39 nM) and **22** (dark blue; 0.2, 1, and 5 mg/kg; *in vitro* free uptake IC_{50} = 0.73 nM), n = 5. *TTR* mRNA levels are relative to the PBS group and are expressed as mean ± standard error. (b) Long-term reduction of TTR protein by **22** in wild-type mice during weekly SC dosing at 1 mg/kg (ED_{50}, -♦-, n = 19) and 2.5 mg/kg (ED_{80}, -■-, n = 17). Serum TTR protein levels from individual animals were normalized to their respective individual predose serum protein levels. Controls were treated with PBS (-▲-, n = 5). Blood samples were drawn prior to each dose for protein evaluation by ELISA. Data points are mean relative serum TTR protein concentrations; error bars are standard deviations.

are highly soluble in water (≥200 mg/mL), a 1 mg/kg dose in a 70 kg individual represents a clinically attractive injection volume of 0.35 mL. When **22** was administered chronically (SC, QW) over 280 days, sustained dose-dependent and long-lasting pharmacology, as measured by evaluation of serum TTR levels, was observed in mice (n = 17 and 19 for ED_{80} and ED_{50} dose groups, respectively) without evidence of tachyphylaxis or sensitization (Figure 5b), showing efficient, robust recycling of the receptor.

In conclusion, we have demonstrated that suitably protected synthetic ASGPR ligands derived from GalNAc are compatible with solid-phase oligonucleotide synthesis, thereby providing an efficient manufacturing process to enable rapid lead identification and optimization. Optimal design of multivalent GalNAc-conjugated siRNAs can elicit robust RNAi-mediated gene silencing in hepatocytes *in vitro* and *in vivo* without the aid of drug delivery agents. The delivery is mediated by specific binding of the GalNAc ligands to the ASGPR, and suitably stabilized siRNAs were found to inhibit target gene expression in mice with single-dose ED_{50} values of 1 mg/kg. Importantly, this delivery approach shows improved tissue-specific delivery and efficacy after SC dosing relative to IV administration. The dose-dependent sustained pharmacological effects without adverse effects from chronic dosing for over 9 months indicate the efficiency of the ASGPR for chronic SC treatment. Chemically modified siRNA−GalNAc conjugates represent a novel class of RNAi therapeutics with demonstrated preclinical efficacy *in vivo*. Further studies evaluating these RNAi-based agents in preclinical animal models and patients with numerous genetically defined disease targets are ongoing.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods, characterization and uptake data, and complete refs 3, 4, 6, 7, 19, 20, and 26. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): All Alnylam authors except M.R.A. are current employees with salary and stock options.

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