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

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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 (ED₅₀) 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. 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). Recent clinical data showed that siRNAs formulated in LNPs inhibit expression of therapeutically relevant genes in humans. Alternative approaches for in vivo delivery of siRNA include the use of targeted cationic cyclodextrin-containing polymer NPs and charge-masked polycationic polymers. 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. 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 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. Covalent conjugation of carbohydrates, peptides, and polyamines to oligonucleotides (ONs) for delivery and targeting of potential nucleic acid therapeutics has been reported. 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, is expressed on hepatocytes and facilitates uptake and clearance of circulating glycoproteins with exposed terminal galactose and GalNAc glycans via clathrin-mediated endocytosis. Multi-valency and the presence of Ca²⁺ ions are prerequisites for proper recognition and binding of the ligands to the carbohydrate recognition domain of the receptor. The binding affinity of ligands varies from micromolar to low-nanomolar and depends on the number and spatial orientation of the sugar moieties present. Several novel ASGPR ligand mimics have also been reported recently, 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. 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 were reengineered to facilitate covalent conjugation to siRNAs. Appropriately protected bi- and triantennary GalNAc monomers were synthesized and conjugated to siRNA to demonstrate ligand-receptor interactions and uptake of the siRNA conjugate in vivo. The biantennary glucose moiety was introduced between the synthesized from the corresponding solid supports triantennary GalNAc (GalNAc₃)-conjugated sense (S) strands and triantennary GalNAc (GalNAc₂) and glucose (Glc₂) S strands were synthesized using standard SPSD conditions. The 3′-end cap (E) to provide 3′-exo-nuclease protection. AS strand 9 was synthesized from solid support 44 and Alexa Fluor 647 dye (section 3 in the SI). Annealing of equimolar amounts of S and AS strands yielded siRNAs.

Table 1. siRNAs Used in This Study

<table>
<thead>
<tr>
<th>siRNA</th>
<th>S/AS</th>
<th>sequence (5′-3′)</th>
<th>target</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>5/9</td>
<td>GGAUCuaAaUugUACuAaA/ waGGAuACAAuAAaGAuACuAcU-F</td>
<td>ApoB100</td>
</tr>
<tr>
<td>17</td>
<td>6/9</td>
<td>GGAUCuaAaUugUACuAaGlc/ waGGAuACAAuAAaGAuACuAcU-F</td>
<td>ApoB100</td>
</tr>
<tr>
<td>18</td>
<td>7/9</td>
<td>GGAUCuaAaUugUACuAaGlc/GlcAcU/F</td>
<td>ApoB100</td>
</tr>
<tr>
<td>19</td>
<td>8/9</td>
<td>GGAUCuaAaUugUACuAaGlc/GlcAcU/F</td>
<td>ApoB100</td>
</tr>
<tr>
<td>20</td>
<td>10/11</td>
<td>GGAUCuaAaUugUACuAaGlc/ApoB100</td>
<td>ApoB100</td>
</tr>
<tr>
<td>21</td>
<td>12/14</td>
<td>AaCasGuaGiuCUCuGCuUAcUuAcA (GalNAc)</td>
<td>mTTR</td>
</tr>
<tr>
<td>22</td>
<td>13/15</td>
<td>AaCasGuaGiuCUCuGCuUAcUuAcA (GalNAc)</td>
<td>mTTR</td>
</tr>
</tbody>
</table>

*S and AS represent sense and antisense strands, respectively. **Upper-case, italicized upper-case, and lower-case letters indicate 2′-OH, 2′-deoxy-2′-fuo, 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), biantennary glucose; (GalNAc₁), biantennary GalNAc; (GalNAc₂), triantennary GalNAc; (Tyr*-GalNAc₁), ¹²⁵I-radiolabeled biantennary GalNAc (see Table S1 in the SI for details). ● indicates a phosphorothioate (PS) linkage. ○ indicates a triphosphothioate (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′-exo-nuclease protection. AS strand 9 was synthesized from solid support 44a and Alexa Fluor 647 dye (section 3 in the SI). The solubilities of the conjugates in water and aqueous buffers were comparable to those of the unmodified siRNAs. Uptake of the siRNA–GalNAc conjugates was evaluated in freshly isolated primary mouse hepatocytes (Figure 2). 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 Ca²⁺, which is crucial for binding of the ligand to the receptor. Furthermore, preincubation of the receptor with...
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. 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 2](image1.png)

**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. Error bars are standard deviations.

![Figure 3](image2.png)

**Figure 3.** Uptake of 125I-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 (▲) 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 4](image3.png)

**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 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
design of conjugate 22 with additional PS linkages, which provide improved protection against S′-exonucleases. Conjugate 22 elicited robust gene silencing in mice, with a single-dose median effective dose (ED$_{50}$) of $\sim$1 mg/kg, a 5-fold in vivo potency improvement over 21 (Figure 5a). As 21 and others in this class are highly soluble in water ($\geq$200 mg/mL), a 1 mg/kg dose in a 70 kg individual represents a clinically attractive injection volume of 0.35 mL. When 22 was administered chronically (SC, QW) over 280 days, sustained dose-dependent and long-lasting pharmacology, as measured by evaluation of serum TTR levels, was observed in mice ($n = 17$ and 19 for ED$_{50}$ and ED$_{10}$ 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.

### ACKNOWLEDGMENTS
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### REFERENCES


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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.