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Serial incorporation of a monovalent GalNAc phosphoramidite unit into hepatocyte-targeting antisense oligonucleotides

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ABSTRACT

The targeting of abundant hepatic asialoglycoprotein receptors (ASGPR) with trivalent *N*-acetylgalactosamine (GalNAc) is a reliable strategy for efficiently delivering antisense oligonucleotides (ASOs) to the liver. We here experimentally demonstrate the high systemic potential of the synthetically-accessible, phosphodiester-linked monovalent GalNAc unit when tethered to the 5′-terminus of well-characterised 2′,4′-bridged nucleic acid (also known as locked nucleic acid)-modified apolipoprotein B-targeting ASO via a bio-labile linker. Quantitative analysis of the hepatic disposition of the ASOs revealed that phosphodiester is preferable to phosphorothioate as an interunit linkage in terms of ASGPR binding of the GalNAc moiety, as well as the subcellular behavior of the ASO. The flexibility of this monomeric unit was demonstrated by attaching up to 5 GalNAc units in a serial manner and showing that knockdown activity improves as the number of GalNAc units increases. Our study suggests the structural requirements for efficient hepatocellular targeting using monovalent GalNAc and could contribute to a new molecular design for suitably modifying ASO.

1. Introduction

Several recent animal and clinical phase studies indicate that therapeutic antisense oligonucleotides (ASOs) can operate systemically without encapsulation if chemically modified to simultaneously exhibit higher binding affinity to the target transcripts and enhanced pharmacokinetics. In this context, we and others have unequivocally shown the benefits of utilizing conformationally constrained nucleotides such as 2′,4′-bridged nucleic acid (2′,4′-BNA) (also known as locked nucleic acid, LNA)[1,2] in combination with phosphorothioate (PS) chemistry. Our primary target to date has been severe dyslipidemia originating from the genetic dysregulation of lipids and cholesterol metabolism in the liver.[3–6] We have shown the strong knockdown activity of 2′,4′-BNA/LNA-modified ASOs against in vivo dyslipidemia models, compared to the conventional phosphorothioate ASOs. However, several quantitative studies revealed that less than 3% of an ASO dose is distributed to the target liver and much of this hepatic fraction is eliminated.[4,7] To ameliorate this situation, we have focused on developing novel bridged nucleic acid-type building blocks with hydrophobic chains to alter the distribution pattern of ASOs, but this strategy has met limited success.[8] Therefore, the pharmacokinetic and pharmacodynamic manipulation of high affinity and stable ASOs still represent a major challenge.

The direct or indirect attachment of liver-avid functional molecules (e.g., α-tocopherol and *N*-acetylgalactosamine (GalNAc)) onto ASOs helps improve target knockdown activity in the liver. Nishina et al. tethered α-tocopherol to 2′,4′-BNA/LNA-modified ASOs and found that the use of biodegradable linkers improved the potency of ASOs in murine liver.[9] Our group also recently reported that an ASO duplex with an α-tocopherol-conjugated complementary RNA markedly enhanced potency in vivo, presumably due to liberation of the parent ASO by intrinsic cellular mechanisms.[7] Separately, Prakash et al. showed that ASOs containing 3′-triantennary GalNAc enhanced hepatic knockdown 7-fold in mouse models.[10] Further synthetic and structural optimization by Østergaard et al. revealed that 5′-modification of GalNAc is more potent than modification of the 3′-congener, consistent with the observation that 5′-conjugated ASO is quickly metabolized, efficiently liberating the parent ASO. In general, the success of these bioconjugation strategies for ASO depends on tethering liver-targeting molecules using bio-labile linkers.

The trivalent GalNAc-mediated binding of ASOs to abundant hepatic asialoglycoprotein receptors (ASGPR) is a reliable strategy for improving ASO potency in the liver. However, the synthesis of GalNAc clusters is cumbersome, hampering use of the GalNAc

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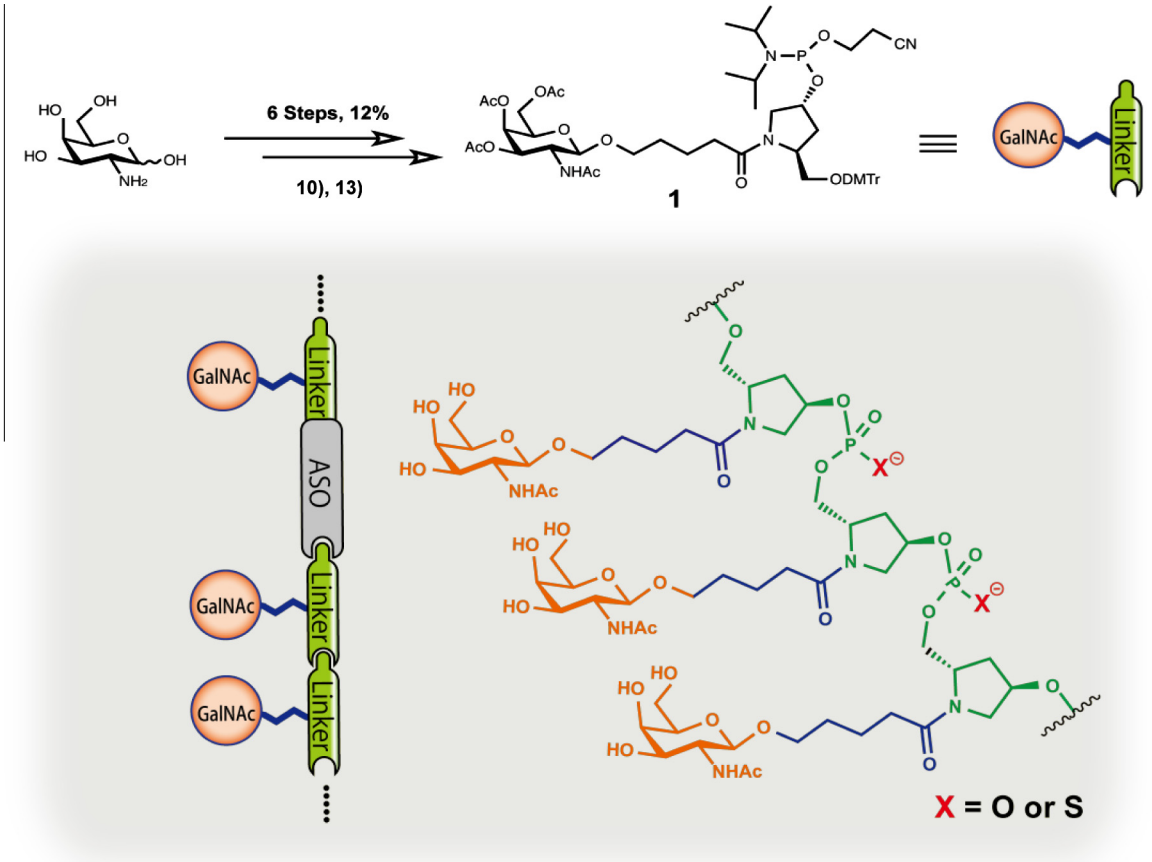

Figure 1. Design and synthesis of GalNAc-conjugated ASO. GalNAc phosphoramidite (compound **1**) was synthesized from *N*-acetylgalactosamine in 6 steps and an overall yield of 12%.[10,13] GalNAc-conjugated ASO is composed of one or several monovalent GalNAc sugar units (orange) attached by short tethers (blue) to ASO through a pyrrolidine linker (green). The potency of these monovalent GalNAc-conjugated ASOs could be altered by changing the number of units, their direction, and the linker structure.

cluster conjugation strategy. Several approaches to simplify their synthesis have been recently reported;[10–13] of particular interest is the elegant work of Rajeev et al. on a simplified GalNAc for siRNA (Fig. 1).[13] They constructed a triantennary GalNAc cluster by serial (1+1+1) incorporation of simplified monovalent GalNAc unit **1** using a standard solid-phase oligonucleotide synthesis approach. Surprisingly, this trivalent GalNAc enhanced both hepatic siRNA uptake and target knockdown in liver. We here described another application of the identical monovalent GalNAc phosphoramidite unit **1** for enhancing the activity of 2′,4′-BNA/LNA-modified ASOs in liver. The synthesis of monomeric GalNAc **1** is particularly fast and straightforward compared to the synthesis of a conventional three-in-one type GalNAc unit, allowing us to extensively explore approaches for tethering **1** to ASOs by altering the number of building blocks, direction (3′-end or 5′-end), and linker structure (PS or phosphodiester).

2. Results and discussion

2.1. Synthesis of monomeric GalNAc phosphoramidite unit 1

Our group designed and began synthesis of the simplified monovalent GalNAc unit **1**, but Rajeev et al. very recently independently reported the synthesis of the identical molecule.[13] Thus, our synthesis and structural determination of **1** was conducted according to the publication of several reports by other researchers (Supporting information).[10,13,14]

2.2. Design of ASOs with simplified GalNAc-conjugations to the 3′- or 5′-terminus and affinity comparison

Significant evidence of the superiority of the triantennary structure for GalNAc conjugation[15] prompted us to conjugate a trimer of **1** at the 3′-end of a well-characterized apolipoprotein B (apoB)-targeting ASO and explore whether this simplified GalNAc **1** improves ASO potency in the liver. Our initial choice of three linker structures (Table 1) was based on available information regarding bioconjugation. **GN1** is a fully phosphorothioated apoB-targeting ASO and should be the most bio-stable of the three (**GN1**–**GN3**). **GN2** has a single bio-labile phosphodiester site between the parent ASO and the GalNAc cluster, allowing the cluster to be liberated. **GN3** was designed as the most bio-labile of the three linkers, in which all three GalNAc–GalNAc and 2′,4′-BNA/LNA-GalNAc internucleotide linkages are a native phosphodiester linkage. Three 5′-versions of GalNAc-terminated ASOs (**GN4**–**6**) were also designed for comparison. Accessibility to the flexible elongation of the simplified monomeric GalNAc **1** on ASO is a clear advantage and was maximized by synthesizing the monomer and pentamer of GalNAc **1** tethered to the 5′-end with phosphodiester bonds (**GN7** and **GN8**).

The target mRNA affinities of these compounds were estimated from the melting temperature (T_m) of the complexes. The attached units may slightly reduce affinity, but all the GalNAc-conjugated ASOs maintained binding affinity toward complementary RNA (T_m = 57–60 °C) comparable to **ApoB**, a parent ASO (T_m = 61 °C) (Table 1).

Table 1
Sequences and T_m values of GalNAc-conjugated or -unconjugated ASOs used in this study

ID	Sequence (5′–3′)	T_m (°C)
ApoB	G^C^a^t^t^g^g^t^a^t^T^C^A	61
GN1	G^C^a^t^t^g^g^t^a^t^T^C^A^R^R^R	59
GN2	G^C^a^t^t^g^g^t^a^t^T^C^AR^R^R	59
GN3	G^C^a^t^t^g^g^t^a^t^T^C^ARRR	60
GN4	R^R^R^G^C^a^t^t^g^g^t^a^t^T^C^A	57
GN5	R^R^RG^C^a^t^t^g^g^t^a^t^T^C^A	57
GN6	RRRG^C^a^t^t^g^g^t^a^t^T^C^A	57
GN7	RG^C^a^t^t^g^g^t^a^t^T^C^A	60
GN8	RRRRRG^C^a^t^t^g^g^t^a^t^T^C^A	58

Conditions: for a duplex formation with targeted RNA, 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl. The sequence of target; 5′-CACUGAAUACCAAUGCUGAA-3′. Upper and lower case indicate 2′,4′-BNA/LNA and nature DNA, respectively. R represents monovalent GalNAc unit and ^ indicates a phosphorothioate (PS) linkage. T_m values were determined by averaging three measurements, which were accurate to within 1 °C.

2.3. Enhanced in vivo efficacy of phosphodiester-linked simplified GalNAc-conjugated ASO

A superior tethering strategy for the simplified GalNAc conjugation was identified by first comparing the in vivo knockdown activities of **GN1**, **GN2** and **GN3**. Normal chow-fed C57Bl/6J mice (8-weeks old, male, $N = 4$ per group) were subcutaneously injected with **ApoB**, **GN1**, **GN2** or **GN3** at a dose of 35, 70 or 140 nmol/kg and the expression level of apoB mRNA in the liver was analyzed 72 h post-injection. As shown in Figure 2a, the knockdown activities of **GN1** and **GN2** were similar to or lower than that of the parent **ApoB**-ASO, whereas **GN3** exhibited significantly improved in vivo potency. In addition, serum total cholesterol levels were consistent with the hepatic mRNA levels of **GN1**, **GN2** and **GN3**, supporting their systemic activity (Fig. 2b). These results indicate that the simplified monovalent GalNAc **1** can strongly activate ASO in liver provided that three **1**s are tethered to ASO in a serial manner with bio-labile linkers.

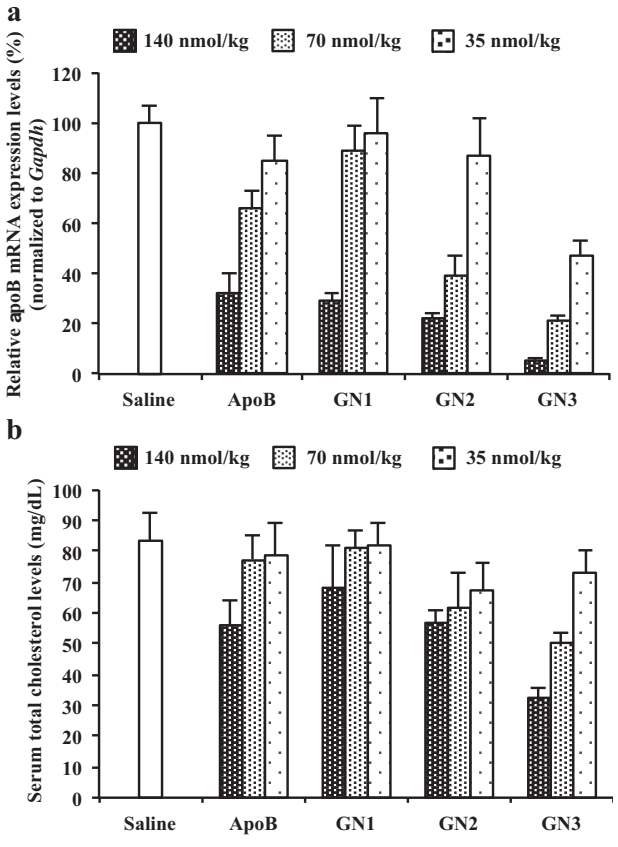

Figure 2. Efficacy of GalNAc-conjugated or unconjugated ASOs. (a) Quantitative RT-PCR analysis of apolipoprotein B (apoB) mRNA levels normalized to *Gapdh* mRNA levels following subcutaneous injection of **ApoB**, **GN1**, **GN2** or **GN3** (35, 70 or 140 nmol/kg). (b) Decreased levels of serum total cholesterol after subcutaneous injection of ASOs. Sera were harvested 3 days after the injection. Data shown are presented as mean values + SD, $N = 4$.

2.4. Enhanced effect of 5′-conjugation over 3′-conjugation

Studies using a conventional triantennary GalNAc with a single branching point[11] demonstrated that a different attachment direction (3′-end or 5′-end) of the monovalent GalNAc **1** can affect activity. The in vivo efficacies of **GN4**–**6** were compared to determine in which direction **1** should be elongated and how the three **1**s should be linked to each other. **ApoB**-ASO, **GN4**, **GN5** and **GN6** were singly administered to male mice at doses of 35–140 nmol/kg. On day 3, the livers were harvested and apoB expression was measured, and blood was collected and total cholesterol was analyzed. GalNAc-conjugated ASOs with bio-labile phosphodiester linkers (**GN5** and **GN6**) surprisingly enhanced apoB mRNA degradation (Fig. 3a). **GN6** was the most potent of the six ASOs (**GN1**-**GN6**) and provided 93% knockdown even at a dose of 35 nmol/kg (~0.2 mg/kg). In contrast, the 5′-trimer linked using PS chemistry, represented by **GN4**, did not significantly enhance the potency of the parent ASO. Serum total cholesterol levels were consistent with the mRNA levels (Fig. 3b). The advantage of 5′-GalNAc over 3′-congeners has been reported for a conventional 3-in-1 type GalNAc, and a possible mechanism underlying the superiority of 5′-conjugation with phosphodiester bonds suggests that their potential metabolic susceptibility affects their fast liberation kinetics.[11]

2.5. Comparison of hepatic distribution of GalNAc-conjugated ASOs

The activity differences between **GN1**, **GN2** and **GN3** and **GN4**, **GN5** and **GN6** could be due to the fact that a specific and/or non-specific interaction between ASOs and endogenous substances increases as the number of PS linkages increases, which may interfere with a specific interaction of GalNAc with ASGPR.[16] The inhibitory effect of PS linkages on the receptor binding of GalNAc was estimated by fixing the amount of intact ASO accumulated in the liver using a previously described ELISA method.[5] As shown in Figure 3c, GalNAc conjugation of **GN5** and **GN6** provides an ~4-fold activation of hepatic uptake of ASO, but only a 2.5-fold increase using fully phosphorothioated **GN4**, indicating that PS can interfere with receptor binding. Calculations showed that 3% of the **ApoB** and 13% of the **GN6** dose was distributed in the liver. **GN5** and **GN6** were similarly distributed in liver, but **GN5** was less potent than **GN6**, indicating that efficient GalNAc must be liberated after the GalNAc-terminated ASO is taken up through the ASGPR pathway. Further information regarding the precise subcellular dynamics of GalNAc-terminated ASOs is required to optimize the linker chemistry.

2.6. Onset of hepatotoxicity

The hepatotoxicity of chemically-modified ASOs is a major concern regarding ASOs in clinical development[17–20] but its underlying mechanisms are unknown. We hypothesized that the above-mentioned large influx of GalNAc-conjugated ASOs in the liver may affect the toxicology of ASO; consequently, serum liver transaminases (alanine-aminotransferase, ALT and aspartate-aminotransferase, AST) were measured as biomarkers for acute

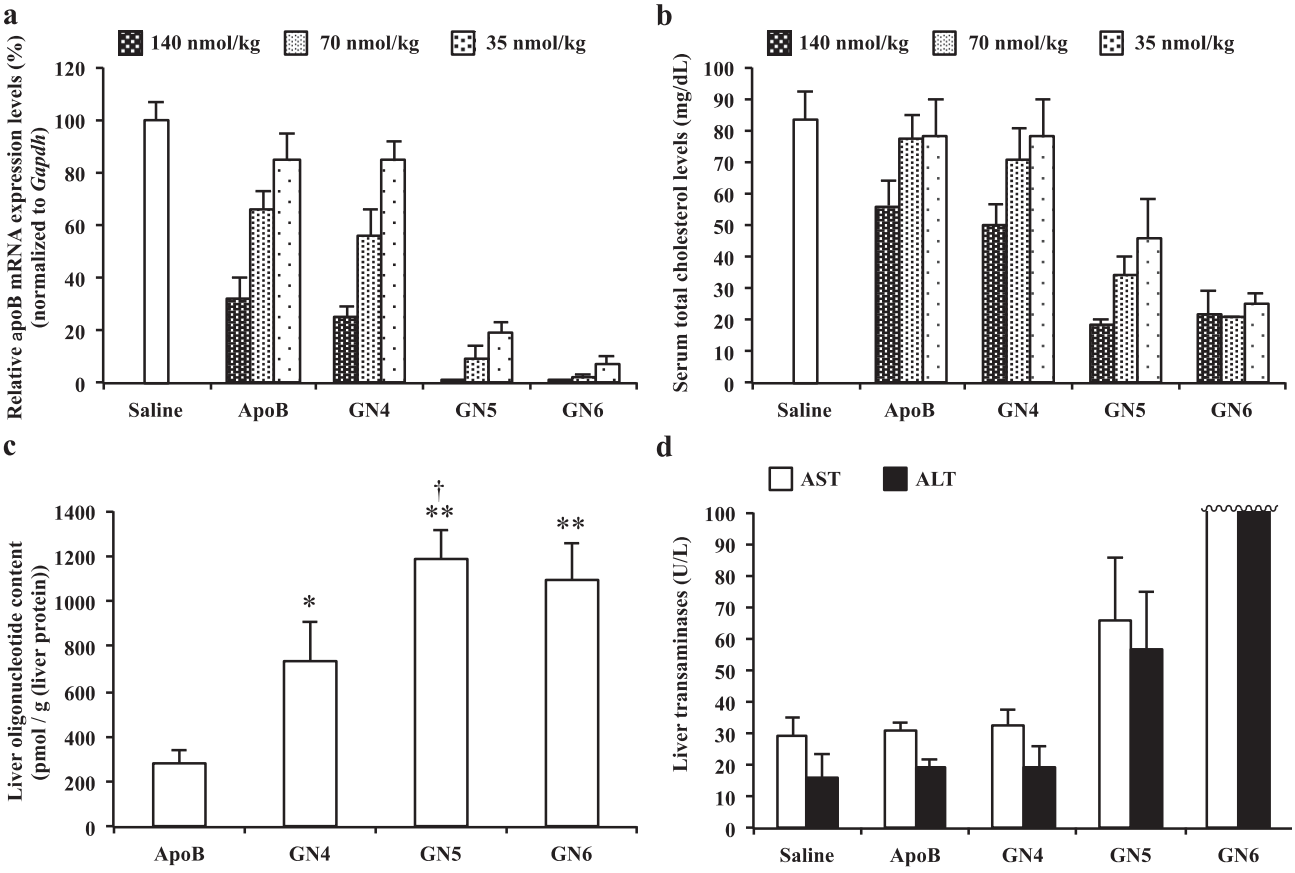

Figure 3. Effects of GalNAc-conjugated ASOs. (a) Dose-dependent reduction of the gene by GalNAc-conjugated ASOs. Quantitative RT-PCR analysis of apolipoprotein B (apoB) mRNA levels normalized to *Gapdh* mRNA levels 3 days after subcutaneous injection of **ApoB**, **GN4**, **GN5** or **GN6** (140, 70 or 35 nmol/kg), $N = 4$. (b) Decreased levels of serum total cholesterol after subcutaneous injection of ASOs, $N = 4$. (c) Concentration of GalNAc-conjugated or unconjugated ASOs in liver 3 days after subcutaneous injection of 140 nmol/kg of one of the ASOs ($N = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs **ApoB**, $^{\dagger}P < 0.05$ vs **GN4**). (d) Serum liver transaminases (alanine-aminotransferase, ALT and aspartate-aminotransferase, AST) levels. The transaminase levels of **GN6** represent >1000 U/L. Data shown are presented as mean values + SD.

hepatocellular injury after injection of the GalNAc-conjugated ASOs into mice. Transaminases were in the normal range for **ApoB**-ASO and **GN4**, but were elevated moderately for **GN5** and severely for **GN6** at a dose of 140 nmol/kg (Fig. 3d). As mentioned above, the hepatic ASO levels of **GN5** and **GN6** were comparable, but the knockdown activity of **GN6** overwhelmed that of **GN5**, despite their similar composition. This indicates that one risk factor for hepatotoxicity is which organelle in a hepatocyte ASOs migrate to, and not how much ASO is distributed to the liver as a whole. Indeed, cytosolic or subnuclear overexposure to ASO may increase both hybridization-dependent and -independent off-target effects, which are now more critical factors for ASO-dependent hepatotoxicity than they used to be.[21,22] Our observations may contribute to a new approach to the molecular design of compounds for inhibiting the onset of hepatotoxicity.

2.7. Preferred number of simplified GalNAc monomers for serial conjugation

We next screened the preferred number of monovalent GalNAc units for serial conjugation in terms of efficient hepatocellular delivery and knockdown activity. **GN6**, **GN7** and **GN8** were administered to mice as described above and knockdown activity in the liver was evaluated 3 days post-injection. As shown in Figure 4a, even a single incorporation of **1** (**GN7**) demonstrated greatly improved activity over the parent compound. We also showed facile accessibility to serial elongation up to the pentamer, and systematically evaluated the structure–activity relationship of this family of compounds in terms of GalNAc-conjugation to ASO. Specifically, the results show that knockdown activity is positively correlated with the number of modifications; for example, pentameric **GN8** was more potent than trivalent **GN6**, although trivalent GalNAc is well suited as a putative trimeric ASGP receptor.[23] As we expected, serum total cholesterol levels were fully consistent with their mRNA levels (Fig. 4b). Figure 4c shows the low dose (17.5 nmol/kg) **GN6** was distributed in liver with quite similar efficiency compared to higher dose (140 nmol/kg) **GN6** shown in Figure 3c. More interestingly, when compared under the same dosage, the liver ASO content was shown to keep increasing without saturation up to at least pentamer and no toxicity was observed (Fig. 4c and d). Simplified GalNAc **1** provides opportunities to identify more efficient ways of targeting the reliable ASGPR uptake pathway.

3. Conclusion

In conclusion, we experimentally demonstrated the high hepatocellular delivery potential of the synthetically-accessible monovalent GalNAc **1** at the 5′-end of ASO and revealed that the conjugation of this GalNAc unit with bio-labile phosphodiester linkers are preferable to have the great potency of ASO. We also here showed the flexibility of this monomeric unit by attaching a serial number of this unit and concluded that the gene-silencing effects are positively correlated to the number of GalNAc. Our study could contribute to unclear the structural requirement for efficient hepatocellular targeting using monovalent GalNAc **1** and

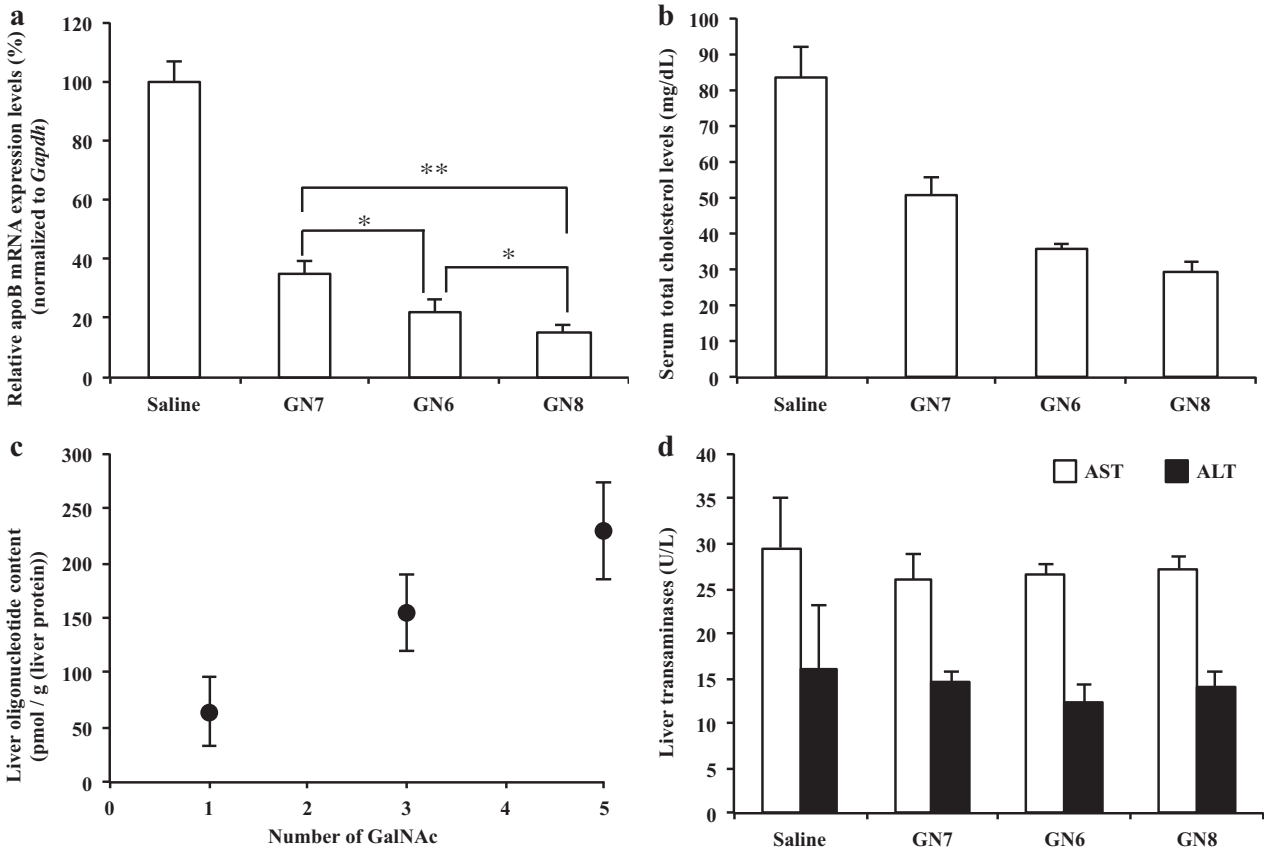

Figure 4. Effects of a serial number of GalNAc unit-conjugated ASOs. (a) Quantitative RT-PCR analysis of apolipoprotein B (apoB) mRNA levels normalized to *Gapdh* mRNA levels following subcutaneous injection of **GN7**, **GN6** or **GN8** at a dose of 17.5 nmol/kg. These GalNAc-conjugated ASOs differ in the number of monovalent GalNAc units. Data represent mean values + SD, $N = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$. The differences between **GN7**, **GN6** and **GN8** versus the saline group ($P < 0.01$) are statistically significant. (b) Decreased levels of serum total cholesterol after subcutaneous injection of ASOs. (c) Concentration of GalNAc conjugated ASOs in liver 3 days after subcutaneous injection of 17.5 nmol/kg each ASOs. Data represent mean values ± SD, $N = 4$ (d) Serum liver transaminases (alanine-aminotransferase, ALT and aspartate-aminotransferase, AST) levels.

may also shed light on the mechanisms of the onset of hepatotoxicity.

4. Experimental

4.1. UV melting experiments

UV melting experiments were performed on a SHIMADZU UV-1800 spectrometer (SHIMADZU, Kyoto, Japan) equipped with a T_m analysis accessory. Two single-stranded oligonucleotides were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to give a 2.0 μM oligonucleotide solution. The mixture was annealed by heating at 90 °C and then slowly cooled to room temperature. The T_m was calculated as the temperature at which the formed duplexes were half-dissociated. The melting profile was recorded at 260 nm and the scan rate was 0.5 °C/min from 5 to 90 °C. The final values were determined by averaging three independent measurements.

4.2. Design and synthesis of ASOs

Variants of 2′,4′-BNA/LNA-antisense oligonucleotides designed to target apoB mRNA were synthesized by Gene Design Inc (Osaka, Japan). ASOs were designed with complementary target sites for both mouse and human apoB mRNA sequences and have been reported previously.[4] The syntheses were carried out by standard phosphoramidite procedures and purified using HPLC. The composition and purity of each compound was analyzed by MALDI-TOF mass spectrometry.

4.3. Mouse study

All animal procedures were performed with the consent of the Animal Care Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan). C57Bl/6J mice were purchased from SLC Japan (Tokyo, Japan). All mice were male, and studies were initiated when the animals were 8 weeks old. The mice were kept on a 12-hour light/dark cycle, with free access to food and water. The mice were fed a normal chow (CE-2, CLEA Japan, Tokyo, Japan) for one week prior to the experiments. ASOs were administered to the mice subcutaneously at a dose range of 17.5–140 nmol/kg/injection. At the time of sacrifice 72 h after the single administration, the mice were anesthetized with Isoflurane (Escain®, Pfizer Japan, Tokyo, Japan) and the livers and kidneys were harvested. Liver tissues were stored in RNA*later*® (Life Technologies) or snap-frozen in liquid nitrogen. Peripheral blood was collected in BD microtainer® tubes (BD, Franklin Lakes, NJ) for separation of serum.

4.4. Quantitative real-time PCR

Total RNA was extracted from mouse liver tissues by using 500 μL of Lysis Buffer (LRT, wako Japan, RT-S2). Gene expression was evaluated by a quantitative reverse transcription-PCR method. RNA samples were performed by using a High Capacity cDNA

Reverse-Transcription Kit (Applied Biosystems, Foster City, CA), and quantitative PCR was performed by using Taqman Gene Expression Assays (Applied Biosystems). The apoB mRNA levels were normalized to the *Gapdh* mRNA levels. The probes for *apoB* (Mm01545156_m1_FAM) and *Gapdh* (Mm99999915_g1_VIC) genes were obtained from Applied Biosystems.

4.5. ASO concentration in liver

The hepatic concentration of ASOs was determined using a previously described ELISA method.[5] The template was 22-mer DNA complementary to the ASOs with biotin at the 3′-end. The ligation probe DNA was a 9-mer DNA with phosphate at the 5′-end and digoxigenin at the 3′-end. Both DNA compounds were purchased from Gene Design (Osaka, Japan). Nunc immobilizer streptavidin F96 black plates were purchased from Thermo Fisher Scientific. The template DNA solution (100 nM) and the ligation probe DNA solution (200 nM) contained 3.5 units/well T4 DNA ligase (TaKaRa, Shiga, Japan) with 66 mM Tris–HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, and 0.1 mM ATP. The washing buffer used throughout the assay contained 25 mM Tris–HCl (pH 7.2), 0.15 M NaCl, and 1.0% Tween 20. Anti-digoxigenin-AP Fab fragments were obtained from Roche Diagnostics. A 1:2000 dilution of the antibody with 1:10 super block buffer in TBS containing 0.1% Tween 20 (TBST) was used in the assay. The alkaline phosphatase luminous substrate was prepared using 150 μL/well AttoPhos Substrate (Promega, Madison, WI).

4.6. Assay procedures

Frozen liver tissue was placed in a 2 mL tube containing 100 μL of RIPA buffer (Sigma–Aldrich, St Louis, MO) in 400 μL PBS and a zirconia ball (ø 5 mm; Irie, Tokyo, Japan) and mechanically homogenized for 2 min at 30 oscillations per second using a TissueLyser II apparatus (Qiagen, Haan, Germany). Total protein concentrations were measured with a detergent compatible assay kit (Bio-Rad, Hercules, CA). Hybridization-ELISA assay was performed at the concentration range of 128 pM–1000 nM in duplicate. More than six standard solutions were prepared for generating the standard curve for this assay. Liver homogenates prepared from ASO-untreated mice were added to ApoB-ASO or each GalNAc-ASO solutions to generate standard samples over the concentration range 128 pM–1000 nM. The template DNA and probe DNA solution (100 μL) and standard solution (10 μL) or liver homogenates (10 μL) containing the ASOs were added to Nunc immobilizer streptavidin F96 black plates and incubated at 37 °C for 1.5 h to allow the binding of biotin to the streptavidin-coated wells and hybridization. After hybridization, each well was washed three times with 200 μL of washing buffer, then 200 μL of a 1:2000 dilution of anti-digoxigenin-AP was added and the plate was incubated at 37 °C for 1.5 h. After washing four times with the washing buffer, AttoPhos solution was added to the plate and after 15 min, the luminescence intensity was determined using a SpectraMax M2e microplate reader (Molecular Devices, Japan). The linear range of 128–1000 pM in this ELISA system was determined as $r > 0.98$.

4.7. Serum chemistry

Blood was collected from the inferior vena cava following sacrifice and centrifuged for 20 min at 2300×*g* to obtain the serum. Serum levels of total cholesterol and two hepatic toxicity biomarkers, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were measured using a DRI-CHEM 7000 chemistry analyzer (Fujifilm Tokyo, Japan).

4.8. Statistics

The data are reported as means + SD or ±SD (Fig. 4c). All statistical analyses were performed using the Holm test to assess statistical significance between pairs of groups in the ELISA assay (Fig. 3c) and qRT-PCR assay (Fig. 4a).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.11.036.

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