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Conjugation of mono and di-GalNAc sugars enhances the potency of antisense oligonucleotides via ASGR mediated delivery to hepatocytes

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ABSTRACT

Antisense oligonucleotides (ASOs) conjugated to trivalent GalNAc ligands show 10-fold enhanced potency for suppressing gene targets expressed in hepatocytes. Trivalent GalNAc is a high affinity ligand for the asialoglycoprotein receptor (ASGR)—a C-type lectin expressed almost exclusively on hepatocytes in the liver. In this communication, we show that conjugation of two and even one GalNAc sugar to single stranded chemically modified ASOs can enhance potency 5–10 fold in mice. Evaluation of the mono- and di-GalNAc ASO conjugates in an ASGR binding assay suggested that chemical features of the ASO enhance binding to the receptor and provide a rationale for the enhanced potency.

Antisense oligonucleotides bind their cognate mRNA in cells by Watson–Crick base pairing. Upon binding, they modulate function of the mRNA via one or more antisense mechanisms to produce a pharmacological effect.[1] Second generation ASOs are phosphorothioate (PS) backbone modified with a central gap region of 7–14 DNA nucleotides flanked on either end with 2′-modified nucleotides.[2] The DNA gap region promotes cleavage of the targeted mRNA by RNase H1 mediated hydrolysis,[3] while the 2′-modified nucleotides enhance ASO affinity for cognate RNA.[4] We recently showed that the potency of second generation ASOs for suppressing gene targets expressed in hepatocytes can be enhanced 10-fold by targeted delivery via the Asialoglycoprotein receptor (ASGR).[5,6]

The ASGR is a C-type lectin which is abundantly expressed on hepatocytes of all mammals and regulates levels of plasma glycoproteins terminating with sialic acid α2,6 galactose and *N*-acetyl galactosamine (GalNAc) sugars.[7,8] The functional receptor in mammals is comprised of two subunits (ASGR1 and ASGR2) which form a hetero-oligomeric complex with varying ratios (2–5:1).[9] The ASGR is internalized via clathrin mediated endocytosis in coated pits on the basolateral membrane of hepatocytes.[10] Upon internalization, the ligand–receptor complex is transported to endo-lysosomal compartments.[11] Acidification of endosomal compartments promotes dissociation of the ligand–receptor complex. The receptor is recycled back to the plasma membrane while the cargo is sorted to lysosomes for degradation.

Mice lacking the ASGR2 subunit are viable and fertile but express the ASGR1 subunit at reduced levels.[12] Cells expressing ASGR1 alone are capable of binding ligand but substantial binding is dependent on the level of expression of the protein.[13] In contrast, mice lacking the ASGR1 subunit do not bind ligand and do not express ASGR2 on the plasma membrane.[14] The ASGR1 possesses the carbohydrate recognition domain (CRD) for calcium mediated sugar binding[15] and the cytoplasmic signal for binding clathrin adaptor proteins within coated pits.[16]

Elegant work by Lee showed that synthetic glycosides with branched tethers bind the ASGR with high affinity.[17,18] Binding affinity was dependent on the nature of the sugar (GalNAc > galactose), number of sugars (4 = 3 > 2 > 1) and the geometrical spacing between the sugar moieties.[19] Given the complexity of ASGR binding and trafficking pathways, we wished to determine optimal valency of GalNAc sugars required for efficient ASGR-mediated delivery of ASOs to hepatocytes. In this manuscript, we show that second generation ASOs of different configurations can be effectively delivered to hepatocytes in mice using two and even a single GalNAc sugar. Our results suggest that the chemical features of

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second generation ASOs enhance interactions of the sugar ligand with the ASGR which facilitates delivery to hepatocytes.

For the current study, we prepared GalNAc-ASO conjugates with three (GN3), two (GN2) and one (GN1) sugars using a lysine-based scaffold similar to that described by Valentijn for preparation or tri-antennary GalNAc clusters (Fig. 1).[20] Briefly, the syntheses commenced with the mono-GalNAc structure derived from N^{α}-acetyl-N^{ε}-Boc-lysine (**1**), the di-GalNAc compound derived from N^{α}-Boc-N^{ε}-Boc-L-lysine (**10**) and the tri-GalNAc cluster derived from the HBTU facilitated condensation of compound **10** with N^{ε}-Boc-L-lysine methyl ester (**17**) (Scheme 1). The methyl ester of **17** was saponified to prepare the free acid **18**. At which point, compounds **1**, **10** and **18**, were subjected to analogous synthetic routes. Uronium mediated coupling of the lysine derivatives to benzyl 6-aminohexanoate afforded compounds **3**, **11**, and **19**. Subsequent acid treatment provided the mono, di and tri-amine structures **6**, **12** and **20**. GalNAc hexanoic acid (**4**)[21] as well as the corresponding pentafluorophenyl (Pfp) ester (**5**) were utilized to prepare **7**, **13** and **21** according to Scheme 1. The benzyl esters were removed via catalytic hydrogenation and the Pfp esters were prepared utilizing PFPTFA to synthesize the mono, di and tri-GalNAc clusters **9**, **15** and **23**.[22]

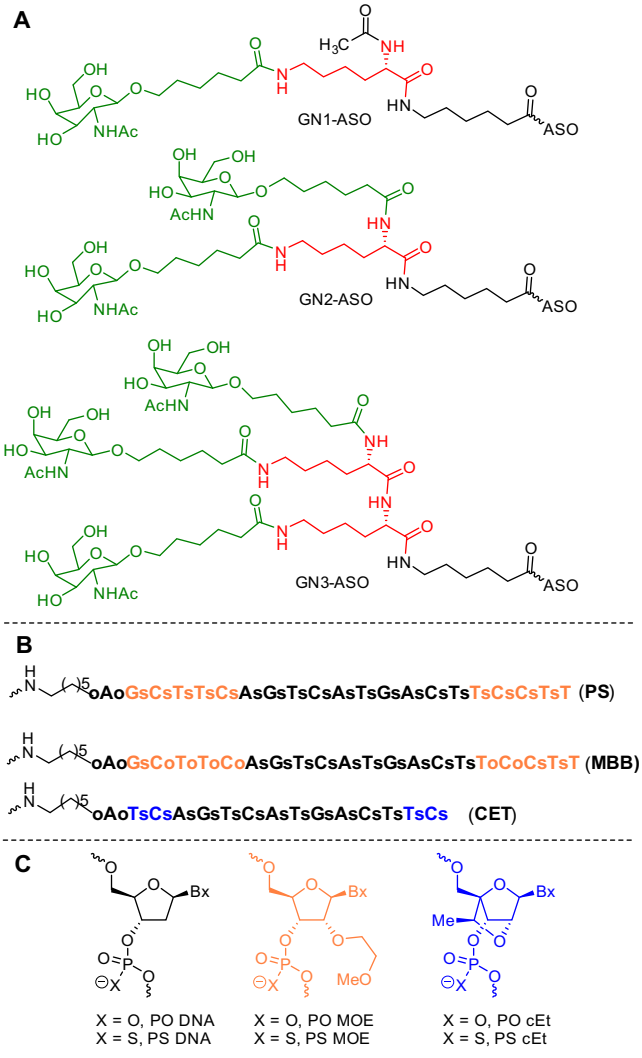
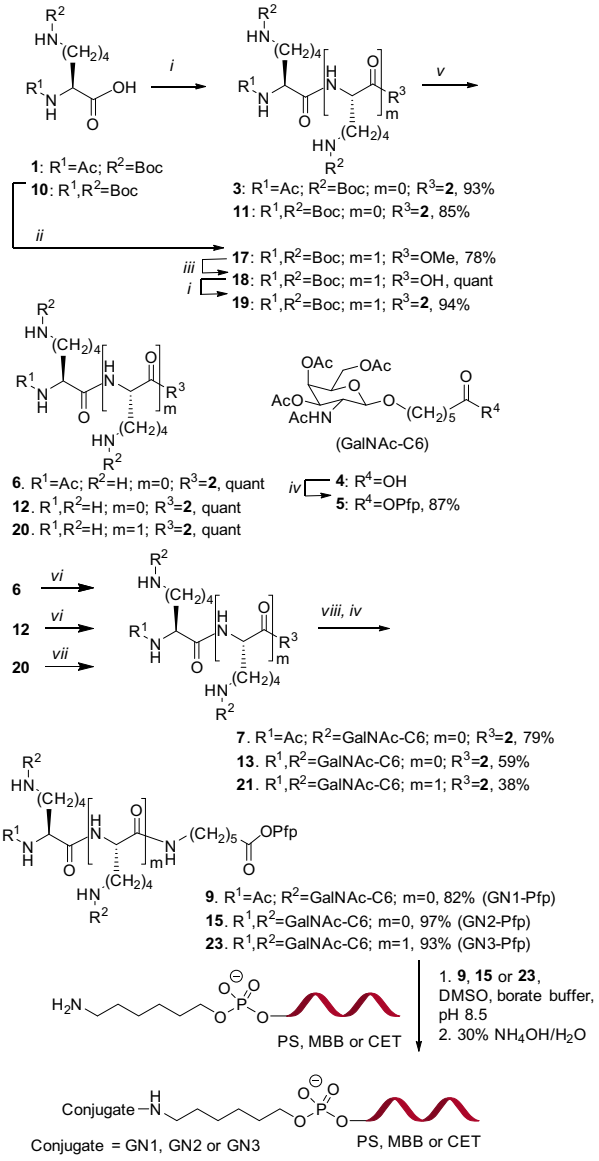


Figure 1. (A) Structures of mono-, di- and tri-GalNAc conjugated ASOs (B) sequence and backbone composition of ASOs and (C) structures of nucleoside modifications used in ASOs.



Scheme 1. Reagents and conditions: (i) Benzyl 6-aminohexanoate (**2**), HBTU, iPr$_2$EtN, DMF; (ii) H-Lys(Boc)-OMe, HBTU, Et$_3$N, DMF; (iii) LiOH, MeOH, THF, H$_2$O, quant; (iv) PFPTFA, iPr$_2$EtN, CH$_2$Cl$_2$; (v) 50%TFA/CH$_2$Cl$_2$; (vi) **5**, iPr$_2$EtN, DMF; (vii) **4**, HATU, iPr$_2$EtN, DMF; (viii) H$_2$, Pd/C, MeOH.

The pentafluorophenyl activated peracetylated GalNAc clusters **9**, **15** and **23** were conjugated to 5′-aminohexyl-modified ASOs followed by hydrolysis of the acetate protecting groups to provide the conjugates represented by GN3-ASO, GN2-ASO and GN1-ASO, respectively (Scheme 1). Three distinct ASO designs targeting the scavenger receptor B-1 mRNA (SRB-1) were employed for the initial evaluation (Fig. 1).[23] These included a fully phosphorothioate (PS) modified 5-10-5 MOE gapmer (**PS0**) and its mixed-backbone (**MBB**) variant where six PS linkages in the flanks were replaced with natural phosphodiester (PO) linkages. MBB ASOs show reduced non-specific binding to intra and extra-cellular proteins and GalNAc conjugation enhances potency of these designs relative to their full PS parents.[6] We also investigated a truncated version of ASO **PS0** where the MOE nucleotides in the wings were replaced with higher affinity constrained Ethyl (cEt) nucleotides (**CET0**).[24] Shorter cEt ASOs show enhanced potency in animal models relative to their longer 5-10-5 MOE counterparts.[5,23] Presumably, the reduced charge and molecular weight facilitates escape of shorter ASOs from endosomal compartments.