Lipid nanoparticles incorporating a GalNAc ligand enable in vivo liver *ANGPTL3* editing in wild-type and somatic *LDLR* knockout non-human primates

Lisa N. Kasiewicz[1], Souvik Biswas[1], Aaron Beach[1], Huilan Ren[1], Chaitali Dutta[1], Anne Marie Mazzola[1], Ellen Rohde[1], Alexandra Chadwick[1], Christopher Cheng[1], Kiran Musunuru[2], Sekar Kathiresan[1], Padma Malyala[1], Kallanthottathil G. Rajeev[1], and Andrew M. Bellinger[1]*

** Denotes corresponding author*
[1]Verve Therapeutics, 500 Technology Square, Suite 901, Cambridge, MA, 02139, USA.
[2]Division of Cardiovascular Medicine, Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA.
Corresponding author email: abellinger@vervetx.com

Abstract

Standard lipid nanoparticles (LNPs) deliver gene editing cargoes to hepatocytes through receptor-mediated uptake via the low-density lipoprotein receptor (LDLR). Homozygous familial hypercholesterolemia (HoFH) is a morbid genetic disease characterized by complete or near-complete LDLR deficiency, markedly elevated blood low-density lipoprotein cholesterol (LDL-C) levels, and premature atherosclerotic cardiovascular disease. In order to enable in vivo liver gene editing in HoFH patients, we developed a novel LNP delivery technology that incorporates a targeting ligand—*N*-acetylgalactosamine (GalNAc)—which binds to the asialoglycoprotein receptor (ASGPR). In a cynomolgus monkey (Macaca fascicularis) non-human primate (NHP) model of HoFH created by somatic knockout of the *LDLR* gene via CRISPR-Cas9, treatment with GalNAc-LNPs formulated with an adenine base editor mRNA and a guide RNA (gRNA) targeting the *ANGPTL3* gene yielded ~60% whole-liver editing and ~94% reduction of blood ANGPTL3 protein levels, whereas standard LNPs yielded minimal editing. Moreover, in wild-type NHPs, the editing achieved by GalNAc-LNPs compared favorably to that achieved by standard LNPs, suggesting that GalNAc-LNP delivery technology may prove useful across a range of in vivo therapeutic applications targeting the liver.

Delivery of nucleic acid therapeutics to the liver via LNPs relies on LDLR-mediated uptake into hepatocytes.[1,2] HoFH is a genetic disease characterized by complete or near-complete absence of LDLR and, therefore, LNP treatment necessitates the use of alternative receptor pathways besides LDLR (Fig. 1). One such pathway is ASGPR, a receptor system almost entirely expressed on the surfaces of hepatocytes.[3] Previous delivery technologies taking advantage of this receptor system include ASGPR-specific ligand conjugated antisense oligonucleotides[4,5], siRNAs[6], and GalNAc-LNPs constituted with siRNA[1,7,8,9,10].

The work presented here expands ASGPR-mediated delivery to other nucleic acid therapeutic modalities, specifically to liver delivery of base editors[11]. We describe the development of GalNAc-LNP technology comprising a novel, potent GalNAc ligand and a scalable formulation process that together yield robust base editing in two NHP models—*LDLR* somatic knockout (KO) and wild-type (WT).

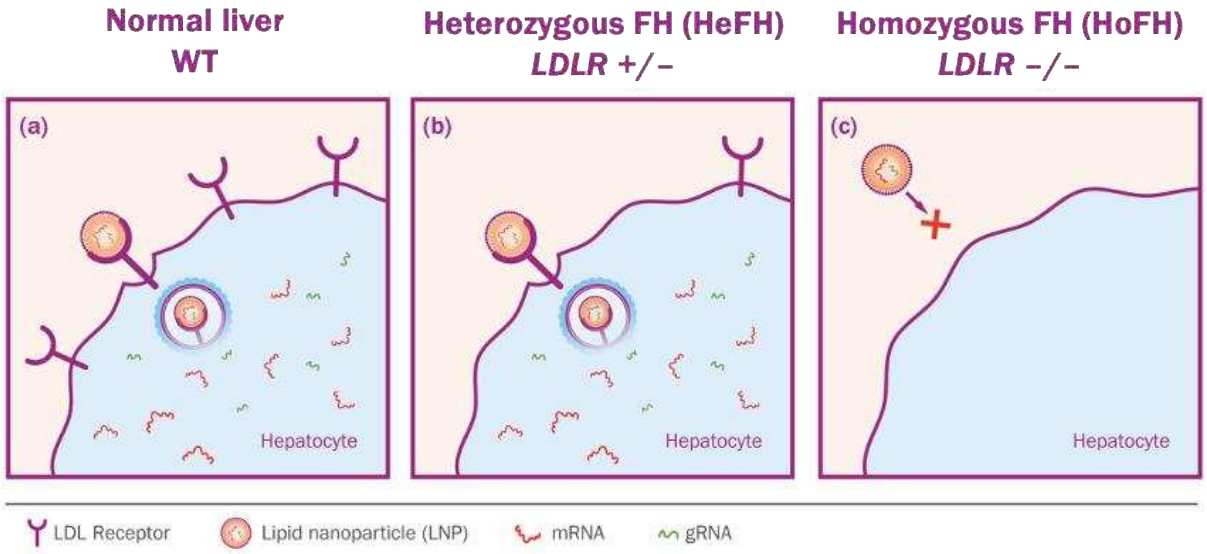

FIGURE 1: Depicts LNP uptake into hepatocytes with (a) normal LDLR expression, (b) partial deficiency as in heterozygous FH (*LDLR* +/–) and (c) complete or near-complete deficiency as may occur in homozygous FH (*LDLR* –/–). The LDLR on wild-type and *LDLR* +/– hepatocytes facilitates efficient LNP uptake into the liver, whereas the lack of LDLR on *LDLR* –/– hepatocytes stymies uptake of a standard LNP.

In order to enable in vivo liver base editing in the LDLR-deficient HoFH population, we designed two novel GalNAc-based ligands (Fig. 2a) and conjugated them to a series of lipid anchors (select structures shown in Fig. S1).[12] The lysine-based trivalent ligand (Design 2) outperformed the TRIS-based ligand (Design 1) when targeting *Ldlr* –/– mice (Fig 2b, Fig. S2, Table S1). We then undertook structure-based rational design, varying the spacer length between the trivalent ligand moiety and the lipid chain, as well as incorporating three different lipid anchors (Fig. S1). We formulated GalNAc-LNPs with an adenine base editor 8.8-m (ABE8.8) mRNA and a gRNA targeting the mouse *Angptl3* gene, screened for in vivo editing activity in *Ldlr* –/– mice (Fig. S3, Table S1), and identified GalNAc-Lipid GL6 as a high performing ASGPR-targeting moiety (Fig. 2c).

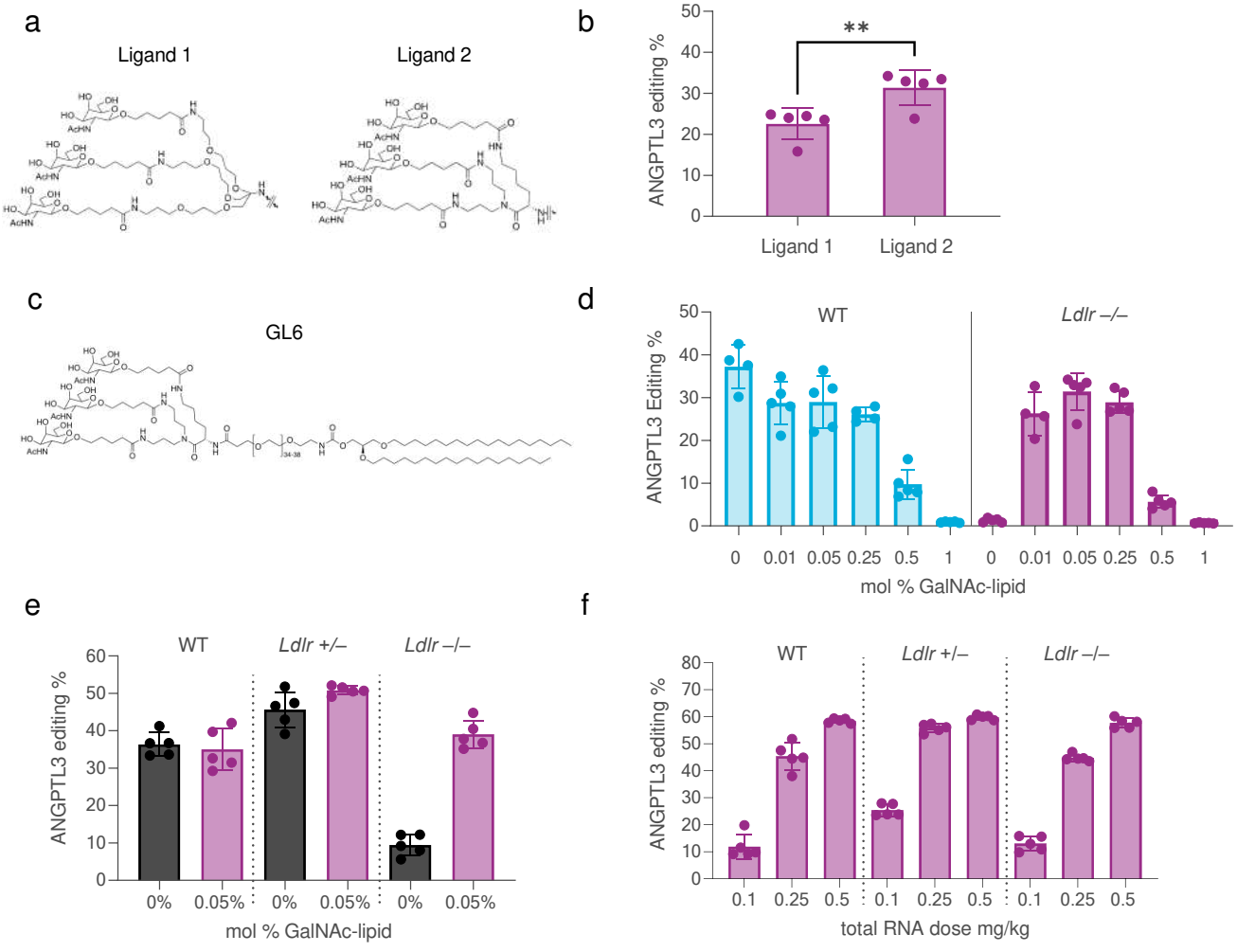

FIGURE 2: GalNAc-lipid optimization in LDLR-deficient mouse model. (a) ASGPR ligand designs 1 and 2; (b) in vivo performance in *Ldlr* –/– mice of ligand designs 1 and 2 at 0.1 mg/kg, where the PEG spacer and lipid chain were kept the same; (c) GalNAc-lipid GL6 comprising a PEG spacer and 1,2-di-*O*-octadecyl-*sn*-glyceryl lipid anchor; (d) titration of the surface density of GalNAc ligand demonstrated a low density near 0.05 mol % of GalNAc-lipid to optimally rescue liver editing in *Ldlr* –/– mice while preserving editing in WT mice at a low overall RNA dose of 0.1 mg/kg; (e) LNPs constituted with 0.05 mol % GL6 achieved rescue of *Angptl3* editing in the liver and reduction of blood ANGPTL3 protein levels in *Ldlr* –/– mice at 0.25 mg/kg; (f) demonstration of near-identical dose response of liver *Angptl3* editing using the optimized GalNAc-LNPs (constituted with 0.05 mol % GL6) in three genotypes: WT, *Ldlr* +/–, and *Lldr* –/–. Error bars represent standard deviations (s.d.), and individual data points for each animal are displayed as symbols within the bar. $n = 5$ for all panels.

Next, we developed and evaluated various options for incorporating GalNAc-lipid into the LNP during the manufacturing process. Several strategies of post-addition incorporation of GalNAc-lipid following LNP formulation resulted in a non-uniform distribution of GalNAc-lipid in the drug product based on a lectin binding assay (Fig. S4). As an alternative, we observed that mixing the GalNAc-lipid with other lipid excipients prior to LNP particle formation generated stable particles which produced similar efficacy in mice (Fig. S5) and allowed for efficient scale-up to larger batch sizes.

It has been unclear what concentration of GalNAc-lipid decorating an LNP surface would be optimal for in vivo liver delivery. LNPs targeting the *Angptl3* gene were prepared with increasing mol % of GalNAc-lipid and assessed in WT and *Ldlr* –/– mice (Fig. 2d). As little as 0.01 mol % GalNAc-lipid substantially rescued editing in *Ldlr* –/– mice without affecting Z-average size, payload entrapment, or editing performance in WT animals (Table S1). Efficacy in both mouse models remained high until about 0.3 mol % GalNAc-lipid of the total lipid content, with 0.05 mol % producing the highest editing. We confirmed the rescue of *Angptl3* editing activity of GalNAc-LNPs with 0.05 mol % GalNAc-lipid in multiple experiments across WT, *Ldlr* +/–, and *Ldlr* –/– mice (Fig. 2e, Table S1). The corresponding reductions in blood ANGPTL3 protein levels (Fig. S6) correlated well with the observed editing percentages. Dose-

response comparisons in WT, *Ldlr* +/–, and *Ldlr* –/– mice demonstrated that there were near equivalent potencies in all three genotypes (Fig. 2f, Fig. S6B, Table S1) and that a 0.25 mg/kg total RNA dose with 0.05 mol % GalNAc-lipid rescued editing in *Ldlr* –/– mice and maintained editing as effectively as standard LNPs in WT and *Ldlr* +/– mice. Notably, these data indicate our ASGPR ligands may be 10-fold more potent than any GalNAc-LNP reported previously.[1,7]

Next, in order to evaluate the potency of GalNAc-LNPs in a species which might better predict human translation, we created a model of HoFH in NHPs (Fig. 3a). Two gRNAs targeting different locations on the *LDLR* gene were co-formulated with *Streptococcus pyogenes* Cas9 mRNA in a standard LNP formulation and intravenously administered to WT NHPs at a 2 mg/kg dose. With dual gRNAs targeting nearby sites in the *LDLR* gene, Cas9-mediated high-efficiency deletion of approximately 34 base pairs in the *LDLR* gene in the liver (Fig. 3b). This editing effectively knocked out liver LDLR expression (Fig. 3c) and conversely increased blood LDL-C levels about 6-fold on average (Fig. 3d), thus generating a model of HoFH in NHPs.

We next tested both standard LNPs and GalNAc-LNPs for the ability to deliver gene editing cargo in this HoFH NHP model. When *LDLR* KO NHPs were treated with standard LNPs (not formulated with GalNAc-lipid) (Table S2) at a dose of 2 mg/kg, minimal editing occurred at the target site in the *ANGPTL3* gene (Fig. 3e) and, thus, there was little reduction in blood ANGPTL3 protein levels (Fig. 3f). In contrast, treatment with GalNAc-LNPs (Table S2) completely rescued therapeutic efficacy in *LDLR* KO NHPs. At a 2 mg/kg dose, we observed 60% whole-liver editing of the *ANGPTL3* gene (Fig. 3e), leading to >90% lowering of blood ANGPTL3 protein (Fig. 3f), ~55% lowering of blood triglycerides (data not shown), and ~35% lowering of blood LDL-C (Fig. 3g) that in absolute terms was ~100 mg/dL of LDL-C reduction in the HoFH NHP model.

We assessed whether GalNAc-LNPs are also effective in WT NHPs in which LDLR is present. In WT NHPs, we compared GalNAc-LNPs versus standard LNPs, with each carrying ABE8.8 mRNA and a gRNA targeting *ANGPTL3*. GalNAc-LNPs achieved editing that lowered blood ANGPTL3 protein by 90%, whereas standard LNPs achieved a 75% reduction of blood ANGPTL3 (Fig. 3h). These data suggest that GalNAc-LNPs are effective across a range of genotypes including WT animals.

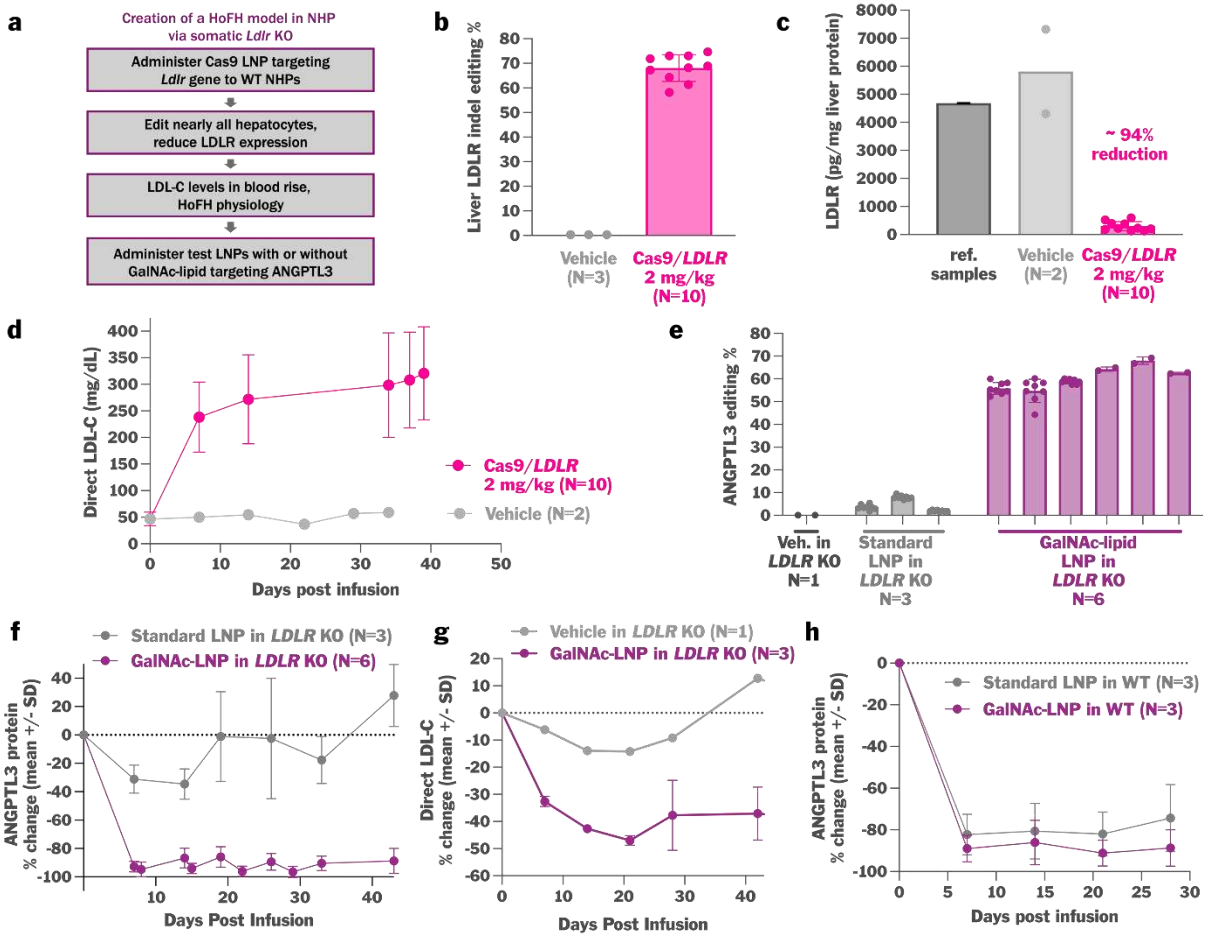

FIGURE 3: Generation of a novel somatic *LDLR* KO NHP model using CRISPR-Cas9 gene editing and successful demonstration of adenine base editing by GalNAc-LNPs targeting *ANGPTL3* in the monkey liver. (a) Schematic detailing the creation of the somatic *LDLR* KO model in NHPs. WT animals were treated with Cas9 dual gRNA LNPs targeting the *LDLR* gene, thus editing and disrupting *LDLR* in the liver. (b) A 2 mg/kg dose of Cas9 LNPs targeting *LDLR* resulted in editing of ~70% of all alleles in a liver biopsy. (c) LDLR protein levels assayed by ELISA on a second liver biopsy were markedly reduced by ~94%, and (d) blood LDL-C increased from ~50 mg/dL to ~300 mg/dL. LNPs without GalNAc-lipid were not effective in *LDLR* KO NHPs, yielding (e) <10% *ANGPTL3* editing and (f) minimal or no ANGPTL3 protein reductions at a 2 mg/kg dose. GalNAc-LNPs at a 2 mg/kg total RNA dose and with 0.05 mol % GL6 resulted in rescue of (e) high-efficiency liver *ANGPTL3* editing and (f) durable >90% reductions in blood ANGPTL3 protein. In panel (e), each column represents an NHP, and editing results reflect liver biopsy (1-2) or necropsy (8) samples. In the HoFH NHP model with

markedly elevated baseline LDL-C levels of ~300 mg/dL, editing of *ANGPTL3* with the GalNAc-LNPs (g) lowered LDL-C (~35%, or ~100 mg/dL in absolute terms). (h) Comparison of blood ANGPTL3 reductions in WT NHPs dosed with either standard LNPs or GalNAc-LNPs at 2 mg/kg. Error bars represents s.d., and individual data points for each animal are displayed in panels (b), (c), and (e).

In summary, we have developed LNP delivery technology incorporating novel GalNAc-based ASGPR-targeting ligands and tested that technology in a NHP model of HoFH characterized by somatic LDLR deficiency in the liver. In HoFH NHPs, administration of GalNAc-LNPs carrying a adenine base editing cargo resulted in efficient editing of the target *ANGPTL3* gene in the liver, whereas standard LNPs did not. The same GalNAc-LNPs effectively delivered a base editing cargo to the livers of WT NHPs. GalNAc-LNPs provide a potent new tool for effective in vivo delivery of nucleic acid therapies.

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