

Clinical Proof of Concept for a Novel Hepatocyte-Targeting GalNAc-siRNA Conjugate

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Advancement of RNAi-based therapeutics depends on effective delivery to the site of protein synthesis. Although intravenously administered, multi-component delivery vehicles have enabled small interfering RNA (siRNA) delivery and progression into clinical development, advances of single-component, systemic siRNA delivery have been challenging. In pre-clinical models, attachment of a triantennary *N*-acetylgalactosamine (GalNAc) ligand to an siRNA mediates hepatocyte uptake via the asialoglycoprotein receptor enabling RNAi-mediated gene silencing. In this phase 1 study, we assessed translation of this delivery approach by evaluating the safety, tolerability, pharmacokinetics, and pharmacodynamics of a GalNAc-siRNA conjugate, revusiran, targeting transthyretin (TTR). Subjects received a placebo or ascending doses of revusiran subcutaneously ranging from 1.25–10 mg/kg in the single and 2.5–10 mg/kg in the multiple ascending dose phases. Revusiran was generally well tolerated, with transient, mild to moderate injection site reactions the most common treatment-emergent adverse events. Doses of 2.5–10 mg/kg revusiran elicited a significant reduction of serum TTR versus the placebo ($p < 0.01$), with mean TTR reductions of approximately 90% observed with multiple dosing. These results demonstrate translation of this novel delivery platform, enabling clinical development of subcutaneously administered GalNAc-siRNAs for liver-based diseases.

INTRODUCTION

RNAi is an evolving approach for the potential treatment of genetic, metabolic, infectious, and malignant disease through its ability to selectively suppress disease-causing genes. The utility of this approach relies on productive delivery of the small interfering RNA (siRNA) to the site of protein synthesis, where the RNA-induced silencing complex (RISC) resides.^{1,2} Initial advances in siRNA delivery were made through the use of intravenously (i.v.) administered, multi-component delivery systems such as lipid nanoparticles (LNPs) and polymers, resulting in a clinical proof of concept for systemic RNAi.^{3,4} Although clinical progress has been made with these multi-component systems, they are encumbered by the need for i.v. administration and, in the case of LNPs, pre-medication with steroids to mitigate infusion-related reactions.³ As such, development of a single-component siRNA delivery platform amenable to subcutaneous (s.c.) administration is an attractive approach.

The incorporation of a ligand to facilitate uptake via a cell surface receptor has been explored for efficient drug delivery. Because of its abundant expression on hepatocytes, ability to support multiple rounds of uptake, and ligand specificity, the asialoglycoprotein receptor (ASGPR) has been utilized for liver-specific drug and gene delivery in animals⁵ and for delivery of radiopharmaceuticals to hepatocytes in humans.⁶ More recently, attachment of a *N*-acetylgalactosamine (GalNAc) ligand directly to an siRNA has been shown to mediate uptake into hepatocytes, resulting in potent and durable target mRNA knockdown in pre-clinical species (Figure 1).⁷

Revusiran is an investigational RNAi therapeutic agent for the treatment of transthyretin (TTR)-mediated amyloidosis (ATTR amyloidosis), a rare, multisystemic disease affecting ~50,000 people worldwide, resulting from the deposition of insoluble TTR amyloid fibrils in various organs and tissues.^{8,9} Autosomal dominant mutations of the *TTR* gene destabilize the liver-expressed native tetramer, yielding amyloidogenic TTR monomers and oligomers in circulation that form amyloid deposits in the heart, peripheral nerves, and gastrointestinal tract, leading to either cardiomyopathy and/or polyneuropathy. More than 100 different *TTR* mutations leading to hereditary ATTR amyloidosis (h-ATTR amyloidosis) have been described, and clinical presentation varies according to the *TTR* gene mutation.^{10,11} In addition to the inherited form of the disease, deposition of wild-type (WT) TTR in the heart can also occur, leading to cardiomyopathy.¹² Regardless of the form of ATTR amyloidosis, disease progression following symptom onset is associated with a substantial deterioration in patients' quality of life and, ultimately, leads to death within approximately 5–15 years of diagnosis.^{8,12}

Revusiran is comprised of a 2'-deoxy-2'-fluoro- and 2'-O-methyl-containing siRNA directed against a region of the human *TTR* mRNA shown to be conserved in WT and all documented variants of the *TTR* gene, conjugated to GalNAc, and is amenable to subcutaneous delivery.^{7,13} This hepatocyte-selective GalNAc-mediated

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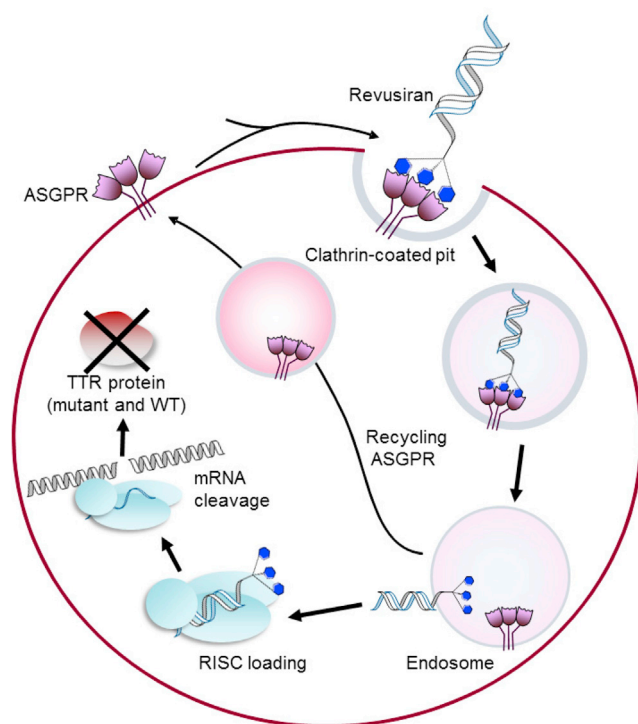


Figure 1. Mechanism of Hepatocyte Uptake and Action of Revusiran

The siRNA targeting *TTR* mRNA is conjugated with a triantennary GalNAc. GalNAc binds to the ASGPR, which is highly expressed on hepatocytes, thus targeting revusiran to the liver. The revusiran-ASGPR complex is then taken into hepatocytes by clathrin-mediated endocytosis, where the siRNA causes *TTR* mRNA destruction through the RISC in the cytoplasm. The ASGPR is recycled to the cell surface for multiple rounds of siRNA uptake.

delivery approach is well suited to targeting TTR, given that the majority of circulating TTR (>95%) is derived from hepatocytes.¹⁴ Potent, specific, and durable RNAi-mediated TTR suppression has been demonstrated in rodents and non-human primates (NHPs; cynomolgus monkeys) by employing this GalNAc-siRNA platform,^{7,13} however, translation of this approach in humans had not been demonstrated.

Here, we report the first ever clinical translation of this GalNAc-siRNA delivery platform in a phase 1 trial of revusiran. We assessed the safety, tolerability, pharmacokinetics (PK), and pharmacodynamics (PD) of single and multiple doses of revusiran in a phase 1 healthy volunteer trial designed to determine the activity and optimal dosing regimen for subsequent trials in patients with ATTR amyloidosis.

RESULTS

These data describe the weight-based dose escalation part of this randomized, double-blind, placebo-controlled phase 1 trial in healthy volunteers. A total of 41 subjects were recruited across the single ascending dose (SAD) and multiple ascending dose (MAD) phases of the study from February 25, 2013 to August 29, 2014. Twelve sub-

jects were randomly assigned to receive revusiran and four to receive a placebo in the SAD phase (Figure 2). In the MAD phase, 19 subjects received revusiran (three subjects in each of the 2.5, 5, and 7.5 mg/kg once every 2 weeks [Q2W] and 10 mg/kg cohorts and seven in the 7.5 mg/kg once weekly [QW] cohort), and six received a placebo (Figure 2). Overall, 39 subjects (95%) completed the study; two subjects withdrew consent (one each in the 1.25 mg/kg SAD and 7.5 mg/kg QW MAD cohorts). All 41 study participants were included in the data analyses. The baseline characteristics for all study participants are listed in Table 1.

Single and multiple doses of revusiran were generally well tolerated, with no dose-limiting toxicities (DLTs), flu-like symptoms, serious adverse events (SAEs), or treatment discontinuations because of treatment-emergent adverse events (TEAEs). The majority of TEAEs were of mild to moderate intensity. Severe events were reported in only two subjects. One case of syncope occurred in the 5.0 mg/kg SAD cohort that was deemed unrelated to the study drug, and one case of an injection site reaction (ISR) that was considered severe (erythema > 10 cm) occurred in a subject in the 7.5 mg/kg QW cohort in the MAD phase. The most commonly observed TEAEs were mild to moderate ISRs (defined as two or more mild signs or symptoms or one moderate or severe sign or symptom; Table S1); these were only reported in subjects who received multiple doses of revusiran (13 of 19 subjects; Table 2; Table S2). The most frequently reported ISR signs and symptoms were transient erythema (47.4%), pain (31.6%), and swelling (36.8%) that self-resolved. Aside from events associated with the injection site, headache was the only TEAE observed in more than 10% of subjects receiving revusiran as well as the placebo. There were no clinically relevant abnormalities recorded for renal function (creatinine), hematological parameters including platelets, thyroid function tests, C-reactive protein (CRP), cytokines, or urinalysis during either phase of the study (data not shown). Mild, transient, asymptomatic elevations in aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) ($\leq 2 \times$ upper limit of normal [ULN]) were observed in 9 of 19 subjects who received multiple doses of revusiran and 1 of 6 subjects who received the placebo (Figure S1). These transaminase elevations were not clinically significant, did not concur with elevations in total bilirubin and alkaline phosphatase (ALP), and showed no obvious dose relationship. All subjects with transaminase elevations were recovering toward baseline either during treatment or within several weeks after the treatment was completed (Figure S1). Anti-drug antibodies were not observed after multiple dosing with revusiran (data not shown).

The plasma exposure of subcutaneously administered revusiran increased in a dose-proportional manner (Figure 3; Table S3) over the dose range tested. Maximum revusiran plasma concentrations were achieved between 2 and 6 hr post-administration, and the mean elimination half-life was typically in the 6- to 10-hr range. Importantly, no plasma accumulation was observed after multiple doses of revusiran, and PK parameters at each dose level were generally similar for single and multiple doses of revusiran (Table S3).

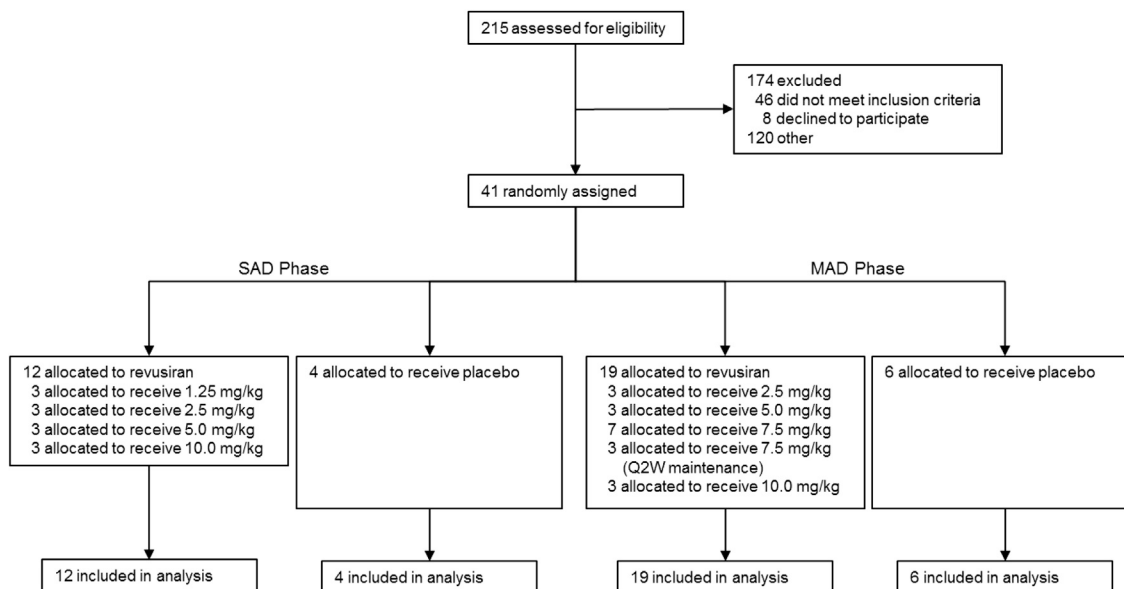


Figure 2. Trial Profile

SAD dose cohorts preceded enrollment of the MAD dose cohorts.

Subcutaneous administration of revusiran led to a significant reduction in serum TTR versus placebo at single doses of 2.5, 5, and 10 mg/kg by day 10 ($p < 0.05$; analysis of covariance [ANCOVA] model) and maximum TTR reductions achieved between days 10 and 14 (Figure 4A). The extent of TTR knockdown increased in a dose-related manner, with the greatest mean (SD) reduction in TTR of 53.3% (8.1%) and maximum knockdown in an individual subject of 59.6% after a single subcutaneous dose of 10 mg/kg revusiran (Table 3).

A significant reduction in TTR versus placebo ($p < 0.01$, ANCOVA model) was also observed across all dose cohorts in the MAD phase (Table 3). For all dose cohorts, mean TTR levels reached a nadir on day ≥ 14 and were maintained during weekly revusiran dosing through approximately 2–3 weeks after dosing cessation before TTR levels began to recover (Figure 4B). The mean (SD) maximal TTR reduction from baseline for weekly revusiran ≥ 5 mg/kg ranged from 87.5% (7.22) to 92.4% (1.50) (Table 3). In comparison with weekly dosing, the mean (SD) maximal TTR reduction was slightly lower when revusiran (7.5 mg/kg) was dosed Q2W (Table 3). Across individual subjects, the maximum reduction in TTR was 96.2%, achieved in the 7.5 mg/kg QW cohort (Table 3); similar levels of robust TTR reduction were observed in the revusiran 5.0, 7.5, and 10 mg/kg weekly dosing cohorts (Table 3). Multiple dosing of revusiran was also tested in NHPs, and comparison with these data revealed a strong association between TTR knockdown in humans and NHPs ($R^2 = 0.83$, $p < 0.001$; Figure S2), demonstrating clinical translation of this subcutaneous GalNAc-siRNA delivery platform.

Consistent with TTR's role in vitamin A transport, reductions in retinol binding protein (RBP) and vitamin A were also observed in

the revusiran dosing cohorts (Table S4). Mean maximal reductions of 68.4%–70.6% and 83.7%–85.6% for RBP and vitamin A, respectively, were achieved in the MAD phase with weekly revusiran ≥ 5 mg/kg. These reductions in RBP and vitamin A were strongly associated with the reductions observed in serum TTR ($R^2 = 0.87$ and 0.94 for RBP and vitamin A, respectively, in the MAD cohorts; both $p < 10^{-15}$; Figure S3).

DISCUSSION

The success of RNAi-based therapies will hinge on the ability to deliver siRNA to the target cell and site of RNAi action, resulting in RISC-mediated target mRNA cleavage and inhibition of protein synthesis. Here we provide the first demonstration in humans of the safety and pharmacodynamic activity of a GalNAc-siRNA conjugate that effectively targets hepatocytes and mediates knockdown of liver-expressed TTR, the pathogenic protein of ATTR amyloidosis. Single and multiple subcutaneous doses of revusiran were generally well tolerated and resulted in potent and durable TTR knockdown in healthy volunteers, demonstrating clinical translation of this novel GalNAc-siRNA delivery platform.

Single subcutaneous doses of revusiran in humans resulted in a dose-related increase in TTR knockdown, with a mean maximal reduction of $>50\%$ achieved with administration of 10 mg/kg. Multiple dosing of revusiran resulted in rapid mean maximal TTR reduction of $>90\%$, which was maintained with weekly doses of ≥ 5 mg/kg. The reduction in TTR was still apparent with revusiran ≥ 5 mg/kg at least 7 weeks after cessation of dosing, demonstrating the lasting effect of this treatment. The strong correlation between human and NHP TTR knockdown suggests that NHPs are predictive of human PD for GalNAc-siRNA conjugates and provides a valuable preclinical model

Table 1. Demographics and Baseline Characteristics

Characteristic	SAD Phase			MAD Phase		
	Placebo (n = 4) ^a	Revusiran (mg/kg) 1.25 (n = 3)	Revusiran (mg/kg) 2.5 (n = 3)	Placebo (n = 6)	Revusiran (mg/kg) 2.5 QW (n = 3)	Revusiran (mg/kg) 5.0 QW (n = 3)
Median age (years) (range)	45.5 (40–53)	25.0 (23–43)	25.0 (20–48)	44.5 (22–54)	26.0 (21–41)	41.0 (36–42)
Male sex (n) (%)	4 (100)	3 (100)	3 (100)	6 (100)	3 (100)	3 (100)
Mean weight (kg) (SD)	82.6 (13.6)	74.0 (5.6)	91.3 (12.8)	75.7 (10.7)	83.0 (6.3)	81.5 (14.8)
Mean TTR (µg/mL) (SD)	322.1 (58.1)	266.4 (19.3)	259.5 (12.0)	258.4 (42.7)	235.7 (26.3)	283.1 (9.9)
					280.6 (24.2)	254.6 (37.3)
					47.0 (23–55)	7.5 Q2W (n = 3)
					7.5 QW (n = 7)	10.0 QW (n = 3)
					3 (100)	23.0 (21–23)
					6 (86)	
					84.5 (9.0)	
					227.6 (60.1)	

^aThe SAD placebo cohort contained one subject of Hispanic or Latino ethnicity. All other subjects were non-Hispanic or non-Latino.

for translational research of other GalNAc-conjugated siRNA-based therapeutic agents.

The GalNAc-siRNA conjugate is the first single-component, subcutaneously administered delivery approach for targeting siRNA to the liver. Prior to this advancement, successful RNAi-mediated TTR knockdown in humans had been demonstrated with intravenously administered TTR siRNA encapsulated in a multi-component LNP (patisiran) co-administered with steroid pre-medication to mitigate the risk of infusion-related reactions.³ These LNPs are bound by apolipoprotein E and taken up by low-density lipoprotein (LDL) receptors expressed on hepatocytes, where they are internalized by endocytosis.¹⁵ In contrast, subcutaneously administered revusiran is targeted to the liver by a simple carbohydrate ligand that enables receptor-mediated uptake by the ASGPR expressed on hepatocytes and does not require any pre-medication. The level of TTR knockdown observed with revusiran administration in this study is comparable with that reported for intravenous patisiran (0.3 mg/kg every 3 weeks¹⁶) and greater than that reported for the antisense oligonucleotide (ASO) agent IONIS-TTR_{RX} in healthy volunteers.^{17,18}

The most common TEAEs for revusiran were mild to moderate ISRs and the signs and symptoms associated with these reactions. These events were transient and resolved without any intervention. Injection site events are not uncommon with subcutaneous drug administration and are among the most frequently reported AEs following dosing of other oligonucleotide-based therapeutic agents.¹⁹ Importantly, there was no evidence of systemic immune stimulation, with no relevant changes in serum levels of CRP or cytokines and no evidence of anti-drug antibody production with revusiran treatment. Furthermore, no clinically relevant changes were observed in hepatic, renal, hematological, or thyroid function tests with revusiran administration.

Because revusiran targets a region within the *TTR* mRNA that is common to both WT and all documented *TTR* mutations, it is expected that this GalNAc-siRNA will lower the levels of all forms of TTR involved in ATTR amyloidosis. The reduction of both WT and mutant TTR is hypothesized to prevent further formation of amyloid deposits and potentially promote their regression. Elimination of mutant TTR is the fundamental driver behind liver transplantation for ATTR amyloidosis with polyneuropathy, and the clinical benefits associated with this procedure highlight the potential effect of therapies that can lower hepatic TTR.^{20,21} Indeed, the high level of TTR knockdown achieved with revusiran (~90%) is comparable with the reduction in mutant TTR following liver transplantation, with the added advantage of affecting both mutant and WT TTR production. The high potency of revusiran is also particularly promising because a reduction in levels of amyloidogenic protein of only 50% has been associated with clinical benefits in light-chain amyloidosis.²² The activity of revusiran described here demonstrates the capacity for GalNAc-siRNA to effectively target the liver, where TTR is predominantly produced, providing a therapeutic strategy for ATTR amyloidosis.

Table 2. Safety and Tolerability: TEAEs Related to the Study Drug Occurring in ≥ 10% Subjects in Either the SAD or MAD Phases

TEAE	SAD Phase										MAD Phase				
	Placebo (n = 4)					Revisiran (mg/kg)					Revisiran (mg/kg)				
	1.25 (n = 3)	2.5 (n = 3)	5.0 (n = 3)	10.0 (n = 3)	Placebo (n = 6)	2.5 QW (n = 3)	5.0 QW (n = 3)	7.5 QW (n = 7) ^a	7.5 Q2W (n = 3)	10.0 QW (n = 3)	All Revisiran (n = 31)				
Injection site reaction ^b	0	0	0	0	1 (17)	1 (33)	1 (33)	5 (71)	3 (100)	3 (100)	13 (42)				
Injection site erythema	0	0	0	0	0	2 (67)	1 (33)	2 (29)	3 (100)	2 (67)	10 (53)				
Injection site pain	0	0	0	1 (33)	0	0	2 (29)	2 (29)	2 (67)	0	5 (16)				
Headache	1 (25)	0	1 (33)	0	2 (33)	0	2 (29)	0	0	1 (33)	5 (16)				
Injection site swelling	0	0	0	0	0	0	0	3 (43)	0	0	3 (10)				

^aOne subject in the 7.5 mg/kg group was excluded from this analysis because only one post-dose data point was available.

^bAn injection site reaction was defined as two or more mild signs or symptoms or one moderate or severe sign or symptom (Table S4).

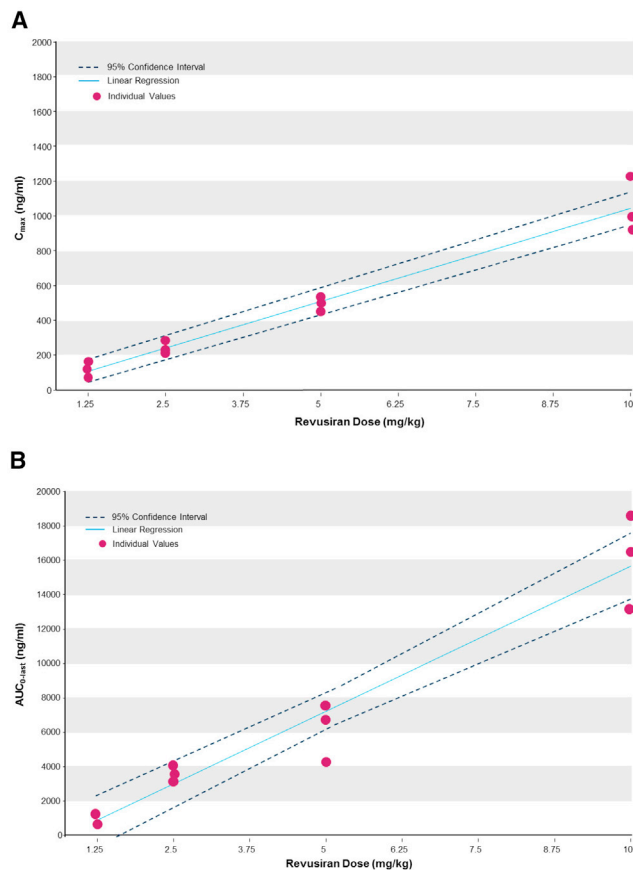


Figure 3. Proportionality of Revisiran Plasma C_{max} and AUC_{0-last} after a Single Administration of Revisiran

(A and B) Proportionality of revisiran plasma observed maximum plasma concentration (C_{max}) (A) and area under the plasma concentration-time curve from zero to the last measurable time point (AUC_{0-last}) (B) after a single administration of revisiran.

The utility of this GalNAc conjugate approach for efficient and potent hepatocellular siRNA delivery has provided a robust platform for the development of RNAi-based therapies in liver-based diseases, with GalNAc-siRNA conjugates in development for the treatment of hemophilia,²³ acute hepatic porphyria,²⁴ and hepatic infectious diseases,²⁵ among others. Indeed, GalNAc-mediated delivery has been deployed with other oligonucleotide-based therapeutic agents, including ASOs²⁶ and anti-microRNAs (miRNAs),²⁷ as demonstrated in preclinical studies and their advancement into clinical development. The data presented in this study provide proof of concept for human translation of this GalNAc-siRNA platform, thus promoting the clinical adoption of this approach for future therapies.

MATERIALS AND METHODS

Study Design and Participants

This phase 1, randomized, double-blind, placebo-controlled study was conducted at two centers in the United Kingdom (Covance

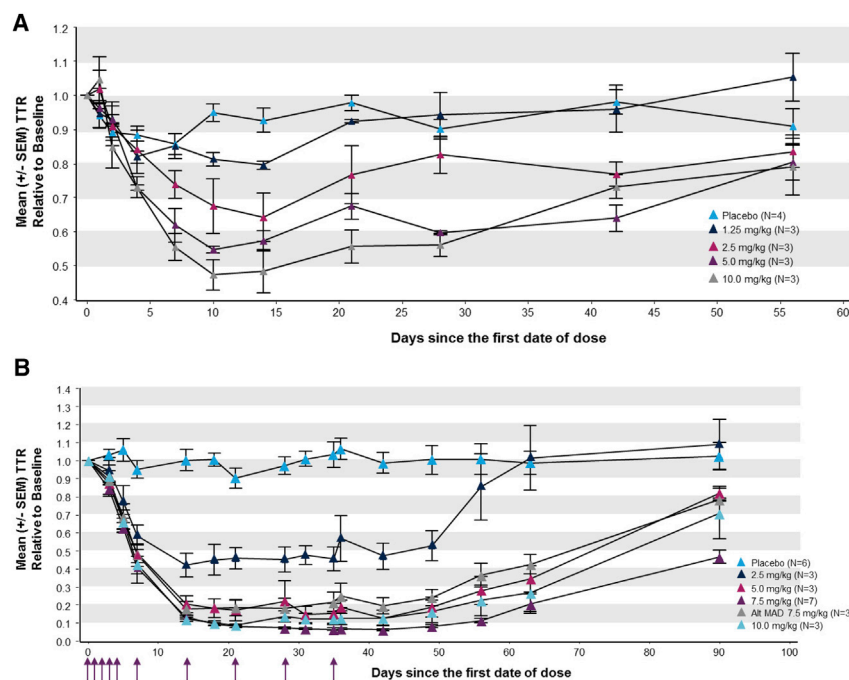


Figure 4. Pharmacodynamic Effect of Revusiran on Serum TTR Levels

(A and B) Mean (\pm SEM) change in serum TTR over time relative to baseline in the SAD (A) and MAD (B) phase. Arrows indicate days of dosing in the multidose cohorts at 2.5, 5, and 7.5 (QW) and 10 mg/kg revusiran (days 0–4 and then weekly from days 7–35). The alternate MAD 7.5 mg/kg cohort was dosed on days 0–4 and then every other week from days 7–35.

For each cohort, dosing was staggered so that dosing of subjects 1 and 2 occurred no sooner than 48 hr apart. Dosing proceeded for the remaining subjects based on safety review committee (SRC) assessment of safety and tolerability and no sooner than 48 hr after subject 2. Dose escalation occurred sequentially in the SAD and MAD cohorts based on SRC review of safety data. Subjects in the SAD cohorts received a single subcutaneous dose of 1.25, 2.5, 5, or 10 mg/kg revusiran or placebo. Subjects in the MAD cohorts received a total of 10 subcutaneous doses of the study drug (one dose per day on days 0–4 and then weekly from days 7 to 35 [maintenance]) at 2.5, 5, and 10 mg/kg. Based on acceptable tolerability, additional MAD cohorts were added to investigate revusiran 7.5 mg/kg with either QW or Q2W maintenance dosing. Subjects were followed at screening (days –45 to –2), admission (day –1), dosing (up to day 35), and follow-up (up to 56 days and 90 days after the first dose in the SAD and MAD phases, respectively).

Clinical Research Unit, Leeds and Hammersmith Medicines Research). Eligible subjects were healthy Caucasian volunteers aged 18–55 years with a body mass index of 18.0–30 kg/m². Subjects had ALT, AST, and gamma-glutamyl transpeptidase levels of $<1.5 \times$ ULN, albumin ≥ 3.9 g/dL, and total bilirubin and ALP levels that were considered clinically normal by the investigator. Women were required to be postmenopausal or surgically sterilized; men had to use effective means of contraception. Subjects with hepatitis B or C or HIV infection were excluded.

This study was performed in accordance with the principles associated with the World Health Organization Declaration of Helsinki and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. Written informed consent was obtained from all participating subjects. The clinical trial protocol was approved by an independent ethics committee. This trial is registered with [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT01814839).

Randomization and Masking

Assignment of treatment was masked from the study subjects, principal investigators, medical monitors, and study site personnel. Subjects were randomized to receive either revusiran or a placebo (normal saline) based on a cohort-specific randomization list generated by the research site's biostatistician. Only the site pharmacist was aware of the treatment assignment.

Procedures

Four subjects were assessed at each dose level, with three receiving revusiran and one receiving the placebo in all SAD and MAD cohorts.

The primary objective was safety and tolerability of single and multiple doses of revusiran. Reported adverse events (AEs) were collected from the first dose until 28 days after the last dose and summarized by the Medical Dictionary for Regulatory Activities.²⁸ AEs were classified as mild, moderate, or severe, and their potential relationship to the study drug was recorded. Treatment-emergent adverse events were defined as events that started after exposure to the study drug or worsened in severity after dosing. An ISR was defined as two or more mild signs or symptoms or one moderate or severe sign or symptom (Table S1). The severity of injection site signs and symptoms was based on a Food and Drug Administration (FDA) guidance document for vaccine trials in healthy volunteers.²⁹ Clinical laboratory tests included hematology, serum chemistries, electrocardiograms, liver function tests, thyroid function parameters, coagulation, CRP, cytokines, and urinalysis. Anti-drug antibody production was assessed using a validated ELISA (Supplemental Materials and Methods).

The secondary objectives were to characterize the pharmacokinetics of revusiran and to assess its pharmacodynamic effect on serum TTR levels. In addition, the effect of revusiran on secondary markers of TTR lowering, vitamin A and RBP, was explored. Samples were

Table 3. Mean Maximal Knockdown and Individual Maximum Knockdown for TTR per Dose Cohort Relative to Baseline values

	SAD Phase					MAD Phase					
	Placebo (n = 4)	Revusiran (mg/kg)				Placebo (n = 6)	Revusiran (mg/kg)				
		1.25 (n = 3)	2.5 (n = 3)	5 (n = 3)	10 (n = 3)		2.5 QW (n = 3)	5 QW (n = 3)	7.5 QW (n = 7)	7.5 Q2W (n = 3)	10 QW (n = 3)
KD											
Maximum TTR KD (%)	22.0	22.7	49.0 ^a	47.7 ^a	59.6 ^a	33.4	70.6 ^b	93.3 ^b	96.2 ^b	91.1 ^b	94.0 ^b
TTR KD at nadir (mean ± SD) (%)	18.3 ± 4.19	21.6 ± 1.19	37.7 ± 12.33	46.7 ± 0.88	53.3 ± 8.11	16.9 ± 10.49	58.2 ± 11.12	87.5 ± 7.22	87.9 ± 16.77	83.4 ± 7.27	92.4 ± 1.50

KD, knockdown.

^aSignificant reduction versus placebo (p <0.05).

^bSignificant reduction versus placebo (p <0.01).

collected at pre-specified times for these measures ([Supplemental Materials and Methods](#)). TTR concentration was evaluated using a validated ELISA (Charles River Laboratories). Biomnis Specialized Medical Pathology evaluated vitamin A using high-performance liquid chromatography (HPLC) and RBP by nephelometry.

Statistical Analysis

This study was not powered for formal hypothesis testing. Sample size was based on the planned dose escalation scheme. All subjects who received at least one dose of the study drug were included in the safety population; those who had at least one post-dose sample for PK and PD parameters were included in these populations, respectively. TTR knockdown at the nadir was calculated per individual subject as the lowest TTR level relative to baseline (average of the three pre-dose measures at screening and days -1 and 0) on any post-dose day. Analysis of variance models (with day and treatment group as factors) and ANCOVA models (with day and treatment group as factors and baseline TTR as a covariate) were used to assess TTR knockdown at the nadir and over time; Tukey's post hoc tests examined pairwise comparisons (i.e., between dose groups at a particular time point or at the nadir). Linear regression was used to assess the correlations between TTR knockdown in human and NHPs and the correlations between TTR knockdown and reductions in circulating RBP and vitamin A. No substitutions were made for missing data points. Continuous variables were described using descriptive statistics, and categorical and ordinal variables were presented as frequencies and percentages. Analyses were conducted using SAS for Windows (version 9.2 or higher; SAS Institute) and/or R for Windows (version 3.0).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods, three figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ymthe.2016.10.019>.

AUTHOR CONTRIBUTIONS

Conceptualization, T.S.Z., V.K., A.C., R.H., B.R.B., S.N., A.V., and J.G.; Methodology, T.S.Z., V.K., A.C., R.H., B.R.B., J.C., M.B., S.N., A.V., and J.G.; Formal Analysis, R.H. and B.R.B.; Investigation and Resources in the Conduct of the Clinical Trial, J.C. and M.B.; Writing – Original Draft, T.S.Z. and J.G.; Writing – Review & Editing, T.S.Z.,

V.K., A.C., R.H., B.R.B., J.C., M.B., S.N., A.V., and J.G.; Supervision, A.V. and J.G.

CONFLICTS OF INTEREST

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Supplemental Information

Clinical Proof of Concept for a Novel

Hepatocyte-Targeting GalNAc-siRNA Conjugate

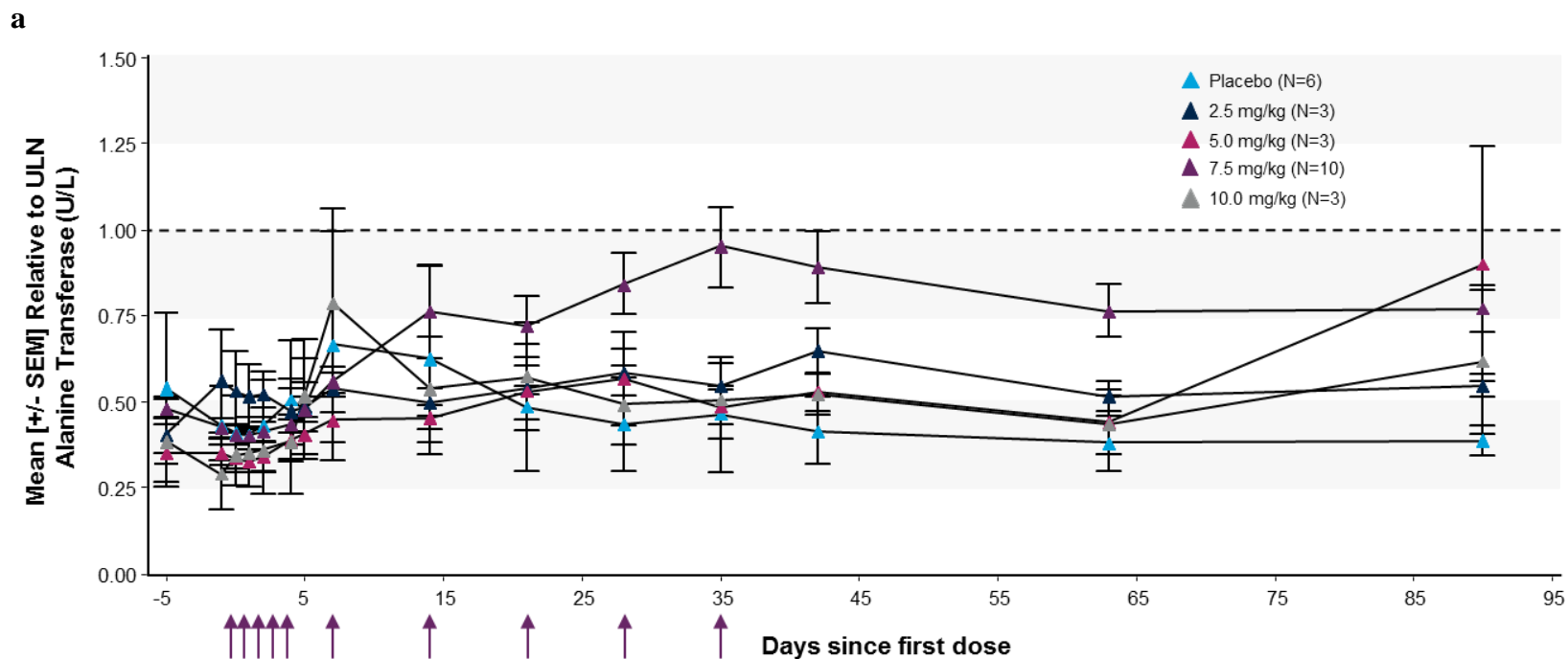
Tracy S. Zimmermann, Verena Karsten, Amy Chan, Joseph Chiesa, Malcolm Boyce, Brian R. Bettencourt, Renta Hutabarat, Saraswathy Nochur, Akshay Vaishnav, and Jared Gollob

Supplemental Figures and Tables

Figure S1 Mean relative to upper limit of normal (a) alanine transferase and (b) aspartate transferase over time after multiple doses of revusiran.

Symbols represent mean \pm SEM. Dotted line corresponds to ULN. Arrows indicate days of dosing in the multidose cohorts at 2.5, 5, and 10 mg/kg (Days 0-4 then weekly from Days 7 to 35 [maintenance]). The combined 7.5 mg/kg MAD cohorts were dosed on Days 0-4 then either once weekly (N=7) or once every two weeks (N=3) during the maintenance phase.

SEM=standard error of the mean; ULN=upper limit of normal; U/L=Units/Liter



b

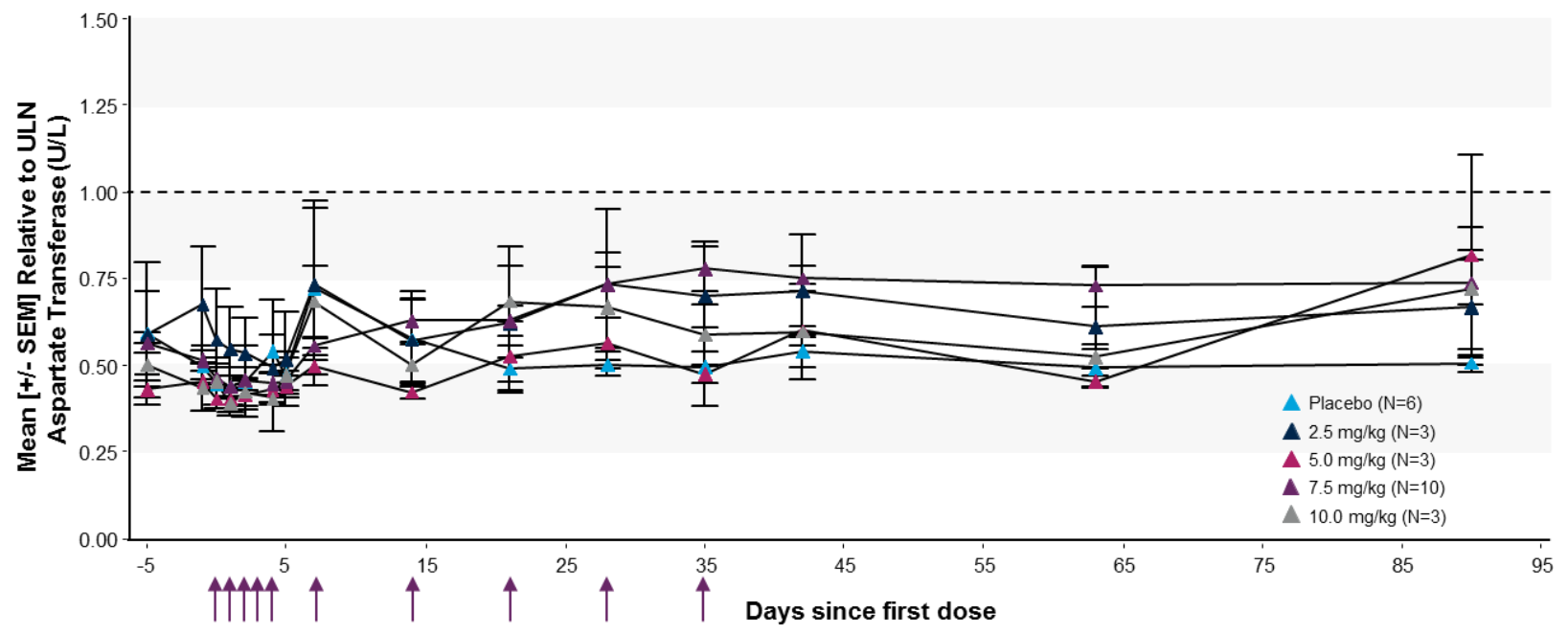


Figure S2 Correlation between human and non-human primate TTR knockdown after multiple doses of revusiran. Relationship between TTR KD in NHPs (x-axis) and humans (y-axis) per subject/timepoint at identical dose levels and paradigms. Symbols represent means \pm SEMs. Line, R^2 , and P value are associated with linear regression of human means on NHP means.

KD=knockdown; NHP=non-human primate (cynomolgus monkeys); SEM=standard error of the mean.

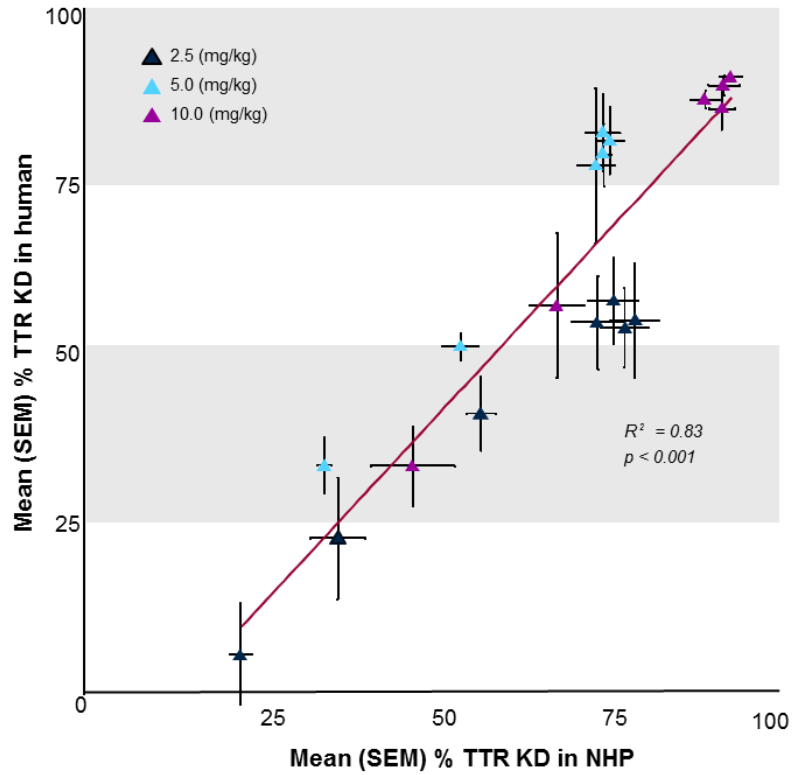


Figure S3 Correlation of changes in serum TTR with changes in RBP and vitamin A in the MAD phase.

Relationships between RBP and vitamin A relative to baseline (squares and triangles, respectively; y-axis), and TTR relative to baseline (x-axis), per subject/timepoint. Colours indicate treatment group (dose). RBP: $R^2 = 0.87$, $p < 10^{-15}$; Vitamin A: $R^2 = 0.94$; $p < 10^{-15}$ from linear regressions of log-transformed data.

MAD= multiple ascending dose; RBP=retinol binding protein; TTR=transthyretin.

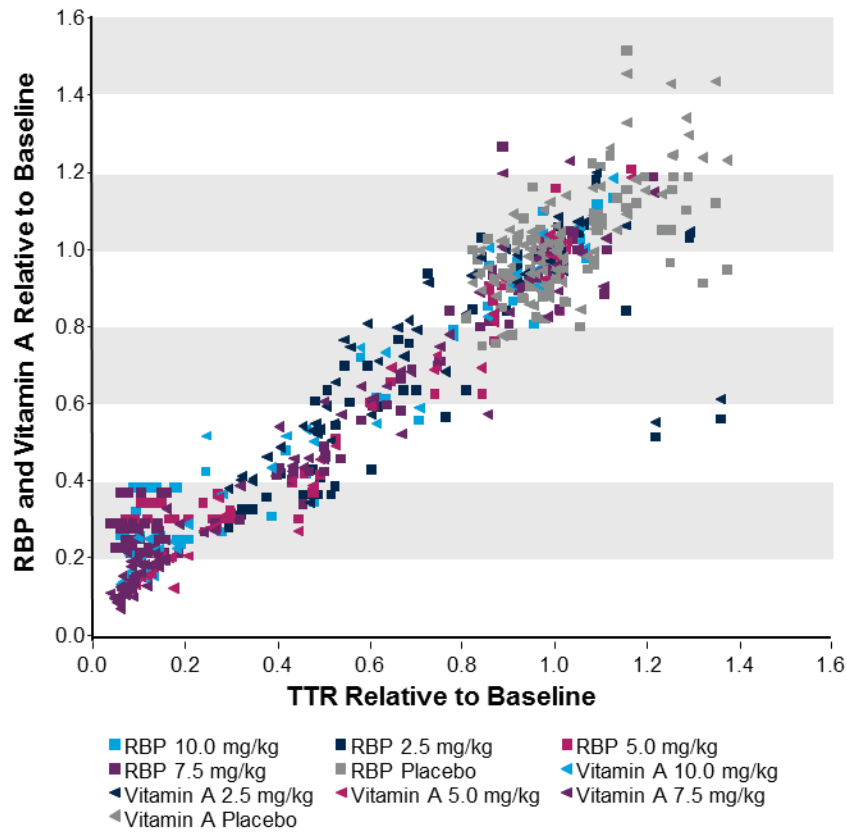


Table S1 Intensity of injection-site signs and symptoms^a

Sign/symptom	Mild	Moderate	Severe
Pain ^b	Does not interfere with activity	Repeated use of non-narcotic pain reliever for >24 h or interferes with activity	Any use of narcotic pain reliever or prevents activity
Tenderness	Mild discomfort with strong palpation	Moderate discomfort with normal palpation	Severe discomfort with light palpation
Erythema	2.5–5 cm	5.1–10 cm	>10 cm
Induration/swelling	2.5–5 cm and does not interfere with ADL	5.1–10 cm or interferes with ADL	>10 cm or prevents ADL
Pruritus	Mild or localised; topical intervention indicated	Intense or widespread; intermittent; skin changes from scratching (eg, oedema, papulation, oozing/crusts); systemically administered drugs other than corticosteroids or other immunosuppressive drugs indicated	Intense or widespread; constant; limiting self-care ADL or sleep; systemically administered corticosteroid or immunosuppressive therapy indicated
Ulceration	Combined area of ulcers <1 cm; non-blanchable erythema of intact skin with associated warmth or edema	Combined area of ulcers 1–2 cm; partial thickness skin loss involving skin or subcutaneous fat	Combined areas of ulcers >2 cm; full thickness skin loss involving damage to or necrosis of subcutaneous tissue that may extend down to fascia
Pigmentation changes	Slight or localised; covering \leq 10% of BSA	Marked or generalised; covering >10% of BSA	—
Other	Asymptomatic or mild	Moderate; minimal local or	Invasive or more significant

symptoms; clinical or diagnostic observation only; intervention not indicated	non-invasive intervention indicated; limiting age- appropriate instrumental ADL	intervention indicated
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ADL=activities of daily living; BSA=body surface area.

^aTable adapted from guidance developed by the Food and Drug Administration for healthy volunteers enrolled in vaccine trials and Common Terminology Criteria for Adverse Events (version 4.0).¹

^bPain is post-injection pain associated with the injection site but not associated with the needle stick.

Table S2 TEAEs occurring in >1 subject across the SAD and MAD phases

TEAEs in >1 subject, n (%)												
TEAE	SAD phase					MAD phase						All revusiran (n=31)
	Placebo (n=4)	Revusiran (mg/kg)				Placebo (n=6)	Revusiran (mg/kg)					
		1.25 (n=3)	2.5 (n=3)	5.0 (n=3)	10.0 (n=3)		2.5 QW (n=3)	5.0 QW (n=3)	7.5 QW (n=7)	7.5 Q2W (n=3)	10.0 QW (n=3)	
Injection-site reaction	0	0	0	0	0	1 (17)	1 (33)	1 (33)	5 (71)	3 (100)	3 (100)	13 (42)
Injection-site erythema	0	0	0	0	0	0	2 (67)	1 (33)	2 (29)	3 (100)	2 (67)	10 (32)
Injection-site hematoma	0	0	0	0	1 (33)	2 (33)	3 (100)	1 (33)	4 (57)	0	0	9 (29)
Headache	1 (25)	0	1 (33)	1 (33)	0	2 (33)	0	0	2 (29)	0	1 (33)	5 (16)
Injection-site pain	0	0	0	0	1 (33)	0	0	0	2 (29)	2 (67)	0	5 (16)
Injection-site swelling	0	0	0	0	0	0	0	0	3 (43)	0	0	3 (10)
Dizziness	0	0	0	0	0	0	0	1 (33)	2 (29)	0	0	3 (10)
Nausea	0	0	0	0	0	0	0	1 (33)	1 (14)	0	0	2 (6)

SAD=single ascending dose; MAD=multiple ascending dose; TEAE=treatment-emergent adverse event; QW=every week; Q2W=every other week.

^bAn ISR was defined as ≥ 2 mild signs or symptoms, or one moderate or severe sign or symptom (Supplemental Table S1).

Table S3 PK parameter estimates at comparable dose levels following a single dose (SAD cohort) and 5 and 10 doses (MAD cohorts) of revusiran

PK parameter	Revusiran 2.5 mg/kg			Revusiran 5.0 mg/kg			Revusiran 10.0 mg/kg		
	SAD	MAD	MAD	SAD	MAD	MAD	SAD	MAD	MAD
	(n=3)	Dose 5 (n=3)	Dose 10 (n=3)	(n=3)	Dose 5 (n=3)	Dose 10 (n=3)	(n=3)	Dose 5 (n=3)	Dose 10 (n=3)
AUC _{0-last} (µg h/ml)	3.60 (14.1)	2.96 (9.64)	3.45 (21.73)	6.13 (27.3)	9.65 (18.79)	8.00 (20.54)	17.01 (8.2)	22.50 (28.14)	22.74 (14.72)
C _{max} (µg/l)	0.24 (9.9)	0.27 (27.99)	0.31 (38.16)	0.50 (7.0)	0.65 (25.33)	0.53 (2.09)	1.05 (15)	1.84 (35.02)	1.80 (23.43)
T _{max} (h)	6.00 (6.0–12.0)	3.33 (34.64)	4.67 (24.74)	2.00 (2.0–2.0)	6.00 (0.00)	4.00 (50.00)	6.02 (0.50–8.0)	1.83 (103.25)	2.17 (81.04)
t _{1/2β}	7.37 (NC)	7.29 (15.99)	5.75 (17.46)	6.02 (NC)	6.41 (29.52)	10.98 (47.66)	9.59 (NC)	7.87 (61.54)	6.01 (43.07)
CL/F (l/h/kg)	0.65 (NC)	0.69 (18.85)	0.70 (19.61)	0.67 (NC)	0.48 (12.74)	0.50 (25.82)	0.56 (NC)	0.41 (36.01)	0.41 (7.14)
V _z /F (l/kg)	6.80 (NC)	7.37 (32.62)	5.88 (36.18)	5.85 (NC)	4.47 (36.66)	7.51 (35.40)	7.82 (NC)	4.42 (47.77)	3.58 (46.78)

AUC_{0-last}=area under the plasma concentration–time curve from zero to the last measurable time point; CL/F=apparent clearance; C_{max}=observed maximum plasma concentration;
CV=coefficient of variation; NC=not calculated because ≤ 2 subjects had data; PK=pharmacokinetic; $t_{1/2\beta}$ =beta half-life; T_{max}=time of observed maximum plasma concentration;
V_z/F=apparent volume of distribution.

Values are mean (CV%) except for T_{max} in the SAD cohort, which is median (range)

Table S4 Mean maximal KD and individual maximum KD for RBP and vitamin A by dose cohort, relative to baseline values

	SAD phase					MAD phase					
	Placebo	Revusiran (mg/kg)				Placebo	Revusiran (mg/kg)				
		1.25	2.5	5.0	10.0		2.5 QW	5 QW	7.5 QW	7.5 Q2W	10.0 QW
	(n=4)	(n=3)	(n=3)	(n=3)	(n=3)	(n=6)	(n=3)	(n=3)	(n=7)	(n=3)	(n=3)
RBP											
Maximum RBP KD, %	28.8	30.5	47.2	43.9	57.1	25.0	71.9	69.8	78.3	75.0	75.0
RBP KD at nadir (mean ± SD), %	24.3 ±	24.1 ±	38.9 ±	42.5 ±	49.6 ±	20.0 ±	60.3 ±	68.4 ±	70.6 ±	72.2 ±	70.3 ±
	4.01	8.88	8.08	1.44	9.96	6.58	13.52	2.3	7.66	2.64	7.37
Vitamin A											
Maximum KD, %	32.0	33.2	37.3	55.7	55.1	33.4	65.6	89.1	92.9	87.6	86.8
KD at nadir (mean ± SD), %	24.2 ±	20.6 ±	31.7 ±	46.4 ±	46.1 ±	15.3 ±	56.1 ±	84.4 ±	85.6 ±	81.3 ±	83.7 ±
	5.56	11.32	7.29	8.88	10.74	12.76	13.51	4.89	13.89	5.55	4.08

KD=knockdown; SAD=single ascending dose; MAD=multiple ascending dose; RBP=retinol binding protein; SD=standard deviation; QW=every week; Q2W=every other week.

Supplemental Methods

Pharmacodynamics

Serum samples for pharmacodynamic assessments including TTR concentrations and secondary markers Vitamin A and RBP were taken at screening, admission (Day -1), and pre-dose on Day 0 for both SAD and MAD cohorts. Additionally, serum samples were collected up to Day 56 in the SAD cohorts (Days 1, 2, 4, 7, 10, 14, 21, 28, 42 and 56) and up to Day 90 in the MAD cohorts (Days 3, 5, 7, 14, 18, 21, 28, 31, 35, 36, 42, 49, 56, 63 and 90). Serum draws on a dosing day were taken prior to dosing in the MAD cohorts (Days 3–35).

Pharmacokinetics

The concentration of TTR siRNA (revusiran) was evaluated in plasma samples using the validated hybridisation-based ATTO-Probe-HPLC assay (Tandem laboratories, Salt Lake City, UT, USA). In the SAD cohorts, comprehensive sampling plasma PK was performed on Day 0 (5 [\pm 1], 10 [\pm 1], and 30 [\pm 2] minutes pre-dose, and 1, 2, 4, 6, 8 and 12 h [all \pm 5 minutes] post-dose); samples were also taken on Days 1, 2, 4, 7, 10, 14, 21 and 28. For the MAD cohorts, plasma samples were taken on Days 0–5 and up to Day 90 in the maintenance phase (Days 7, 14, 21, 28, 35, 36, 42, 49, 56, 63 and 90). On Days 0, 4 and 35, samples were taken Day 0 (5 [\pm 1], 10 [\pm 1], and 30 [\pm 2] minutes pre-dose and 1, 2, 4, 6, 8 and 12 h [all \pm 5 minutes] post-dose); on all other dosing days, plasma was collected approximately 1 h prior to dose and 2 h after dosing. Noncompartmental and/or compartmental models were used to estimate PK parameters.

Anti-Drug Antibody

Anti-drug antibody production was assessed using a validated ELISA. In brief, ~1 ng of revusiran was coupled to each well of an ELISA plate as a modified 5'-phosphate duplex (5'-phosphate on sense strand). Serum samples from subjects were then tested for binding to the revusiran duplex in this sandwich ELISA with detection by anti-immunoglobulin G/M coupled with horseradish peroxidase. A rabbit anti- keyhole limpet haemocyanin-revusiran polyclonal antibody was used as a positive control. Serum samples were first screened for potentially positive samples. A potentially positive sample was defined as a sample that produced mean $A_{450\text{nm}} \geq$ the assay plate specific cut-point (PSCP). All serum samples considered potentially positive (Mean $A_{450\text{nm}} \geq$ PSCP) during the screening analysis were evaluated in a confirmatory assay. To be considered as a confirmed positive sample, the %signal inhibition between the spiked (spiked with revusiran)

and the unspiked sample had to be \geq the confirmatory cut point. All serum samples from the multiple dose cohorts were negative in the screening assay ($A_{450\text{nm}} < \text{PSCP}$) and therefore negative for anti-drug antibodies (data not shown).

References

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