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Safety, Pharmacokinetic, and Pharmacodynamic Evaluation of a 2′-(2-Methoxyethyl)-D-ribose Antisense Oligonucleotide–Triantenarry *N*-Acetyl-galactosamine Conjugate that Targets the Human Transmembrane Protease Serine 6[S]

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ABSTRACT

Cellular uptake of antisense oligonucleotides (ASOs) is one of the main determinants of in vivo activity and potency. A significant advancement in improving uptake into cells has come through the conjugation of ASOs to triantenarry *N*-acetyl-galactosamine ($GalNAc_3$), a ligand for the asialoglycoprotein receptor on hepatocytes. The impact for antisense oligonucleotides, which are already taken up into hepatocytes, is a 10-fold improvement in potency in mice and up to a 30-fold potency improvement in humans, resulting in overall lower effective dose and exposure levels. 2′-Methoxyethyl–modified antisense oligonucleotide conjugated to $GalNAc_3$ (ISIS 702843) is specific for human transmembrane protease serine 6 and is currently in clinical trials for the treatment of β-thalassemia. This report summarizes a chronic toxicity study of ISIS 702843 in nonhuman primates (NHPs), including pharmacokinetic and pharmacology assessments. Supratherapeutic doses of ISIS 702843 were well tolerated in NHPs after chronic dosing, and the data indicate that the overall safety profile is very similar to that of the unconjugated 2′-(2-methoxyethyl)-D-ribose (2′-MOE) ASOs. Notably, the $GalNAc_3$ moiety did not cause any new toxicities nor exacerbate the known nonspecific class effects of the 2′-MOE ASOs. This observation was confirmed with multiple $GalNAc_3$-MOE conjugates by querying a data base of monkey studies containing both $GalNAc_3$-conjugated and unconjugated 2′-MOE ASOs.

SIGNIFICANCE STATEMENT

This report documents the potency, pharmacology, and overall tolerability profile of a triantenarry *N*-acetyl-galactosamine ($GalNAc_3$)-conjugated 2′-(2-methoxyethyl)-D-ribose (2′-MOE) antisense oligonucleotide (ASO) specific to transmembrane protease serine 6 after chronic treatment in the cynomolgus monkey. Collective analysis of 15 independent $GalNAc_3$-conjugated and unconjugated 2′-MOE ASOs shows the consistency in the dose response and character of hepatic and platelet tolerability across sequences that will result in much larger safety margins for the $GalNAc_3$-conjugated 2′-MOE ASOs when compared with the unconjugated 2′-MOE ASOs given the increased potency.

Introduction

Single-strand antisense oligonucleotides (ASOs) containing 2′-*O*-methoxyethyl–modified sugars and phosphorothioate linkages [2′-(2-methoxyethyl)-D-ribose (2′-MOE)-ASOs] have achieved proof of concept in clinical trials for a range of diseases using either local or systemic routes of administration (Crooke et al., 2018). The majority of the systemically administered 2′-MOE ASOs target RNA transcripts in hepatocytes, a cell type proven to take up ASOs (Crooke 2008). Recent studies have shown that conjugation with triantennary *N*-acetyl-galactosamine ($GalNAc_3$), a high-affinity ligand for the hepatocyte-specific asialoglycoprotein receptor, can improve drug distribution to hepatocytes (Prakash et al., 2014; Henry et al., 2017; Tanowitz et al., 2017), thus enhancing the potency of the antisense oligonucleotides by 10- fold in mouse models (Prakash et al., 2014) and up to 30-fold in humans (Crooke et al., 2018). Achieving effective hepatocyte concentrations at lower doses has the potential to greatly improve the margins between pharmacologic and toxicologic exposure levels. However, these conjugated 2′-MOE ASOs must also be tested to determine whether there are unique safety issues to be considered.

β-Thalassemia and hemochromatosis are genetic disorders characterized by low levels of hepcidin, the hormonal regulator

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ABBREVIATIONS: ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; ASO, antisense oligonucleotide; AUC, area under the curve; $GalNAc_3$, triantenarry *N*-acetyl-galactosamine; IMTD, Integrated Monkey Toxicology Database; ISIS 702843, 2′-methoxyethyl–modified antisense oligonucleotide conjugated to $GalNAc_3$; 2′-MOE, 2′-(2-methoxyethyl)-D-ribose; NHP, nonhuman primate; PLT, platelet; T_{max}, time to reach C_{max}; TMPRSS6, transmembrane protease serine 6; TSAT, transferrin saturation; ULT, upper limit of normal.

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of iron homeostasis (Ganz and Nemeth, 2011). Suppressed hepcidin levels result in excessive dietary iron absorption and iron overload, the leading cause of morbidity and mortality in these patients. The transmembrane protease serine 6 (TMPRSS6) is predominantly expressed in hepatocytes and is a crucial negative regulator of hepcidin expression (Silvestri et al., 2008). Mouse and human genetic data indicate that reducing *TMPRSS6* in a nondiseased state causes iron-refractory iron-deficient anemia (Finberg et al., 2008). However, in mouse models of β-thalassemia and hemochromatosis, the genetic ablation of *TMPRSS6* has been shown to upregulate hepcidin expression and alleviate many of the disease symptoms that are also exhibited by patients (Finberg et al., 2010). Furthermore, the administration of a mouse-specific *Tmprss6* ASO to mouse models of β-thalassemia and hemochromatosis resulted in increases in hepcidin expression and consequential decreases in serum iron, transferrin saturation, and liver iron concentrations, reducing iron overload in these animals (Hung et al., 2013).

2′-Methoxyethyl–modified antisense oligonucleotide conjugated to $GalNAc_3$ (ISIS 702843) is a drug targeted to human *TMPRSS6*. The oligonucleotide portion of ISIS 702843 is a synthetic oligomer of 20 nucleotides that are connected sequentially by phosphorothioate and phosphodiester linkages (mixed backbone design). The mixed backbone design reduces the total number of phosphorothioate linkages, which reduces nonspecific interactions with proteins and further enhances the potency of $GalNAc_3$-conjugated ASOs (Levin et al., 2008; Geary, 2009; Wang et al., 2019). The nucleotide sequence of ISIS 702843 is complementary to a base-pair position 3162–3181 of the human *TMPRSS6* sequence Genbank NM_153609.2, which lies within exon 18. The ISIS 702843 nucleobase sequence is also homologous with an equal-length portion of monkey *TMPRSS6* mRNA, and the compound is active in this species. The five nucleotides at each of the 5′ and 3′ ends of ISIS 702843, which are also referred to as wings, have 2′-*O*-methoxyethyl–modified sugars. These wings flank a "gap" of ten 2′-deoxyribonucleosides that is necessary to support enzymatic cleavage of the mRNA (the chimeric design). This sugar modification pattern is also referred to as a 5-10-5 MOE gapmer. The hybridization of ISIS 702843 to *TMPRSS6* mRNA results in RNase H-mediated degradation of the cognate mRNA, reduced synthesis of the TMPRSS6 protein, and increased hepcidin levels. Therefore, ISIS 702843 could potentially be used in the clinic to reduce iron overload in patients with hemochromatosis and anemia in patients with β-thalassemia.

ISIS 702843 is representative of a total of 13 $GalNAc_3$-conjugated 2′-MOE ASOs that have now completed chronic monkey toxicology studies. All of the numerical serum chemistry and hematology data from these studies are entered into the Ionis internal Integrated Monkey Toxicology Database (IMTD) (Crooke et al., 2016; Henry et al., 2017). These data were queried to assess differences in select parameters often observed in the unconjugated 2′-MOE ASO monkey data base as well as for any potential new toxicology findings.

The primary objectives of this study in monkeys were to 1) assess the toxicologic effects associated with a high degree of *TMPRSS6* inhibition by ISIS 702843, 2) identify potential target organ effects specific for repeated administration of ISIS 702843 monkeys, 3) assess the reversibility of treatment-induced clinical and histopathological changes with ISIS 702843, and 4) query the available toxicology data base of common effects on clinical pathology parameters for $GalNAc_3$-conjugated versus unconjugated 2′-MOE ASOs.

This manuscript describes the results of a chronic toxicology study performed in cynomolgus monkeys in advance of the initiation of human clinical studies. This study also documented the pharmacokinetic and pharmacodynamic relationships.

Materials and Methods

Oligodeoxynucleotide Characteristics and Preparation. ISIS 702843 [5′-*O*-(6-{5-*N*-[tris({6-[(2-acetamido-2-deoxy-β-*D*-galactopyranosyl)oxy]-hexylamino}-3-oxopropoxymethyl)methyl]amino-5-oxopentanoyl}aminohexyl-1-phosphatyl)-2′-*O*-(2-methoxyethyl)-5-methyl-*P*-thiocytidylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)-5-methyluridylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)-5-methyluridylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)-5-methyluridylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)adenylyl-(3′-*O*→5′-*O*)-*P*-thiothymidylyl-(3′-*O*→5′-*O*)-*P*-thiothymidylyl-(3′-*O*→5′-*O*)-2′-deoxy-5-methyl-*P*-thiocytidylyl-(3′-*O*→5′-*O*)-2′-deoxy-5-methyl-*P*-thiocytidylyl-(3′-*O*→5′-*O*)-2′-deoxy-*P*-thioadenylyl-(3′-*O*→5′-*O*)-2′-deoxy-*P*-thioadenylyl-(3′-*O*→5′-*O*)-2′-deoxy-*P*-thioadenylyl-(3′-*O*→5′-*O*)-2′-deoxy-*P*-thioguanylyl-(3′-*O*→5′-*O*)-2′-deoxy-*P*-thioguanylyl-(3′-*O*→5′-*O*)- 2′-deoxy-*P*-thioguanylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)-5-methylcytidylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)adenylyl-(3′-*O*→5′-*O*)-2′-*O*-(2-methoxyethyl)-*P*-thioguanylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)-5-methyl-*P*-thiocytidylyl-(3′-*O*→5′-*O*)-2′-O-(2-methoxyethyl)-5-methyluridine, 20 sodium salt] is a $GalNAc_3$-conjugated 2′-MOE ASO (molecular formula $C_{296}H_{416}N_{80}Na_{20}O_{155}P_{20}S_{13}$; molecular mass 9071.13 Da). The oligonucleotide portion of the drug consists of 20 nucleotides with the sequence 5′-CTTTATTCCAAAGGGCAGCT-3′. Of the 19 internucleoside linkages, 13 are 3′-*O* to 5′-*O* phosphorothioate diesters, and 6 are 3′-*O* to 5′-*O* phosphate diesters. Ten of the 20 sugar residues are 2-deoxy-D-ribose, whereas the remaining 10 are MOE. The residues are arranged such that five 2′-MOE nucleosides at the 5′ and 3′ ends of the molecule flank a gap of ten 2′-deoxynucleosides. Each of the five cytosine bases are methylated at the 5′-position. ISIS 702843 targets exon 18 of human mRNA for *TMPRSS6* and is homologous to monkey *TMPRSS6* mRNA. The 5′ end of ISIS 702843 is conjugated via a phosphodiester bond to a triantennary cluster of $GalNAc_3$ sugars (Prakash et al., 2014). ISIS 702843 has a molecular mass of 9071.13 Da. The oligonucleotide was designed and synthesized by Ionis Pharmaceuticals (Carlsbad, CA) and then formulated in PBS at concentrations such that the appropriate amount of the test agent was delivered in a subcutaneous dose of 0.2 ml/kg b.wt. to monkeys. Subcutaneous injection sites were rotated between four designated sites on the back.

Animals, Husbandry, and Experimental Design. Forty-four male and 44 female cynomolgus monkeys (2–5 years of age, of Vietnamese origin) were used for this study. Animals were group-housed in pairs or trios of the same sex under conditions of controlled light, temperature, humidity, and room air circulation. Monkeys were acclimated to laboratory conditions during which time they were accustomed to handling/manipulation. The study was conducted at Covance Preclinical Services GmbH, Munster, Germany in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. These studies were approved by the Covance Institutional Animal Care and Use Committee prior to study initiation.

The main study and toxicokinetic monkeys were randomized to the experimental groups detailed in Table 1 and administered ISIS 702843 or vehicle. The initial 2 weeks of study served as a "loading period," during which animals received subcutaneous doses (0, 2, 6, 12, or 30 mg/kg) twice per week. After that, the monkeys received once-weekly subcutaneous injections for a total of 13 (0, 2, 6, 12, or 30 mg/kg) or 39 (0, 2, 6, or 12 mg/kg) weeks, and this was followed by a 26-week treatment-free recovery period. The 30-mg/kg dose level was only

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TABLE 1
Protocol outline and animal disposition for 13-wk toxicity studies of ISIS 702843 in male and female cynomolgus monkeys

Group	Number M/F	Dose (*mg/kg*, s.c.)[a]	Dose Regimen[a]	Number of Animals Sacrificed (M/F)		
				Interim (Day 93)	Terminal (Day 275)	Recovery (Day 455)
Monkeys, main study						
1	9/9	0	biw/q1w	3/3	4/4	2/2
2	7/7	2	biw/q1w	3/3	4/4	
3	7/7	6	biw/q1w	3/3	4/4	
4	9/9	12	biw/q1w	3/3	4/4	2/2
5	5/5	30	biw/q1w	3/3	—	2/2[b]
Monkeys, toxicokinetics						
6	7/7	2	biw for 2 wk	1/sex/interval on days 3, 16, 23, 30, 37, 51, and 72		

F, female; M, male.
[a]ISIS 702843 was administered by subcutaneous injection twice per week (biw) for the first 2 wk (days 1, 5, 9, and 14) and then once weekly (q1w) for the remainder of the study.
[b]Group 5 recovery animals were sacrificed after a 6-mo recovery period on the same day as the terminal animals from groups 1 to 4 (study day 275, recovery day 184).

dosed for 13 weeks because it was well above (~90-fold) the planned clinical dose. It was included to document any adverse effects but not necessary to carry out such a high dose for the full chronic duration of 39 weeks.

A single group of monkeys (seven of each sex) was designated for toxicokinetic analyses. Each monkey was administered 2 mg/kg twice per week for 2 weeks on days 1, 5, 9, and 14, and then they were sacrificed (one per sex per interval) for tissue collection on experimental days 3 (after a single dose), 16, 23, 30, 37, 51, and 72.

Toxicokinetic Analyses. Plasma concentrations of ISIS 702843 were determined at multiple time points after administering the drug to cynomolgus monkeys (detailed in Table 1). Plasma samples were analyzed using a hybridization enzyme-linked immunosorbent assay (Yu et al., 2002). Briefly, plasma samples (25 μl) were first treated with proteinase K to mitigate potential interference from antidrug antibodies. The full sequence ISIS 702843 in samples was hybridized to a complementary cutting probe (702843 cutRP), which was biotinylated at the 5′ end and labeled with digoxigenin on the 3′ end of the sequence. The hybridized solution was then placed in a NeutrAvidin-coated ELISA plate. After binding had taken place, S1 nuclease was added to digest the unhybridized probes. An anti-digoxigenin antibody conjugated to alkaline phosphatase was used to detect the hybridized analyte by binding to digoxigenin. AttoPhos was used as a substrate for fluorometric readout. Sample concentrations were determined by interpolation from the standard curve, which was fit using a four-parameter logistic function with a $1/\text{response}^2$ weighting to fit the response (Relative Fluorescence Units) versus concentration of ISIS 702843. Tissues from monkeys were collected and analyzed for ISIS 702843 concentrations using a validated HPLC-MS/MS (high-performance liquid chromatography tandem mass spectrometry) method (Murphy et al., 2005). Briefly, using 25 mg of tissue in 250-μl aliquot volume of monkey tissue homogenate, tissue samples were prepared with an initial phenol/chloroform extraction followed by a solid phase extraction and then analyzed using HPLC-MS/MS. Chromatographic separation was performed with a gradient system at a flow rate of 0.300–0.425 ml/min using an ACQUITY UPLC OST C18 column heated to 60°C with 400 mM hexafluoroisopropanol/15 mM TEA in water and methanol as the mobile phase with a 5-μl injection volume. Using a similar ASO as an internal standard, ISIS 758113 concentrations were calculated using Analyst software 1.5.1/1.5.2 and Watson Bioanalytical LIMS 7.4.1 with a quadratic regression using the least squares method (with $1/x^2$ weighting). Plasma sample analyses were conducted at PPD, Inc. (Richmond, VA), and tissue sample analyses were conducted at CMIC Co Ltd. (Hoffman Estate, IL). Plasma and tissue sample analyses were performed based on the principles and requirements described in 21 CFR (Code of Federal Regulations) part 58. The lower limits of quantitation were 1.295 ng/ml and 0.700 μg/g in plasma and tissue, respectively.

ISIS 702843 plasma concentration-time data were analyzed by noncompartmental (individual animal profiles) methods using the computer program WinNonlin Professional, Version 5.2 (Pharsight Corporation, Mountain View, CA). The C_{max} and the time to reach C_{max} (T_{max}) were obtained directly from the concentration-time data. The plasma disposition half-life ($t_{1/2\lambda z}$) associated with the terminal elimination phase was calculated using the equation, $t_{1/2\lambda z} = 0.693/\lambda_z$, wherein λ_z is the rate constant associated with the terminal elimination phase. A minimum of three data points was used to define the rate constants (α or λ_z), and the correlation of regression (rsq) had to be greater than 0.8 for the estimate to be accepted. The area under the plasma concentration-time curve from zero time (predose) to 48 hours ($AUC_{0\rightarrow 48\ h}$) was calculated using the linear trapezoidal rule. Tissue concentrations were reported as observed by dose and time of sample collection, and summary statistics of the concentration data are reported.

Pharmacologic Demonstration. The effect of ISIS 702843 on the expression of hepatic *TMPRSS6* mRNA was assessed in liver samples collected during the interim (week 13) and terminal (week 39) necropsies. Approximately 100 mg of frozen liver tissue was homogenized in guanidinium isothiocynate with 8% β-mercaptoethanol. The resulting lysate (30 μl) was applied to Qiagen RNeasy 96-well plate for mRNA purification according to the manufacturer's instructions. After purification, the mRNA was subjected to reverse-transcription polymerase chain reaction analysis. The Life Technologies ABI StepOne Plus Sequence Detection System, which uses real-time fluorescence polymerase chain reaction, was used to quantify TMPRSS6 mRNA levels. The assay is based on a target-specific probe labeled with a fluorescent reporter and quencher dyes at opposite ends. The probe is hydrolyzed through the 5′ exonuclease activity of Taq DNA polymerase, leading to an increasing fluorescence emission of the reporter dye that can be detected during the reaction. *TMPRSS6* mRNA level was normalized to total mRNA using RiboGreen fluorescence from the same mRNA sample. The following primer/probe set was used to measure *TMPRSS6* mRNA levels: forward primer 5′-CAA AGCCCAGAAGATGCTCAA-3′, reverse primer 5′-GGAATAGACGGA GCTGGAGTTG-3′, and probe 5′-FAM-ACCAGCACCCGCCTGG-GAACTT-IOWA-BLACK-3′.

Toxicology Assessments. Antemortem evaluative criteria included survival, clinical signs, injection site observation, body weight and food consumption, ophthalmologic examinations, clinical pathology (hematology, clinical chemistry, coagulation, and urinalysis tests), immunotoxicology assessment (complement analysis, cytokine/chemokine analysis, and T cell–dependent antibody response), and toxicokinetic analyses.

Monkeys also received ECG and blood pressure evaluations prior to initiation of dosing, during weeks 13 and 39 and at the end of the recovery. ECG tracings were examined for qualitative abnormalities, heart rate; RR, PR, QRS, and QT intervals were determined; and corrected QT (QT_{cB}) values were derived.

The conduct and data collection for all routine observations and physical and clinical examinations were accomplished in compliance with the principles of Good Laboratory Practices according to the standard operating procedures established at the performing laboratory.

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Necropsy and tissue collection for microscopic examinations of main study monkeys at the interim, terminal, and recovery phases were conducted on study days 93, 275, and 455, respectively. The numbers of animals necropsied on specific study days are shown in Table 1. Animals were fasted overnight, sedated with ketamine, and terminated by a pentobarbitone overdose before exsanguination.

Grossly visible lesions, injection sites, and representative sections of approximately 45–50 organs/tissues were collected from each animal. Testes and epididymides were fixed in modified Davidson's fluid, and the eyes and optic nerves were fixed in Davidson's fixative. All other tissue samples were preserved in neutral buffered formalin. Specimens were routinely processed, and the full range of tissues was examined microscopically.

Statistical Analyses. Data are presented by the incidence of events and descriptive summary statistics of laboratory test results. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test to detect differences among groups. Outcomes were considered statistically significant when the P value was less than 0.05 ($P < 0.05$), with the exception of the analysis of *TMPRSS6* mRNA data, in which statistical significance was indicated by $^{**}P < 0.001$ and $^{*}P < 0.01$ (Fig. 2).

Monkey Toxicology Database. Composition of the proprietary IMTD (Crooke et al., 2016; Henry et al., 2017). The data contained within the IMTD are comprised of studies with unconjugated 2′-MOE ASOs and $GalNAc_3$-conjugated 2′-MOE ASOs. In general, each study consisted of four to five groups (three to nine animals per sex per group), including a vehicle control (PBS) group. The route of administration was primarily subcutaneous injection; however, intravenous infusion (1 hour) was used in a few studies. The dose regimen varied from study to study from primarily once-weekly administration (2′-MOE ASOs and $GalNAc_3$-conjugated 2′-MOE ASOs) to once-monthly administration ($GalNAc_3$-conjugated 2′-MOE ASOs only). The maintenance doses in animals ranged from 0.25 to 40 mg/kg per week for up to 52 weeks of repeat-dose treatment. During the treatment period, animals were observed daily for clinical signs of toxicity, and body weight was collected weekly. Alanine aminotransferase (ALT) activities and platelet (PLT) counts were determined at least once before the first dose and at several time points during the dosing and recovery phases of the study using established methods. ALT activities were deemed elevated if they were higher than the upper limit of normal (90 IU/l, calculated as two S.D. over the mean of all NHPs prior to treatment in the IMTD). For animals that experienced marked reductions in PLT count (e.g., <50 K cells/μl), dosing was withheld to assess the recovery of PLT counts, and in some cases, dosing recommenced as PLT counts recovered to levels supportive of normal hemostasis. Individual animal data numerical values are used to populate the IMTD.

Nonclinical NHP study data were imported from an IMTD and analyzed using JMP 15 (SAS, Cary, NC). The data base consists of ALT activities and PLT counts measured from NHPs treated with 127 different 2′-MOE chimeric ASOs (2234 NHPs) and 15 different $GalNAc_3$-conjugated 2′-MOE ASOs (852 NHPs), with lengths ranging from 14 bases up to 20 bases long, and tested in a total of 97 studies. A total of 3869 NHPs included in the data base were treated with either saline or ASOs (783 and 3086 animals, respectively). All of the data in the IMTD are from studies conducted at multiple contract research organizations with Asian-sourced NHPs (from China, Cambodia, and Vietnam).

Results

Systemic Exposure of Monkeys to ISIS 702843. Toxicokinetic analyses verified systemic exposures of monkeys to ISIS 702843 (Table 2). There were no sex-related differences in monkeys as reported previously ((Yu et al., 2007)Geary *et al.*, 2003; Yu *et al.*, 2007b; Wang *et al.*, 2019), and data were pooled across the sexes to simplify graphic and tabular representation.

ISIS 702843 was readily absorbed from subcutaneous administration sites with C_{max} values attained in 1.0–4.0 hours postdose and then decreased in an apparent multiexponential fashion with time. Systemic exposure, as quantified by $AUC_{0-48\ h}$, increased supradose proportionally as the dose increased from 2 to 30 mg/kg. The estimated mean terminal elimination plasma half-life value ($t_{1/2}$) was approximately 16.7 days after four doses of 2-mg/kg dose on days 1, 5, 9, and 14. Plasma ISIS 702843 concentration profiles for the cynomolgus monkeys are presented in Fig. 1. The plasma concentration profiles on day 1 and day 91 were similar, and there was no evidence of significant accumulation of the drug based on plasma AUC or C_{max}. However, it should be noted that there was drug accumulation in plasma trough and tissue concentrations due to the long half-life of the drug in plasma and in tissues as reported for this class of drugs (Wang et al., 2019).

After subcutaneous injection, ISIS 702843 primarily distributed to the kidney cortex and liver (Table 3), where the

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TABLE 2

Summary of selected plasma toxicokinetic parameters of total full-length ASO (ISIS 702843 equivalent) after single and multiple subcutaneous administrations in cynomolgus monkeys (sexes combined)

Group	Dose Level (mg/kg)[a]	N	Study Day	No. of Doses	C_{max} ($\mu g/ml$)	T_{max} (h)	$AUC_{0\to48\ h}$ ($\mu g*h/ml$)
2	2	14	1	1	2.19 ± 0.749	1 (1, 2)	5.21 ± 1.21
		6	91[bb]	15	1.82 ± 0.947	1 (1, 2)	5.41 ± 1.11
		8	273[cc]	41	1.13 ± 0.623	2 (1, 2)	5.24 ± 2.77
3	6	14	1	1	9.63 ± 2.39	2 (1, 2)	34.7 ± 7.09
		6	91[b]	15	8.00 ± 2.27	2 (1, 2)	28.4 ± 4.92
		8	273[c]	41	6.43 ± 2.94	2 (1, 2)	38.2 ± 16.4
4	12	18	1	1	20.5 ± 3.88	2 (1, 4)	96.8 ± 14.4
		6	91[b]	15	15.0 ± 5.87	1.5 (1, 2)	74.8 ± 19.7
		12	273[c]	41	20.5 ± 11.6	3 (2, 4)	185 ± 145
5	30	10	1	1	51.5 ± 10.6	4 (2, 4)	310 ± 29.8
		10	91[b]	15	31.3 ± 6.15	2 (2, 6)	263 ± 34.6
		NSC	NSC	NSC	NSC	NSC	NSC

NSC, no sample collected.
Values are presented as mean ± S.D. except T_{max}, which is presented as median (minimum, maximum).
[a]Monkeys were dosed by subcutaneous administration on days 1, 5, 9, and 14 only, and this was followed by once-weekly doses thereafter.
[b]Last dose before interim necropsy in interim-necropsied animals only.
[c]End of treatment; samples collected from all remaining animals.

parent drug (i.e., the unconjugated form of ISIS 702843) was released rapidly upon hydrolysis once internalized. Concentrations of unconjugated ISIS 702843 in the kidney cortex were dose-dependent. However, the increase was less than dose-proportional over the dose range studied. Concentrations of unconjugated ISIS 702843 in the kidney cortex increased approximately 4- to 8-fold over a 15-fold increase in dose, suggesting a saturation of the kidney uptake (Table 3), whereas concentrations of unconjugated ISIS 702843 in the liver increased approximately 5- to 6-fold over the dose range studied. The elimination of unconjugated ISIS 702843 from tissues was slow. The tissue half-lives of unconjugated ISIS 702843 were 23.2 and 17.5 days in the monkey kidney cortex and liver, respectively, which were estimated based on tissue concentration data from monkeys in the toxicokinetic group after multiple 2 mg/kg subcutaneous drug administration (Supplemental Fig. 1).

Pharmacologic Effects. Monkeys exhibited dose-dependent decreases in hepatic *TMPRSS6* mRNA levels, reflecting the intended pharmacologic activity of ISIS 702843. After 13 weeks of treatment, *TMPRSS6* mRNA levels were reduced by approximately 60%–90% when compared with control group mean (40%–10% of control mRNA levels) over the dose range tested (2–30 mg/kg per week; Fig. 2). After 39 weeks of treatment, greater hepatic *TMPRSS6* mRNA reduction was observed at the low dose of 2 mg/kg per week (approximately 80% reduction versus 60% at 13 weeks). Similar levels of reduction were achieved after 3 and 9 months of treatment at the higher doses (Fig. 1; Table 1). After a 26-week treatment-free recovery period, animals that had been treated with 12 mg/kg per week for 39 weeks or with 30 mg/kg per week for 13 weeks still exhibited a reduced *TMPRSS6* mRNA level of approximately 40% (Fig. 2).

Consistent with reduced *TMPRSS6* mRNA, the administration of ISIS 702843 caused decreases in serum iron and changes in other iron-related and hematology parameters. These changes included dose-dependent decreases in the indicators of circulating red cell mass (red blood cell, hemoglobin, hematocrit, etc.) and dose-dependent changes in iron metabolism parameters [serum iron concentration, percent transferrin saturation (TSAT), and unsaturated iron-binding capacity] (Figs. 3 and 4). Decreased iron concentration was observed in both sexes as early as day 23 (down to −69.5% and −75.8% in males and females, respectively) at ≥2 mg/kg per week. There was a corresponding decrease in %TSAT and a corresponding increase in unsaturated iron-binding capacity (Fig. 3). Secondary to these changes were dose-dependent decreases in various hematology parameters [hemoglobin, hematocrit (unpublished data), mean corpuscular hemoglobin, mean corpuscular volume, and increases in absolute reticulocyte count] (Fig. 4).

Toxicology Assessments. No changes attributable to administration of ISIS 702843 occurred in clinical observations, body weights, food consumption, ECG and blood pressure evaluations, ophthalmology, cytokine/chemokine, and T cell–dependent antibody response. Other than the changes in hematology and serum iron parameters mentioned above, which were related to the pharmacologic action of ISIS 702843, there were no significant changes in serum chemistry or hematology parameters. Only minimal changes in liver enzymes (ALT/AST activities) and no decreased platelet count (no count below $200 \times 10^3/\mu l$) in any monkey throughout the 9-month treatment period or during recovery were observed (Fig. 5; Supplemental Tables 1 and 2).

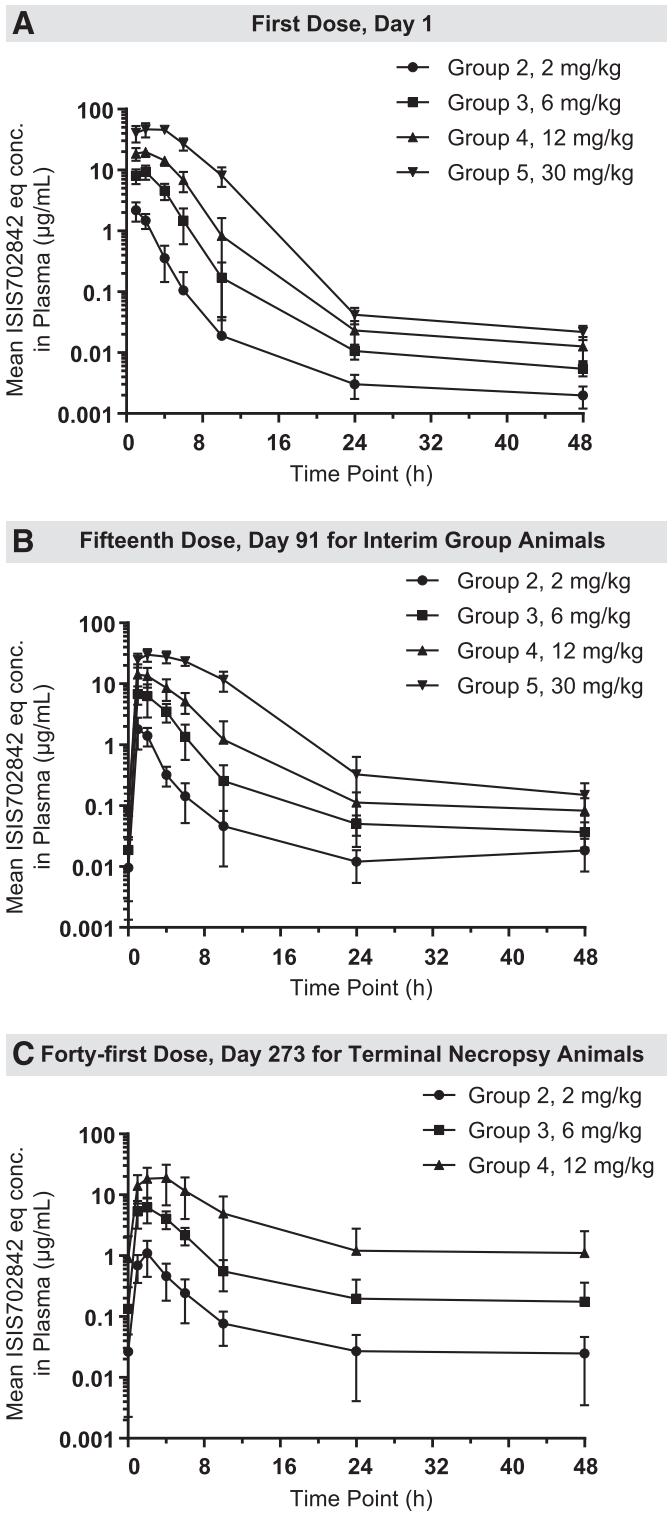

Fig. 1. Mean plasma concentrations of total full-length ISIS 702843 in monkeys (sexes combined) for up to 48 hours after subcutaneous administration at 2–30 mg/kg after single (A) and multiple doses on days 91 (B) and 273 (C). Symbols represent group means, and error bars represent S.D. (n = 6–14).

Transient prolongation of activated partial thromboplastin time (APTT) and alternative complement pathway activation are commonly observed in monkeys treated with oligonucleotides having phosphorothioate internucleoside linkages at doses ≥12 mg/kg per week (Henry et al.,1997, 2008; Sheehan

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TABLE 3
Tissue conc. (microgram per gram)[a] of unconjugated ISIS 702843 measured 48 h after multiple subcutaneous dose administration(s) in cynomolgus monkeys

Group	Dose Level (*mg/kg*)	No. of Doses	Day	Time after Last Dose (*days*)	*N*	Unconjugated ISIS 702843 (*μg/g*)[a]	
						Kidney Cortex	Liver
2	2	15	93	2	6	138 ± 40.7	248 ± 82.7
		41	275	2	8	155 ± 61.7	354 ± 94.8
3	6	15	93	2	6	445 ± 26.3	622 ± 60.2
		41	275	2	8	484 ± 140	906 ± 112
4	12	15	93	2	6	578 ± 58.3	853 ± 128
		41	275	2	8	882 ± 400	1684 ± 663
		41	455	182	4	6.48 ± 3.83	29.9 ± 17.5
5	30	15	93	2	6	1137 ± 177	1496 ± 348
		15	275	184	4	15.4 ± 7.16	71.2 ± 50.0

[a]Values are presented as mean ± S.D.

and Phan, 2001). There was a minimal transient prolongation of APTT and no acute alternative complement pathway activation (no increase in Bb, a split product of complement factor B) after administration of ISIS 702843 (unpublished data). The absence of the typical effects on APTT or alternative complement pathway activation is likely due to the lesser degree of plasma protein binding as a result of the mixed backbone design of ISIS 702843 compared with other unconjugated 2′-MOE ASOs (Yu et al., 2007; Wang et al., 2019). ISIS 702843 has 6 phosphodiester (PO) and 13 PS linkages versus 19 PS linkages in typical unconjugated 2′-MOE ASOs and in vitro protein binding of 702843 in monkey was 97.4% versus >99% for typical 2′-MOE ASOs (Watanabe et al., 2004). Upon macroscopic examination at the 13-week interim necropsy, one male monkey that received a dose of 30 mg/kg had enlarged draining lymph nodes (correlating microscopically with moderate accumulation of macrophages containing granular material), and another male at the same dose revealed increased hepatic size (corresponding to microscopically evident moderate Kupffer cell hypertrophy). At the terminal (9-month) necropsy, one female that received a dose of 12 mg/kg was observed to have liver mottling (correlating with hepatocyte vacuolation), whereas enlargement of draining lymph nodes was observed in one female that received 2 mg/kg (correlating with increased lymphocytes) and another female that received 6 mg/kg (correlating with granular macrophages).

Administration of 30 mg/kg per week of the drug also caused a 1.3-fold increase in mean liver weights, which resolved after a 13-week recovery period (Supplemental Table 3).

Treatment-related microscopic changes were observed, mainly in animals treated with ≥6 mg/kg per week. In the liver, these findings were comprised of basophilic granules in Kupffer cells and hepatocytes and Kupffer cell hypertrophy (Table 4). Basophilic granules in Kupffer cells were observed in males and females in the 6, 12, and 30 mg/kg groups, whereas hepatocellular granules occurred in one female in the 30 mg/kg group. Kupffer cell hypertrophy occurred in animals treated with 6 to 30 mg/kg in both sexes and showed a dose-dependent increase of severity. In the kidney, basophilic granules in tubular cells (minimal) were observed in both sexes in the 30 mg/kg group. Accumulation of macrophages containing granular material occurred in mesenteric, mandibular, and draining (axillary) lymph nodes. Generally, there was a dose-dependent increase of incidence and severity of this finding. Macrophages containing granular material were observed at all dose levels in the mesenteric and axillary lymph nodes and at 6, 12, and 30 mg/kg in the mandibular lymph nodes (Supplemental Table 4). These findings were considered consistent with uptake and accumulation of the oligonucleotide at peripheral organ storage depots and are not considered adverse (Henry et al., 2008; Lenz et al., 2018).

Subcutaneous injection sites in this study were characterized by the presence of minimal mononuclear cell infiltrates oriented primarily around small dermal blood vessels. Across chronic studies in monkeys, injection sites consistently have a perivascular mononuclear cell infiltrate of minimal or slight severity with variable incidence and severity of hemorrhage, edema, and mixed inflammatory cell infiltrates that tend to be related to injection trauma rather than to the particular antisense oligonucleotide.

Thirty-nine consecutive weeks of treatment with ISIS 702843 at doses up to 12 mg/kg per week resulted in similar

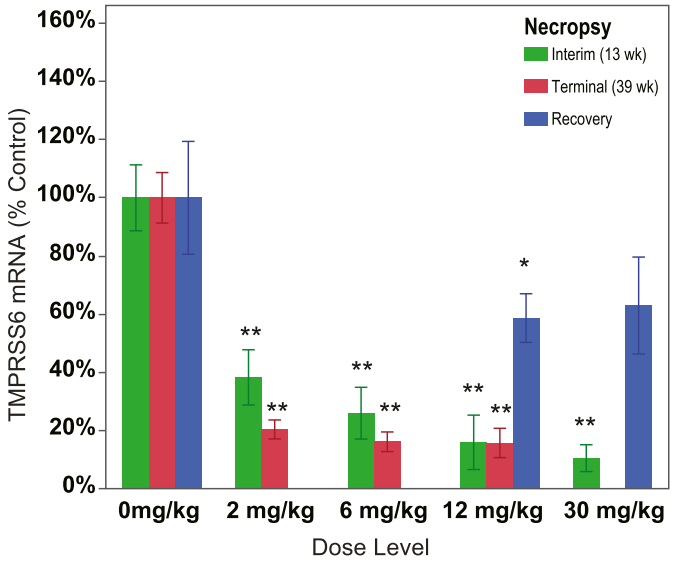

Fig. 2. *TMPRSS6* mRNA reduction in liver in male and female (sexes combined) cynomolgus monkeys administered ISIS 702843 13 or 39 weeks and after a 26-week treatment-free recovery period. Error bars represent S.D. (n = 6 at 13 weeks, n = 8 at 39 weeks, and n = 4 at recovery). Data were analyzed by one-way ANOVA with post hoc Dunnett's method, $^{**}P < 0.001$; $^{*}P < 0.01$.

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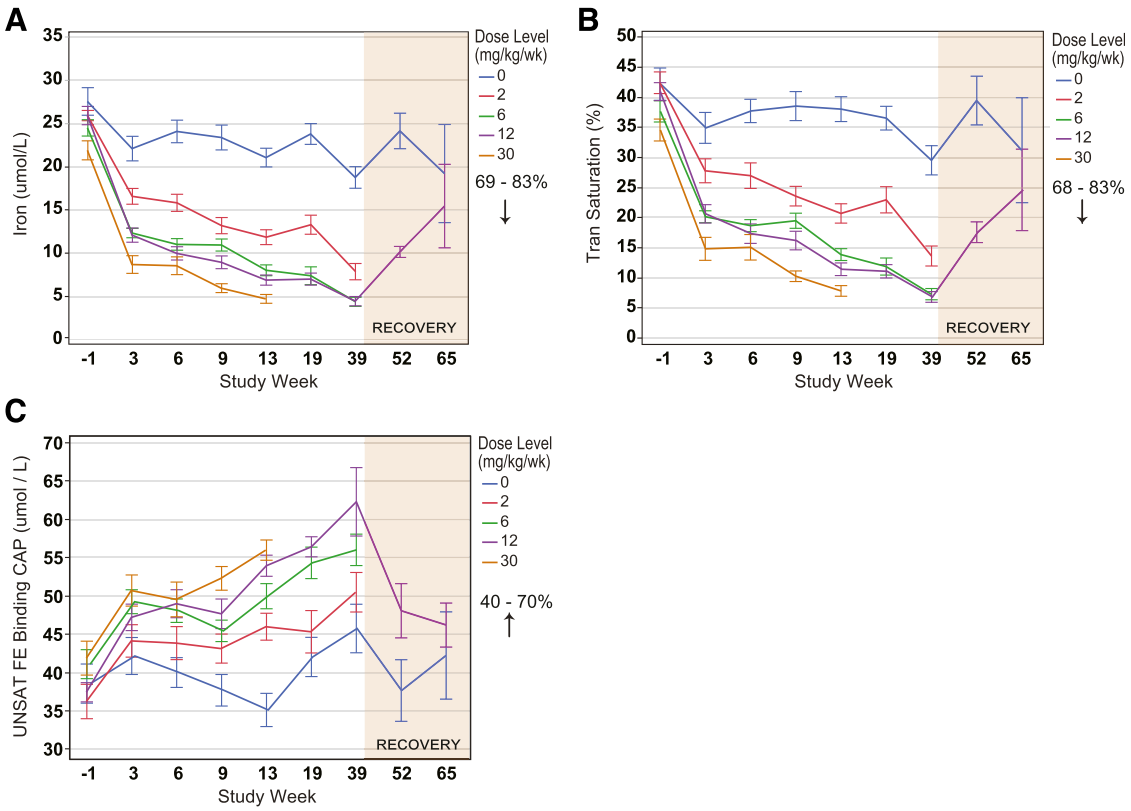

Fig. 3. Changes in serum iron parameters due to *TMPRSS6* inhibition. Treatment of monkeys with ISIS 702843 resulted in changes in (A) serum iron (69%–83% decrease), (B) transferrin saturation (68%–83% decrease), and (C) unsaturated iron-binding capacity (UNSAT FE Binding CAP) (40%–70% increase) over the dose range tested (2–30 mg/kg per week). Symbols and lines represent group mean values, and error bars represent S.D. (n = 6 to 14 per time point).

findings to those observed at 13 weeks with no obvious progression of lesions.

Reversibility was assessed by a 26-week treatment-free recovery period in a subset of animals that were treated with 30 mg/kg per week for 13 consecutive weeks or with 12 mg/kg per week for 39 consecutive weeks. All test article–related findings had resolved except for basophilic granules in lymph nodes and hepatocytes. These findings were of reduced incidence and severity, indicating partial recovery, and are evidence of the prolonged half-life of the ASO.

IMTD Queries. The IMTD currently has data from 852 total monkeys treated with 1 of 15 individual GalNAc$_3$-conjugated ASOs, with exposure durations ranging from 3 to 9 months. This data base also has data from 2234 total monkeys treated with 1 of 127 unconjugated ASOs. Data from vehicle-treated monkeys across multiple studies were used to determine the control reference ranges (785 individual monkeys). This data base contains the individual animal clinical pathology (hematology and serum chemistry) data at baseline and at multiple time points during treatment. For the purposes of this paper, the data base was analyzed to determine the incidence of increased ALT activities or reduced PLT count. Since ISIS 702843 was dosed once-weekly, only those compounds in the data base that were dosed weekly (4× per month) were compared. The ALT data were queried to address the impact of targeted delivery of ASOs to hepatocytes, which impacts the proportional distribution between parenchymal and nonparenchymal cells as well as the hepatocyte concentrations. It is important to remember that the compounds tested were screened and selected for clinical development, so they represent sequences with optimal tolerability properties (i.e., little to no sequence-specific hepatotoxicity in mice). The queries were performed to look at the incidence of ALT activities that were above the upper limit of normal (ULN) or 3-fold above the ULN (Table 5). Based on the available vehicle control serum chemistry data, the average ALT activity was 44 U/l, the ULN was defined as 90 U/l, and 3-fold above the ULN was defined as 270 U/l. For the vehicle control animals, the incidence of above ULN and 3-fold above ULN ALT activity was approximately 12% and 1%, respectively. Among the 15 2′-MOE–GalNAc$_3$ ASO compounds, the doses used in the toxicology studies covered the same range as studied for the unconjugated MOE ASOs despite the lower effective doses used in clinical studies. At doses below 10 mg/kg per week, the incidence of above ULN and 3-fold above ULN were approximately the same as the control for both the unconjugated MOE and the GalNAc$_3$-conjugated MOE ASOs. At doses ≥10 mg/kg per week, there was a slight increase in the incidence of ALT activities above ULN with an approximate incidence of 20% at doses ≥20 mg/kg per week. However, there was no noticeable increase in the incidence of ALT activities that were 3-fold above ULN (Table 5). Interestingly, there was no apparent difference in the incidence of increased ALT activities when comparing the unconjugated with the GalNAc$_3$-conjugated 2′-MOE ASOs. TheALT data, along with lack of adverse morphologic changes in the liver suggest that, despite the higher concentrations and greater fractional distribution to

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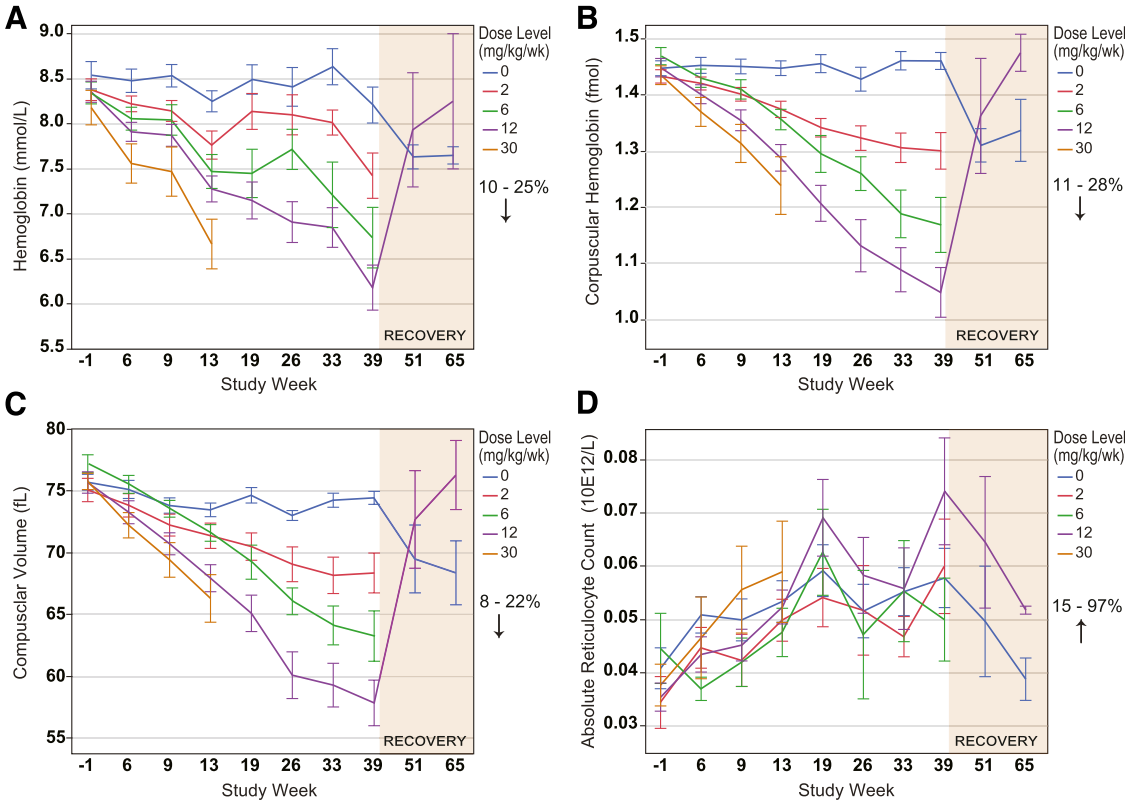

Fig. 4. Changes in hematology parameters due to *TMPRSS6* inhibition. Treatment of monkeys with ISIS 702843 resulted in decreases ranging from 7% to 28% in (A) hemoglobin, (B) corpuscular hemoglobin, and (C) corpuscular volume and increases in (D) absolute reticulocyte count (15%–97% increase) over the dose range tested (2–30 mg/kg per week). Symbols and lines represent group mean values, and error bars represent S.D. (n = 6 to 14 per time point).

hepatocytes for these $GalNAc_3$-conjugated compounds selected for human testing, were tolerated well in monkeys. The PLT values were queried to compare with the PLT data within the unconjugated MOE ASO monkey data base (Table 6). Vehicle-treated monkeys had a 0.5% incidence of PLT counts <50,000 cells/μl and roughly a 3% incidence of PLT counts between 50,000 and 200,000 cells/μl. For $GalNAc_3$-MOE ASOs, there were no cases of severely reduced PLT counts (<50,000 cells/μl) in monkeys treated with <10 mg/kg per week and the 4.4%–5.7% incidence of PLT count between 50,000 and 200,000 cells/μl was similar to the control incidence and the unconjugated MOE incidence (5.1%–5.4%). At doses ≥10 mg/kg/kg, the overall incidence of severely reduced PLT counts (<50,000 cells/μl) for $GalNAc_3$-MOE ASOs (5.3%–6.9%) was similar or less than reported for the unconjugated MOE ASOs (5.6%–7.8%) (Table 6). The incidence of PLT counts between 50,000 and 200,000 cells/μl at doses ≥10 mg/kg/kg was also similar between the $GalNAc_3$-MOE ASOs (9.6%–12.5%) and the unconjugated MOE ASOs (11.5%–12.2%). This observation indicates that the $GalNAc_3$ conjugation does not alter the effect of the MOE ASO on PLT counts and argues that the PLT effect is not related to hepatic distribution and is likely a nonspecific effect of the ASO in individual monkeys (Narayanan et al., 2018).

Discussion

Cellular uptake of antisense oligonucleotides is one of the main determinants of its activity and potency. Cellular uptake of unconjugated 2′-MOE ASOs occurs through receptor-mediated protein-binding interactions in a wide range of cells. However, uptake is often not an efficient process (Bennett et al., 1998). One recent significant advancement in improving uptake into cells has come through the conjugation of ASOs to triantenarry *N*-acetyl-galactosamine, which is a ligand for the asialoglycoprotein receptor on hepatocytes (Prakash et al., 2014, 2016). The impact for ASOs, which are already taken up into hepatocytes, was a 10-fold improvement in potency in mouse models (Prakash et al., 2014) and up to a 30-fold potency improvement in humans (Crooke et al., 2018), resulting in overall lower dose and exposure. Utilizing ligand-receptor–mediated uptake rather than relying on the nonspecific protein binding of the phosphorothioate backbone for uptake of unconjugated 2′-MOE ASOs has also allowed an increase in the phosphodiester content of the oligonucleotide backbones to further reduce the nonspecific protein binding and improve overall safety and efficacy properties without sacrificing activity.

ISIS 702843 represents these compounds with respect to activity, pharmacokinetics, and tolerability in monkeys. ISIS 702843 is optimized for human RNA binding but is homologous and active in monkeys. After 39 weeks of treatment, the *TMPRSS6* mRNA level was reduced by approximately 80% at doses of ≥2 mg/kg per week. The reduction of *TMPRSS6* mRNA was accompanied by dose-related decreases in iron metabolism parameters (serum iron concentration, percent TSAT, and unsaturated iron-binding capacity) and hematology parameters associated with iron levels (hemoglobin,

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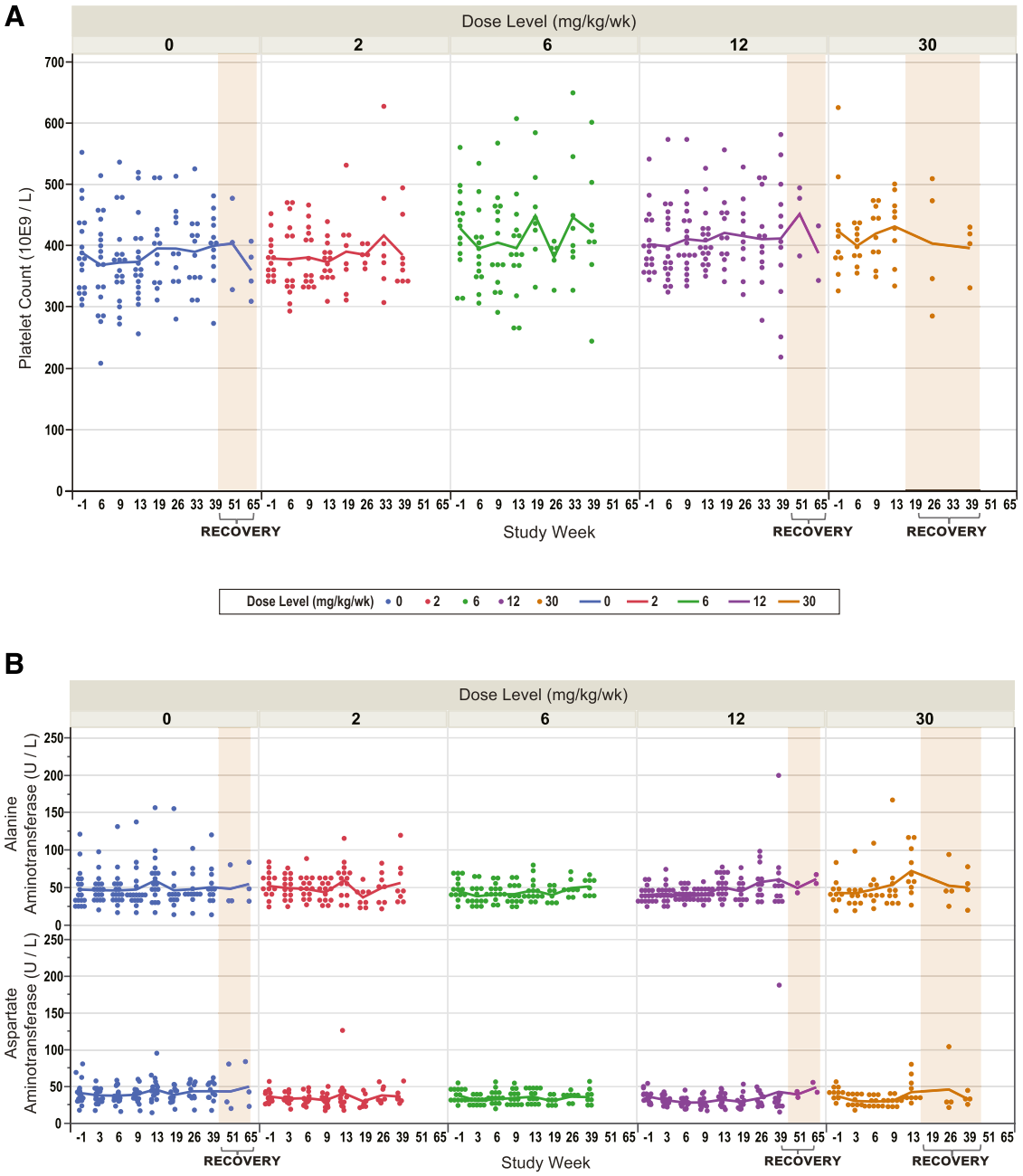

Fig. 5. ISIS 702843 was well tolerated through 39 weeks of dosing. No changes in platelet count (A) or in the liver enzymes ALT/AST (B) were observed in monkeys after 3 months of treatment with ISIS 702843 at doses up to 30 mg/kg per week or after 9 months of treatment at doses up to 12 mg/kg per week. Symbols represent individual animals, and lines represent group means (n = 6 to 14 per time point).

hematocrit, mean corpuscular hemoglobin, etc.). The *TMPRSS6* mRNA levels were also evaluated after 13 weeks of dosing, which indicated reductions of approximately 62%, 74%, 84%, and 90% at doses of 2, 6, 12, and 30 mg/kg per week, respectively. By comparison, an unconjugated 2′-MOE ASO targeting factor XI achieved only a ~50% reduction in monkeys at 4 mg/kg per week after 13 weeks of treatment and a maximum of 80% reduction at 40 mg/kg per week (Younis et al., 2012). More convincing data in terms of potency improvement for GalNAc$_3$ ASOs over unconjugated ASOs came from PD data from multiple clinical studies combined (Crooke et al., 2018). For several GalNAc$_3$-conjugated 2′-MOE ASOs, the ED$_{50}$ is between 0.06 and 0.2 mg/kg per month (4–10 mg per week), which are 20- to 30-fold lower than the ED$_{50}$ for unconjugated 2′-MOE ASOs (Viney et al., 2016; Crooke et al., 2018).

Altered and more-rapid distribution to the hepatocytes is the primary impact of GalNAc$_3$ conjugation on 2′-MOE ASO pharmacokinetics/absorption, distribution, metabolism, and excretion (Wang et al., 2019). This results in a reduced C_{max} and AUC exposure for GalNAc$_3$-conjugated ASOs like ISIS 702843 as compared with the unconjugated 2′-MOE ASOs in monkeys at similar dose levels (Yu et al., 2007; Crooke et al., 2019; Wang et al., 2019). The dose range studied in the monkey toxicology study for ISIS 702843 is very similar to the range of doses studied for unconjugated 2′-MOE ASOs.

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TABLE 4
Microscopic findings in the liver after 13 or 39 wk of treatment with ISIS 702843 and after a 26-wk recovery period

Incidence of Liver Findings: Interim (Day 92/Week 13) Necropsy											
		Males					Females				
Tissue and finding	Level (*mg/kg*)	0	2	6	12	30	0	2	6	12	30
Liver	No. examined	3	3	3	3	3	3	3	3	3	3
Basophilic granules, hepatocyte	Grade, minimal	—	—	—	—	—	—	—	—	—	1
Liver	No. examined	3	3	3	3	3	3	3	3	3	3
Basophilic granules, Kupffer cell	Grade, minimal	—	—	1	2	3	—	—	3	3	3
Liver	No. examined	3	3	3	3	3	3	3	3	3	3
Hypertrophy, Kupffer cell	Grade, minimal	—	—	1	—	—	—	—	2	3	—
	Slight	—	—	—	2	1	—	—	1	—	—
	Moderate	—	—	—	—	2	—	—	—	—	1
	Marked	—	—	—	—	0	—	—	—	—	2
Incidence of Liver Findings: Terminal (Day 275/Week 39) Necropsy											
		Males					Females				
Tissue and finding	Level (*mg/kg*)	0	2	6	12		0	2	6	12	
Liver	No. examined	4	4	4	4		4	4	4	4	
Basophilic granules, hepatocyte	Grade, minimal	—	—	1	3		—	—	4	3	
Liver	No. examined	4	4	4	4		4	4	4	4	
Basophilic granules, Kupffer cell	Grade, minimal	—	—	4	4		—	1	4	4	
Liver	No. examined	4	4	4	4		4	4	4	4	
Hypertrophy, Kupffer cell	Grade, minimal	—	—	2	—		—	1	—	1	
	Slight	—	—	2	—		—	—	1	—	
	Moderate	—	—	—	1		—	—	1	2	
	Marked	—	—	—	3		—	—	2	1	
Incidence of Liver Findings: Terminal (Day 275/Week 39) Necropsy											
		Males					Females				
Tissue and finding	Level (*mg/kg*)	0	2	6	12		0	2	6	12	
Liver	No. examined	2	—	—	2		2	—	—	2	
Basophilic granules, hepatocyte	Grade, minimal	—	—	—	—		—	—	—	1	
Liver	No. examined	2	—	—	2		2	—	—	2	
Basophilic granules, Kupffer cell	Grade, minimal	—	—	—	—		—	—	—	—	
Liver	No. examined	2	—	—	2		2	—	—	2	
Hypertrophy, Kupffer cell	Grade, minimal	—	—	—	—		—	—	—	—	
Incidence of Liver Findings: Recovery (Day 455/Week 65) Necropsy											
		Males					Females				
Tissue and finding	Level (*mg/kg*)	0	2	6	12		0	2	6	12	
Liver	No. examined	2	—	—	2		2	—	—	2	
Basophilic granules, hepatocyte	Grade, minimal	—	—	—	—		—	—	—	1	
Liver	No. examined	2	—	—	2		2	—	—	2	
Basophilic granules, Kupffer cell	Grade, minimal	—	—	—	—		—	—	—	—	
Liver	No. examined	2	—	—	2		2	—	—	2	
Hypertrophy, Kupffer cell	Grade, minimal	—	—	—	—		—	—	—	—	

—, finding not present.

Investigation with other representative $GalNAc_3$-conjugated 2′-MOE ASOs has shown that the molecules circulate in plasma as the conjugated version, but once taken up into hepatocytes, the $GalNAc_3$ sugar moiety is rapidly cleaved and metabolized, liberating the free oligonucleotide (Shemesh et al., 2016). Concentrations in the liver and kidney remain the highest among the tissues examined, indicating both $GalNAc_3$ and phosphorothioate backbone-mediated uptake pathways. The main difference is the relatively higher liver concentrations compared with the kidney for ISIS 702843 across the dose range studied. By comparison, unconjugated 2′-MOE ASOs generally had 3- to 5-times-higher kidney concentrations than liver concentrations (Henry et al., 2012). However, kidney concentrations may still remain higher for other $GalNAc_3$-conjugated ASOs, particularly at higher dose levels.

Administration of suprapharmacological doses of ISIS 702843 of up to 30 mg/kg per week for 12 weeks or up to 12 mg/kg per week for 39 consecutive weeks elicited no grossly observable signs of systemic toxicity in monkeys (doses are approximately 40- to 100-fold greater than the likely human dose). There were no adverse toxicities observed, including no reduction in PLT count, no substantive increase in ALT activities, and little to no effect on APTT or acute activation of the alternative complement pathway. The reduced effect on APTT and complement is likely due to the lesser degree of plasma protein binding as a result of the mixed phosphodiester backbone design of ISIS 702843 compared with other unconjugated 2′-MOE ASO, which are typically full phosphorothioate backbones (Henry et al., 2014). A secondary benefit of targeting uptake with the $GalNAc_3$ conjugate is that the phosphorothioate content can be reduced, reducing some of the nonspecific effects of protein binding (Watanabe et al., 2004).

Hepatic tolerability in monkeys has been one of the main focuses of the nonclinical safety evaluation of ISIS 702843 and other $GalNAc_3$-conjugated 2′-MOE ASOs. Histologically, changes reflecting the uptake and accumulation of ISIS

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TABLE 5
Incidence of ALT values (U/l) above either ULN or 3× ULN: GalNAc$_3$-MOE versus unconjugated MOE ASOs

Conjugation	Dosing Frequency	ULN (0-ULN-3× ULN)	Dose Range (*mg/kg per week*) 0.25 to <5 Column %	*N*	5 to <10 Column %	*N*	10 to <20 Column %	*N*	20 to 40 Column %	*N*
GalNAc$_3$-MOE	4× per mo	3× >ULN	1.1%	2	0.0%	0	2.1%	3	0.5%	1
		>ULN	10.1%	18	12.1%	17	16.2%	23	21.1%	42
		Normal	88.8%	158	87.9%	123	81.7%	116	78.4%	156
		Total *N* =		178		140		142		199
Conjugation	Dosing Frequency	ULN (0-ULN-3× ULN)	Dose Range (*mg/kg per week*) 0.25 to <5 Column %	*N*	5 to <10 Column %	*N*	10 to <20 Column %	*N*	20 to 40 Column %	*N*
MOE	4× per mo	3× >ULN	0.0%	0	0.3%	1	0.7%	3	1.8%	14
		> ULN	11.4%	32	9.4%	28	14.5%	62	20.9%	160
		Normal	88.6%	249	90.3%	270	84.8%	362	77.3%	591
		Total *N* =		281		299		427		765
Conjugation	Dosing Frequency	ULN (0-ULN-3× ULN)	Dose Range (*mg/kg per week*) 0.25 to <5 Column %	*N*						
Vehicle	4× per mo	3× >ULN	0.8%	6						
		>ULN	11.5%	89						
		Normal	87.8%	682						
		Total *N* =		**777**						
ALT (U/l)	Upper limit of normal	90								
	3× ULN	270								
	Avg.	44								
	S.D.	23								
	N animals at baseline	3377								

702843 were observed in the liver at doses ≥6 mg/kg per week. These changes included basophilic granules in Kupffer cells and hepatocytes of the liver. Tissue depots of ISIS 702843 and other antisense oligonucleotides are visualized microscopically as basophilic granules that are most prominent in the kidney, liver, and spleen (Henry et al., 2008). For unconjugated 2′-MOE ASOs, basophilic granules in the liver are typically observed only in Kuppfer cells, but their presence in hepatocytes after ISIS 70243 treatment likely reflects increased uptake of the GalNAc$_3$ conjugate into hepatocytes due to the abundance of the asialoglycoprotein receptor on the cell surface (Prakash et al., 2014; Tanowitz et al., 2017). Despite the high liver concentrations and the observation of basophilic granules in hepatocytes, there were no significant increases in liver enzymes nor were there any degenerative changes in the hepatocytes, thus indicating good hepatocyte tolerability.

The hepatic tolerability of ISIS 702843 is representative of the experience to date of other GalNAc$_3$-conjugated 2′-MOE ASOs that meet the selection criteria for human clinical trials. A low incidence of ALT activities above the ULN in monkeys being handled for dosing and blood collection is not uncommon, with about a 10%–12% incidence being attributed to background or skeletal muscle sources. A data base query of monkeys receiving 20–40 mg/kg per week of various GalNAc$_3$-conjugated 2′-MOE ASOs indicated there was a slight increase in the incidence of ALT activities >ULN but no apparent increased incidence of ALT activities that were >3× ULN. A similar level of increased incidence of ALT activities >ULN was also seen in a query of the unconjugated 2′-MOE ASOs. This slight increase in ALT activities likely reflects a treatment-related effect. However, it is not clear whether this is attributed to greater hepatocyte concentrations

TABLE 6
Incidence of platelet counts <50 K cells/μl: GalNAc$_3$-MOE versus unconjugated MOE ASOs

Conjugation	Dosing Frequency	PLT (0-50-200 K) *cells/μl*	Dose Range (*mg/kg per week*) 0.25 to <5 Column %	*N*	5 to <10 Column %	*N*	10 to <20 Column %	*N*	20 to 40 %	*N*
GalNAc$_3$-MOE	4× per mo	Below 50,000	0.0%	0	0.0%	0	6.9%	10	5.3%	10
		Below 200,000	5.7%	10	4.4%	6	12.5%	18	9.6%	18
		Normal (>200,000)	94.3%	164	95.6%	130	80.6%	116	85.1%	160
		Total *N* =		174		136		144		188
Conjugation	Dosing Frequency	PLT (0-50-200K) *cells/μl*	Dose Range (*mg/kg per week*) 0.25 to <5 Column %	*N*	5 to <10 Column %	*N*	10 to <20 Column %	*N*	20 to 40 %	*N*
MOE	4× per mo	Below 50,000	1.7%	5	3.0%	10	7.8%	37	5.6%	46
		Below 200,000	5.4%	16	5.1%	17	12.2%	58	11.5%	94
		Normal (>200,000)	92.9%	273	92.0%	309	80.0%	380	82.9%	677
		Total *N* =		294		336		475		817

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or possibly reflects other inflammatory effects in the liver at these higher doses.

Tolerability in nonhepatic tissues was typical of the experience with unconjugated 2′-MOE ASOs, which reflects the fact that the high dose across the chemical platforms is the same in this case (high doses of 12–30 mg/kg per week). Kidney, for example, has basophilic granules in the proximal tubular epithelium consistent with tissue concentrations of 800–1100 μg/g.

There were no cases of thrombocytopenia and no trend toward reduced platelet count in monkeys treated with ISIS 702843 at any dose or duration studied. Across all of the $GalNAc_3$-conjugated 2′-MOE ASOs, there was an increased incidence of platelet nadirs below 200,000 cells/μl (slightly below the normal range in our data base) or below 50,000 cells/μl (severely reduced PLT count). This dose-dependent increase was similar to the incidence of low platelet nadirs for unconjugated 2′-MOE ASOs (Henry et al., 2017). These data indicate the potential for moderate or severe thrombocytopenia in monkeys treated with higher doses of 2′-MOE ASOs. However, the incidence and dose response have not been affected by $GalNAc_3$ conjugation. Careful examination of the data revealed the incidence of low platelet count appeared to be in compounds with a full phosphorothioate backbone rather than the mixed phosphodiester backbones, which is consistent with an inflammation-mediated mechanism (Frazier, 2015). As mentioned previously, ISIS 702843 is a mixed backbone ASO in which there are 6 phosphodiester (PO) and 13 PS linkages in the backbone versus 19 PS linkages in typical unconjugated 2′-MOE ASO. The mixed backbone design confers fewer protein-binding properties as compared with a full PS backbone 2′-MOE ASO (Watanabe et al., 2004), which also may also play a role in the decreased incidence of low platelet count observed with ISIS 702843. However, there are still mixed backbone sequences that have had an incidence of PLT count <50,000. Thus, the $GalNAc_3$-MOE ASOs associated with low PLT count likely have greater inflammatory activity whether they are attributed to the sequence or backbone chemistry.

This report summarizes the pharmacokinetics and tolerability profiles of ISIS 702843, a representative $GalNAc_3$-conjugated 2′-MOE ASO, in cynomolgus monkeys. This report also indicates that the overall safety profile, based on the evaluation of two key target organ parameters (ALT and PLT), is very similar to that of the unconjugated 2′-MOE ASOs. Notably, the $GalNAc_3$ moiety did not cause any new toxicities nor exacerbate the known nonspecific class effects of the 2′-MOE ASOs. This observation was confirmed with multiple $GalNAc_3$-MOE conjugates by querying a data base of monkey studies containing both $GalNAc_3$-conjugated and unconjugated 2′-MOE ASOs. These data, along with additional unpublished data from multiple $GalNAc_3$-MOE ASOs, have indicated that the no-adverse-effect levels for the $GalNAc_3$-MOE ASOs have been similar to those for the unconjugated 2′-MOE ASOs. This is largely due to the increased distribution of the $GalNAc_3$-conjugated ASOs to hepatocytes while maintaining similar ASO concentrations in plasma and nonhepatic tissues as compared with the unconjugated ASOs. However, because of increased potency, the human doses of the $GalNAc_3$-MOE conjugates are much lower than the doses of unconjugated 2′-MOE ASOs. Therefore, the use of $GalNAc_3$-MOE–conjugated ASOs results in much larger safety margins when compared with the unconjugated 2′-MOE ASOs.

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Authorship Contributions

Participated in research design: Zanardi, Guo, Henry.

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Performed data analysis: Zanardi, Korbmacher, Boone, Burel, Prill, Guo.

Wrote or contributed to the writing of the manuscript: Zanardi, Korbmacher, Boone, Engelhardt, Wang, Aghajan, Guo, Henry.

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