Integrated Assessment of the Clinical Performance of GalNAc₃-Conjugated 2′-O-Methoxyethyl Chimeric Antisense Oligonucleotides: I. Human Volunteer Experience

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Advances in medicinal chemistry have produced new chemical classes of antisense oligonucleotides (ASOs) with enhanced therapeutic properties. Conjugation of the triantennary N-acetylgalactosamine (GalNAc₃) moiety to the extensively characterized phosphorothioate (PS)-modified 2′-O-methoxyethyl (2′MOE) ASO exemplifies such an advance. This structure-activity optimized moiety effects receptor-mediated uptake of the ASO prodrug through the asialoglycoprotein receptor 1 to support selective targeting of RNAs expressed by hepatocytes. In this study we report the integrated assessment of data available from randomized placebo-controlled dose-ranging studies of this chemical class of ASOs administered systemically to healthy human volunteers. First, we compare the pharmacokinetic and pharmacodynamic profiles of a subset of the GalNAc₃-conjugated PS-modified 2′MOE ASOs to the parent PS-modified 2′MOE ASOs for which plasma analytes are available. We then evaluate the safety profile of the full set of GalNAc₃-conjugated PS-modified 2′MOE ASO conjugates by the incidence of signals in standardized laboratory tests and by the mean laboratory test results as a function of dose level over time. With hepatocyte targeted delivery, the ED₅₀ for the GalNAc₃-conjugated PS-modified 2′MOE ASO subset ranges from 4 to 10 mg/week, up to 30-fold more potent than the parent PS-modified 2′MOE ASO. No GalNAc₃-conjugated PS-modified 2′MOE ASO class effects were identified from the assessment of the integrated laboratory test data across all doses tested with either single or multidose regimens. The increase in potency supports an increase in the safety margin for this new chemical class of ASOs now under broad investigation in the clinic. Although the total exposure is limited in the initial phase 1 trials, ongoing and future investigations in patient populations will support evaluation of the effects of long-term exposure.

Keywords: antisense, oligonucleotide, assessment, clinical trials, triantennary N-acetylgalactosamine

Introduction

Today, five antisense oligonucleotides (ASOs) have been commercialized and one is under review, with indications as diverse as spinal muscular atrophy (SMA) to hereditary transthyretin amyloidosis (hATTR). Scores of other ASOs are progressing in development, representing a number of different chemical and mechanistic classes [1]. ASOs have been administered to humans by intravitreal, intrathecal, intravenous, subcutaneous (SC), and intramuscular injections, orally, and by inhalation and enema [2]. Thus, the potential of antisense technology is being realized while the technology continues to advance and produce steadily better performing agents [3].

Therapeutic oligonucleotides consist of three key elements, the hydrophobic nucleobase, a carbohydrate or modified carbohydrate, and a phosphate or phosphate analog. The nucleobase identifies target sites in RNAs through Watson–Crick hybridization. Optimal affinity and selectivity are conferred by 16–20 nucleotides [4]. In principle, 11–12 nucleotides should confer acceptable selectivity, but even using high RNA affinity oligonucleotides, the affinity is such that potency is inadequate in vivo, for review see Crooke et al. [5]. The carbohydrate is usually a deoxy ribose, a ribose, or a modified ribose. Substitutions at the 2′-position of the ribose alter the structure of the sugar and thus can be used to enhance affinity for target RNA sequences and hence potency. Modified sugars can also contribute a substantial increase in
The fourth, nusinersen (Spinraza), is a fully Synthetic and are administered by weekly SC injection [14–16]. Three, mipomersen, volanesorsen, and inotersen, are designed to exploit RNase H1-mediated RNA target reduction in humans. These ASOs have been the most thoroughly evaluated [1,2]. Three ASOs have been commercialized, and one (volanesorsen) is under review in Europe, Canada, and the United States. Three, mipomersen, volanesorsen, and inotersen, are designed to exploit RNase H1-mediated RNA target reduction and the ability to cross membrane structures because of protein binding [7]. A minimum of 12 PS residues is sufficient to result in plasma protein binding, thus avoiding rapid excretion by the kidney [1]. The PS moiety also results in binding to proteins on the cell surface and in the cell, for review see Refs. [1,2,13].

Double-stranded oligonucleotides, siRNAs, are highly hydrophilic and thus do not enter cells or organs without complex delivery systems, such as lipid nanoparticles, or conjugation of ligands that take advantage of high-capacity receptor systems, such as asialglycoprotein receptors as is the case in hepatocytes for N-acetylgalactosamine (GalNAc), for review see Refs. [3,11,12]. In contrast, single-strand oligonucleotides (ASOs or single-stranded siRNAs) are amphiphatic and thus can cross membranes without the need of formulations or transfection, for review see Refs. [1,2,13].

Of the chemical classes that have been studied, 2’MOE ASOs have been the most thoroughly evaluated [1,2]. Three 2’MOE ASOs have been commercialized, and one (volanesorsen) is under review in Europe, Canada, and the United States. Three, mipomersen, volanesorsen, and inotersen, are designed to exploit RNase H1-mediated RNA target reduction and are administered by weekly SC injection [14–16]. The fourth, nusinersen (Spinraza), is a fully 2’MOE-modified ASO designed to alter the splicing of a specific pre-mRNA and administered intrathecally every 4 months to treat patients with SMA [17–19].

The pharmacokinetics of PS-modified 2’MOE chimeric or “gapmer” ASOs have been extensively characterized in animals and humans. These ASOs are called chimeric or gapmer ASOs because they are designed to exploit RNase H1-mediated cleavage of RNA and thus have a central region (usually 8–10 nucleotides) of PS deoxynucleotides (DNA like) flanked by 2’MOE PS residues on the 5’ and 3’ poles, for review see Refs. [1,10,20]. Absorption and distribution are defined by the PS moieties and thus are similar for PS gapmers and fully 2’-modified PS ASO used to alter splicing or effect translation inhibition or polyadenylate site masking or upstream open-reading frame or translation inhibitory structure-enhanced translation [5,21,22]. After SC dosing, absorption of these ASOs is nearly 100%. The absorption phase half-life is ~60 min in humans and the distribution half-life is 30–60 min, for review see Refs. [1,23]. The pharmacokinetics of these ASOs are remarkably consistent in all species tested [mice, rats, dogs, nonhuman primates (NHPs), and humans].

Plasma protein binding is critical to distribution and these drugs bind to a number of plasma proteins, but the main plasma repository is albumin. The dissociation constant (K_d) for albumin varies from species to species, the degree of lipidation of albumin, the method used to measure binding and, of course, the number of PSs, but for an ASO that has 19 PS residues, the K_d is 140–150 μM. This binding is sufficient to prevent rapid renal clearance and yet the affinity is weak enough to support disproportionation between albumin and capillary, interstitial, and cell surface proteins, for review see Refs. [1,2]. These ASOs are widely distributed at lower doses to liver, kidney, fat cells, the spleen, and bone marrow [7,24]. At higher doses, the primary tissues saturate and these ASOs can then accumulate in secondary tissues [3,23]. Suborgan pharmacokinetics are well characterized in animals for the liver and kidney [25–27]. The liver accumulates ~20% of a total systemic dose achieving ~200–400 μg/g liver in PS 2’MOE ASO concentration at therapeutic doses [28–30]. These ASOs distribute to all the major cell types in the liver with nonparenchymal cells accumulating most of the liver’s ASO levels.

Elimination half-lives from all tissues including the liver are long, 2–4 weeks. Elimination is effected by endonuclease cleavage in the DNA gap for gapmers resulting in half molecules that may be further degraded by exonucleases. As these degradates have only eight to nine PS moieties, they are rapidly cleared by glomerular filtration [7,31,32]. These drugs do not interact with cytochrome p450 enzymes [33]. They do not bind to drug binding sites on albumin, so there are limited drug–drug interactions [34].

Because members of a chemical class share similar properties, we have constructed databases that integrate all safety and tolerability observations from toxicity studies in NHPs and all randomized double-blind placebo-controlled trials for each chemical class of ASOs we are developing. Although the potency of 2’MOE ASOs has increased compared with mipomersen because of more effective screening, the potency of more recently discovered 2’MOE ASOs are consistent with ED_50s of ~150–200 mg/week for targets in the liver [3,35]. Of course, the pharmacokinetic properties of this chemical class are also similar as discussed previously. Similarly, the safety and tolerability profiles have been characterized.

Three publications that summarize the analyses of the integrated safety database that include results from >2,600 subjects treated with 16 2’MOE ASOs systemically in 52 completed clinical trials have been published [36–38]. These publications established the clinical safety of the 2’MOE ASO and highlighted two adverse events, thrombocytopenia and exacerbation of renal dysfunction in patients with hATTR and thrombocytopenia in patients with familial chylomicronemia syndrome that seem to be the result of unique interactions between two different ASOs at doses of 300 mg/week in two unique patient populations. It is important to emphasize that only members of the same chemical
class share similar properties and that even what may seem minor chemical differences, for example, 2’MOE versus 2’-O-methyl (2’OMe), can greatly influence the behavior of ASOs. Therefore, the comments above refer strictly to the properties of members of the 2’MOE chemical class.

More recently, conjugation of triantennary N-acetylgalactosamine (GalNAc3) to 2’MOE ASOs has been shown to increase productive delivery of ASOs to the liver resulting in substantial increases in potency for hepatocyte produced target RNAs [1,39–44]. Today ten 2’MOE ASOs conjugated with a GalNAc3 moiety are in development and 9 have completed at least a 4-week clinical trial in normal volunteers. In total, >600 subjects have been exposed to this chemical class with >200 subjects exposed for 6 months or longer. The purpose, therefore, of this publication is to provide an initial assessment of the performance GalNAc3-conjugated 2’MOE ASOs after weekly or monthly SC dosing in healthy human volunteers. The experience in NHPs with this chemical class will be the subject of a separate report.

Materials and Methods

Clinical trial protocols were approved by the respective institutional review boards, or independent ethics committees. All studies complied with the guidelines of the Declaration of Helsinki and the International Conference on Harmonization Guidelines on Good Clinical Practice. Written informed consent was obtained from all participants before participation in the study. All studies were dose ranging and included a placebo-control group. The route of study drug administration was by SC injection in all protocols.

Bioanalytical methods

Human plasma drug concentrations were determined for each ASO using hybridization-based bioanalytical methods [45]. For GalNAc3-conjugated 2’MOE ASOs, the assay quantitated full-length ASOs (including fully conjugated, partially conjugated with 1, 2, or 3-sugar deletions, and unconjugated ASO). All plasma sample analyses were performed based on the principles and requirements described in 21 Code of Federal Regulations Part 58.

Pharmacokinetic analysis

Noncompartmental analysis methods were used for pharmacokinetic characterization of the plasma concentration data (Phoenix WinNonlin v.6 or higher; Certara, L.P., Cary, NC). Plasma pharmacokinetic parameters reported include peak plasma concentration (C_max) and time to C_max (T_max), AUC_{0-24h}, clearance at steady state (CL_{ss}/F), and apparent terminal elimination half-life (t_{1/2a}).

Dose–response analysis

The relationship between each pharmacodynamic biomarker response (i.e., the individual plasma target protein levels) and dose in human following multiple dose treatments (typically 2 weeks after the last treatment) was analyzed with an inhibitory E_max model using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA) by the following equation: E = 100/(1 + 10^((X - LogED_{50}))), where E is the measured response (% baseline), X is the weekly dose, and ED_{50} is the weekly dose that produced 50% of maximum drug-induced effect.

Safety assessments

Samples were collected before dosing for standard clinical laboratory tests and collected at several time points within a 24-h period after SC dose administration for coagulation tests and complement split products. Data were imported from individual study data sets into one SAS data set for each laboratory test [46].

Injection site reactions (ISRs) were defined as injection site erythema, injection site swelling, injection site pruritus, injection site pain or tenderness that started the day of SC injection, and persisted (start to stop) for 2 days or more. Flu-like symptoms were defined as either (1) influenza-like illness or (2) pyrexia, feeling hot or body temperature increased, plus at least two of the following: chills, myalgia, or arthralgia that started on the day of injection or the next day.

Statistics

Evaluable subjects were those who received at least one dose of study drug. Data are presented by the incidence of events and descriptive summary statistics of laboratory test results. All study data were included for analysis of the incidence of events, except where noted. The baseline was defined as the last nonmissing value before the first dose. An event was defined as data falling outside the normal range or reaching the specified threshold, as defined by protocol stopping rules, standard reporting, or Grade 3 criteria provided by the Food and Drug Administration in Guidance to Industry for healthy adults and adolescents [47].

The over-time analysis of laboratory test results included all study data up to last visit in the single dose regimen cohort, and up to 10 days after the last dose in the multidose regimen cohort. The lower-limit-of-normal and upper-limit-of-normal (ULN) displayed in the mean results over time figures represent the median values (Supplementary Table S1). If there was no reference range available, then the range was calculated as the (mean ±2×SD) of the baseline values in the data set.

A meta-analysis using subject-level data was performed to compare ASO-treated dose groups to placebo group on the multidose regimen test results during the treatment period, defined as the period from first dose to up to 10 days after last dose. The endpoints evaluated were the absolute change from baseline. The data were compared between GalNAc3-conjugated 2’MOE ASO dose groups and placebo using an analysis of covariance model with the dose group and trial as factors and baseline value as a covariate.

Results

To compare the behaviors of the parent PS 2’MOE ASOs with the GalNAc3-conjugated drugs, we performed similar randomized placebo-controlled dose-ranging phase 1 studies in normal volunteers. Single doses of 5–120 mg and multiple doses of 10–120 mg were evaluated in these studies. The multiple dose regimens included six weekly SC doses (Supplementary Table S2), and we also evaluated monthly
doses for the GalNAc₃-conjugated 2'MOE ASOs. Because GalNAc₃-conjugated PS-modified 2'MOE ASOs are substantially more potent for liver targets, they were administered at lower doses than the parent PS-modified 2'MOE ASOs.

Comparison of the pharmacokinetic properties of PS 2'MOE parents with the GalNAc₃ conjugates

Figure 1 gives a schematic of the primary mechanism of entry into hepatocytes for GalNAc₃-conjugated 2'MOE

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| Administered Dose | Parent PS 2'MOE ASO | GalNAc₃ Conjugate | Relative Exposure |
|-------------------|---------------------|-------------------|------------------|
|                   | Plasma C<sub>max</sub> µg/mL | 0.89 | 0.006 |
|                   | AUC µg·h/mL | 166 | 0.5 |
| Liver Distribution | % Total | Hepatocytes | Non-Hepatocytes | Hepatocytes | Non-Hepatocytes |
|                   | | 12% | 88% | 80% | 20% |
| Liver Concentration (est. from NHP) µg/g | 48 | 349 | 14 | 4 |
| Total = 397 µg/g | Total = 18 µg/g |
| Kidney Concentration (est. from NHP) µg/g | 580 | 14 | 14 |
| Total = 580 µg/g | Total = 14 µg/g |

FIG. 1. GalNAc₃-conjugated PS 2'MOE ASOs demonstrate an increased fraction of total liver distribution to hepatocytes compared with parent 2'MOE ASOs. (a) Targeted hepatocyte delivery by the asialoglycoprotein receptor, and (b) estimation of relative exposure at therapeutic doses. 2'MOE, 2'-O-methoxyethyl; ASO, antisense oligonucleotide; PS, phosphorothioate.
Molecular target indicates the RNA target, e.g. mRNA or pre-mRNA. Symbols and nomenclature are based on HUGO standards.

ASOs and the estimated exposure compared with the parent. We have shown that liver concentrations can be estimated by plasma trough concentrations by combining these calculations with liver subfractionate studies conducted in animals to estimate total liver and hepatocyte/nonhepatocyte concentrations [39,48,49]. The lower doses used for GalNAc3-conjugated PS-modified 2MOE ASOs result in lower peak plasma concentrations (Cmax) and plasma concentration area under the curves (AUCs). The fraction of dose distributed to the liver of GalNAc3-conjugated PS 2MOE ASOs is similar to the parent PS-modified 2MOE ASOs; however, ~80% of the total drug in the liver is delivered to the hepatocytes for the GalNAc3 conjugates, in contrast to ~12% with the parent ASOs. This is the reason GalNAc3-conjugated ASOs are so much more potent for hepatocyte targets than the parent ASOs. In addition, GalNAc3-conjugated 2MOE ASOs achieve an equivalent potency at hepatocyte concentrations that are twofold to threefold lower than that of the parent. Therefore, in addition to enhancing total delivery to hepatocytes, more of the ASO is internalized in hepatocyte through “productive” pathways, that is, pathways that deliver ASOs to sites within the cell where target RNAs can be hybridized with the ASO. This has been shown conclusively in vitro and in animals [39,44,48,49].

Table 1 gives the GalNAc3-conjugated PS-modified 2MOE ASOs for which phase 1 data were available for safety and tolerability assessments. Directly assayable plasma analytes were available to assess pharmacodynamic effects for four GalNAc3 conjugates, ANGPTL3-L, Apo(a)-L, ApoIII-L, and FB-L, with data available to support a direct comparison of the properties of the GalNAc3 versions with the parent molecules for three conjugates, ANGPTL3, Apo(a), and ApoIII (volanesorsen). Thus, we focused on these ASOs for pharmacokinetic and pharmacodynamic comparisons.

As given in Table 2, the elimination half-lives of a representative parent PS-modified 2MOE ASOs and the respective GalNAc3 conjugate were similar. Of course, the Cmax
and AUC values were lower for the GalNAc<sub>3</sub> conjugates, reflecting the lower dose, and the times to peak plasma concentrations (T<sub>max</sub>) were similar (as given in Fig. 2). However, distribution from plasma for the GalNAc<sub>3</sub> version was more rapid as shown by the approximately fivefold higher rate of clearance from plasma (CL<sub>ss/F</sub>). The more rapid distribution phase may suggest more rapid extraction from plasma by tissues, but this explanation remains speculative. The extent of plasma protein binding by the GalNAc<sub>3</sub> conjugates was similar to the parent 2′MOE ASOs in all species tested (mouse, 94%–99%; monkey, 97%–99%; humans, 96%–99%). The other pairs of PS-modified 2′MOE ASOs studied behaved similarly (data not shown).

**Pharmacodynamics**

The most striking difference between the parent and GalNAc<sub>3</sub> versions is potency (Fig. 3). The ED<sub>50</sub>s for the parents range from 120 to 210 mg/week, whereas the ED<sub>50</sub>s for the GalNAc<sub>3</sub> versions range from 4 to 10 mg/week. The ED<sub>50</sub> for target reduction for nearly all the PS 2′MOE ASOs are in this range. The consistency of performance of PS 2′MOE ASOs and the GalNAc<sub>3</sub> versions is remarkable and consistent in species ranging from mouse, NHPs, to humans. This consistency in pharmacokinetics and pharmacodynamics supports many drug development efficiencies. To compare the pharmacokinetic properties.
FIG. 4. Pharmacokinetic and pharmacodynamic profile of GalNAc3-conjugated PS 2'MOE ASO in humans after monthly SC doses on study days 1, 29, 57, and 85. (a) Mean plasma drug concentration–time profiles with intensive pharmacokinetic sampling on study days 1 and 85, predose, and ~0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 48-h after the respective first and last SC injections. (b) Mean percent change from baseline over time in plasma prekallikrein levels in subjects who received monthly doses of PKK-L compared with pooled placebo-treated subjects. Blue arrowheads indicate dose days. PKK, prekallikrein.

Table 3. Pharmacokinetic Properties of a GalNAc3-Conjugated Phosphorothioate 2'-O-Methylyethyl Antisense Oligonucleotide Targeting Prekallikrein

| Dose, mg | Study day | N | Cmax, μg/mL | Tmax, h | AUC0–24h, μg·h/mL | AUC0–168h, μg·h/mL | AUC∞, μg·h/mL | CL/F, L/h | t1/2CL, days |
|----------|-----------|---|-------------|---------|-------------------|---------------------|----------------|----------|-------------|
| 20       | 1         | 6 | 0.093 ± 0.035 | 1.5 (1.0, 4.0) | 0.766 ± 0.304 | 0.810 ± 0.326 | — | — | — | — |
| 85       | 6         | 0.145 ± 0.109 | 1.0 (1.0, 2.0) | 0.776 ± 0.190 | 0.872 ± 0.203 | 1.01 ± 0.221 | 20.1 ± 4.8 | 21.1 ± 19.6 | — | — |
| 40       | 1         | 6 | 0.209 ± 0.063 | 1.0 (1.0, 2.0) | 1.65 ± 0.478 | 1.78 ± 0.513 | — | — | — | — |
| 85       | 6         | 0.230 ± 0.130 | 1.8 (1.0, 6.0) | 1.57 ± 0.528 | 1.78 ± 0.586 | 2.18 ± 0.801 | 21.0 ± 0.9 | 38.1 ± 15.8 | — | — |
| 60       | 1         | 6 | 0.458 ± 0.099 | 3.0 (3.0, 4.0) | 3.66 ± 0.787 | 3.90 ± 0.825 | — | — | — | — |
| 85       | 6         | 0.779 ± 0.435 | 2.5 (0.5, 8.0) | 4.82 ± 1.57 | 5.10 ± 1.59 | 5.72 ± 1.72 | 11.3 ± 3.3 | 30.8 ± 3.1 | — | — |
| 80       | 1         | 6 | 0.623 ± 0.195 | 1.0 (1.0, 4.0) | 4.28 ± 0.842 | 4.56 ± 0.826 | — | — | — | — |
| 85       | 6         | 0.807 ± 0.431 | 2.5 (1.5, 6.0) | 5.05 ± 1.25 | 5.47 ± 1.31 | 6.10 ± 1.50 | 13.8 ± 3.3 | NA | — | — |

Values are presented as the mean ± SD, except Tmax, which is presented as median (minimum, maximum). CLss/F is the plasma clearance at steady state after subcutaneous administration. CLss/F was calculated by actual dose/AUC0–s, where AUC0–s refers to the area under the plasma concentration–time curve (AUC) from time 0 to dosing interval τ following the last dose and τ = 672 hours.

NA, not available.
of the parent and GalNAc₃ versions at doses resulting in equivalent target reductions, Supplementary Fig. S1 compares the pharmacokinetics of the parent and GalNAc₃-conjugated 2'MOE ASO for apolipoprotein(a) at 300 mg versus 10 mg, respectively. These doses result in ~60% reduction of plasma Apo(a) levels. With the exception of a significantly lower Cmax, the GalNAc₃ version behaved similarly to the parent. This was true for the other pairs of parent and GalNAc₃-conjugated 2'MOE ASOs (data not given).

Because of the substantial increase in potency conferred by GalNAc₃ conjugation for reducing RNA targets expressed in the hepatocyte, and the elimination half-lives from tissues for these agents range from 3 to 4 weeks, we also evaluated monthly administration of the GalNAc₃-conjugated 2'MOE ASOs. The main purposes of this exercise were to define monthly dose–response curves and to assess if the mid-dose trough level of analytes were reasonably maintained. The monthly dose–response curves and to assess if the mid-dose reductions were maintained between doses as predicted by the single-dose and multiple-dose weekly cohorts, and the target monthly doses behaved similarly to the comparable doses in the trough level of analytes were reasonably maintained. The monthly dose–response curves and to assess if the mid-dose reductions, Supplementary Fig. S1 com-

The demographics of the subjects in the multidose cohort were as expected for normal volunteers (Table 4). As can be seen, there were no effects observed at any dose level on the mean values of any of the analytes measured (Figs. 5–7). To identify individuals who may have had excursions in any analyte, we show the incidences of data falling outside the normal range or reaching a specified threshold. Any abnor-

Safety

Integrated analysis of the safety data for the GalNAc₃-conjugated 2'MOE ASOs included laboratory tests for liver (alanine transaminase, aspartate transaminase, total bilirubin, alkaline phosphatase, and albumin), kidney (serum creatinine, blood urea nitrogen, calculated glomerular filtration rate, and urine total protein), hematology (platelets, absolute neutrophil count, lymphocytes, hemoglobin, and hematocrit), coagulation (activated partial thromboplastin time and prothrombin time) and complement activation (complement split products Bb and C5a).

The results from the multiple ascending weekly doses are given in the primary figures and tables (Figs. 5–9 and Tables 4 and 5) with the respective protocol-specified dose schedules given in Supplementary Table S2; and the results of single ascending doses in the supplementary material (Supplementary Figs. S2–S5 and Supplementary Tables S8 and S9).
FIG. 5. Mean laboratory measurements over time for assessment of liver function in the weekly multidose regimen cohort, (a) alanine transaminase, (b) aspartate transaminase, (c) total bilirubin, (d) alkaline phosphatase, and (e) albumin. Each data point represents data from at least 10 subjects and 3 GalNAc2-conjugated 2′MOE ASOs during the treatment period. Gray dashed lines represent the median reference range values and yellow dashed lines indicate the event criteria. A tabulated summary of results is provided in Supplementary Table S3.
incidence of mild injection site reactions was only 3%. There were no moderate or severe ISRs. With the PS 2’MOE type drugs, the incidence of ISRs ranged from 8% to 48% in similar phase 1 studies [36].

Discussion

GalNAc3 conjugation is the first example of targeted delivery of PS 2’MOE ASOs and represents a significant advance for targets expressed in the liver. The mechanism of targeting does not increase total liver concentrations of the ASOs. Rather, the ligand results in a greater fraction of total liver ASO to be delivered to the hepatocyte without significant changes in the elimination of the ASO [29,39,49]. Since at the low dose employed for GalNAc3 ASOs the total hepatocyte concentration does not exceed the concentrations observed for the parent drugs [39], two important conclusions were obtained. First in humans, as has been demonstrated in animal model studies [39], the GalNAc3 moiety must result in a higher fraction of 2’MOE ASO delivered productively. The second conclusion is also important: For PS 2’MOE ASOs, GalNAc3 conjugation does not increase the total hepatocyte exposure at therapeutic doses arguing that hepatocyte safety should be as attractive as
FIG. 7. Mean laboratory measurements over time for assessment of hematology in the weekly multidose regimen cohort. (a) platelets, (b) hemoglobin, (c) hematocrit, (d) lymphocyte count, and (e) absolute neutrophil count. Each data point represents data from at least 10 subjects and 3 GalNAc$_{3}$-conjugated 2'MOE ASOs during the treatment period. Gray dashed lines represent the median reference range values and yellow dashed lines indicate the event criteria. A tabulated summary of results is provided in Supplementary Table S5.
FIG. 8. Individual subject assessment of platelet counts over time in the weekly multidose regimen cohort by dose level, (a) Placebo, (b) >0–10 mg, (c) >10–20 mg, (d) >20–40 mg, (e) >40–60 mg, and (f) >60 mg. Gray dashed lines represent the median reference range values and yellow dashed lines indicate the event criteria.
FIG. 9. Mean laboratory measurements of coagulation and complement activation over the 24-h period postdose 1 in single and weekly multidose regimen cohorts, (a) activated partial thromboplastin time, (b) prothrombin time, (c) complement split product Bb, and (d) complement split product C5a. Each data point represents data from at least 10 subjects and 3 GalNAc₂⁻conjugated 2'MOE ASOs. Gray dashed lines represent the median reference range values and yellow dashed lines indicate the event criteria. A tabulated summary of results is provided in Supplementary Tables S6 and S7.
Table 5. Incidence of Abnormal Laboratory Test Results in Weekly Multidose Regimen Cohort (N=157)

| Event, n (%) | Placebo | Total ASO | >0–10 | >10–20 | >20–40 | >40–60 | >60 |
|-------------|---------|-----------|-------|--------|--------|--------|-----|
| Liver       |         |           |       |        |        |        |     |
| ALT, n      | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >3×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| >5×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| AST, n      | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >3×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| >5×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Total bilirubin, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| >2×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| >2×ULN AND ALT >3×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Alkaline phosphatase, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| >3×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Albumin, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| <2.5 g/dL, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Kidney      |         |           |       |        |        |        |     |
| Serum creatinine, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| >0.3 mg/dL increase from BSLN AND ≥1.4×BSLN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| BUN, n      | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >31 mg/dL, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| eGFR, n     | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >60 mL/min per 1.73 m², n (%) | 1 (2.8) | 1 (0.8) | 0 (0.0) | 1 (2.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| >30 mL/min per 1.73 m², n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| C5a, n      | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >2×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Platelets, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| <100 K/mm³, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| <75 K/mm³, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hemoglobin, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| M<10.5 g/dL; F<9.5 g/dL, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hematocrit, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| <0.85×BSLN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| <30% (absolute value), n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Lymphocytes, n | 36 | 120 | 32 | 37 | 33 | 12 | 6 |
| <0.5 K/mm³, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Abs. neutrophil count, n | 36 | 121 | 32 | 38 | 33 | 12 | 6 |
| <1.0 K/mm³, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Coagulation  |         |           |       |        |        |        |     |
| APTT, n     | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >1.4×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| PT, n       | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >1.2×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Complement  |         |           |       |        |        |        |     |
| Bb, n       | 36      | 121       | 32    | 38     | 33     | 12     | 6   |
| >2×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| C5a, n      | 30 | 97 | 20 | 26 | 33 | 12 | 6 |
| >2×ULN, or BSLN if >ULN, n (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

Incidence of events is based on confirmed test results for liver, kidney, and hematology parameters during the study period. The incidence of coagulation and complement activation events was determined by a single observation. A confirmed event was defined as a consecutive abnormal laboratory value on a different day. If there was no consecutive test to confirm, then the initial observation was presumed confirmed. If there were multiple values on the same day but different time, the worst value was used. ALT and AST events were confirmed by two consecutive measurements at least 7 days apart with all values between the initial and subsequent test also above the specified threshold.

ALT, alanine transaminase; APTT, activated partial thromboplastin time; AST, aspartate transaminase; BSLN, baseline; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; ULN, upper-limit-of-normal.
the safety of the unconjugated ASOs. This is supported by the absence of liver signals. The reduced doses that can be used for GalNAc3-conjugated 2’MOE ASOs means that peak plasma concentration-related adverse events are likely to be reduced. Lower doses also enhance tolerability and support less frequent than weekly dosing.

The success of GalNAc3 conjugation has stimulated broader efforts to identify ligands that enhance delivery of ASOs to other organs. We and our colleagues at AstraZeneca have reported that conjugation of Glp1 to PS ASOs dramatically increased delivery to the pancreatic islet cells resulting for the first time in robust activity against pancreatic targets [50]. Broader research on targeting ligands may enhance delivery to other organs in due course.

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Author Disclosure Statement

All authors are employees of Ionis Pharmaceuticals.

Supplementary Material

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