Minimal physiologically based pharmacokinetic-pharmacodynamic (mPBPK-PD) model of GalNAc-conjugated siRNA disposition and gene silencing in preclinical species and humans

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List of non-standard abbreviations: ASGPR, asialoglycoprotein receptor; AT, antithrombin; FIH, first-in-human; GalNAc, N-acetylgalactosamine; GSA, global sensitivity analysis; $K_P$, tissue:plasma partition coefficient; mPBPK, minimal physiologically-based pharmacokinetic; PD, pharmacodynamic; PRCC, Partial Rank Correlation Coefficient; QSP, quantitative systems pharmacology; RISC, RNA-induced silencing complex; RNAi, RNA interference; siRNA, small interfering RNA; SC, subcutaneous; TMDD, target-mediated drug disposition

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ABSTRACT

Conjugation of small interfering ribonucleic acid (siRNA) to tris-N-acetylgalactosamine (tris-GalNAc) can enable highly selective, potent, and durable knockdown of targeted proteins in the liver. However, potential knowledge gaps between in vitro experiments, preclinical species, and clinical scenarios remain. A minimal physiologically based pharmacokinetic-pharmacodynamic (mPBPK-PD) model for GalNAc-conjugated siRNA (GalNAc-siRNA) was developed using published data for fitusiran (ALN-AT3), an investigational compound targeting liver antithrombin (AT), to delineate putative determinants governing the whole body-to-cellular PK-PD of GalNAc-siRNA and facilitate preclinical-to-clinical translation. The model mathematically linked relevant mechanisms: i) hepatic biodistribution, ii) tris-GalNAc binding to asialoglycoprotein receptors (ASGPRs) on hepatocytes, iii) ASGPR endocytosis and recycling, iv) endosomal transport and escape of siRNA, v) cytoplasmic RNA-induced silencing complex (RISC) loading, vi) degradation of target mRNA by bound RISC, and vii) knockdown of protein. Physiological values for 36 out of 48 model parameters were obtained from the literature. Kinetic parameters governing (GalNAc)_3-ASGPR binding and internalization were derived from published studies of uptake in hepatocytes. The proposed model well characterized reported PK, RISC dynamics, and knockdown of AT mRNA and protein by ALN-AT3 in mice. The model bridged multiple PK-PD datasets in preclinical species (mice, rat, monkey) and successfully captured reported plasma PK and AT knockdown in a Phase-I ascending dose study. Estimates of in vivo potency (SC_{50}) were similar (~2-fold) across species. Subcutaneous absorption and serum AT degradation rate constants scaled across species by body weight with allometric exponents of -0.29 and -0.22. The proposed mechanistic modeling framework characterizes the unique PK-PD properties of GalNAc-siRNA.
SIGNIFICANCE STATEMENT

(GalNAc)$_3$-conjugated siRNA therapeutics enable liver-targeted gene therapy and precision medicine. Using a translational and systems-based minimal physiologically based pharmacokinetic-pharmacodynamic (mPBPK-PD) modeling approach, drug- and system-specific determinants influencing GalNAc-siRNA functionality in preclinical species (mice, rats, monkeys) and humans were investigated. The developed model successfully integrated and characterized relevant published in vitro-derived biomeasures, mechanistic PK-PD profiles in animals, and observed clinical PK-PD responses for an investigational GalNAc-siRNA (fitusiran). This modeling effort delineates the whole body-to-cellular disposition and liver-targeted pharmacodynamics of GalNAc-siRNA.
INTRODUCTION

Recent advances in RNA-based gene therapy for both rare and prevalent diseases are transforming precision medicine. By harnessing the natural biological process of RNA interference (RNAi) (Hannon, 2002), an important novel class of RNA-based therapeutics are being developed. One such modality is small interfering RNA (siRNA), a therapy which mediates RNAi by acting upstream of most drugs, potently silencing mRNA – the genetic precursors – that encode for targeted proteins, thus preventing their synthesis.

Within cells, unbound cytoplasmic concentrations of siRNA act by engaging the RNA-induced silencing complex (RISC), inducing the RISC-mediated cleavage of the targeted mRNA (Hannon, 2002; Huang, 2017). Historically, efficient in vivo delivery of siRNA has been hampered by multiple factors such as rapid elimination (e.g., nuclease degradation, renal clearance), unfavorable physicochemical properties for cellular uptake (e.g., high molecular weight, large size, negative charge), potential for immunogenicity, and significant endo-lysosomal degradation (Wang et al., 2010; Dowdy, 2017). Recent breakthrough innovations to improve the pharmacokinetic (PK) and pharmacodynamic (PD) properties of siRNA include the use of enhanced stabilization chemistry for oligonucleotide synthesis, 2′-F and 2′-O-methyl (2′OMe) nucleoside modifications to one strand of the siRNA duplex to lower immunogenicity, lipid nanoparticle formulations, and the development of tris N-acetylgalactosamine (GalNAc)_3-conjugated siRNA (GalNAc-siRNA hereafter) (Layzer et al., 2004; Nair et al., 2014; Khvorova and Watts, 2017; Springer and Dowdy, 2018). With the regulatory approvals of two GalNAc-siRNA therapies, OXLUMO™ (lumasiran), GIVLAARI™ (givosiran), and recent clinical successes of many other siRNA compounds, the clinical pipeline for GalNAc-siRNA therapy has increased substantially, with over a dozen late-stage trials to treat diverse liver-related diseases now underway (Khvorova and Watts, 2017; Springer and Dowdy, 2018; Setten et al., 2019; Debacker et al., 2020; Hu et al., 2020).

Administered via the subcutaneous (SC) route, siRNA conjugated to tris-GalNAc maintain potent and durable gene silencing activity in the clinic (e.g., weeks to months), which occurs through a series of
kinetic steps involving i) rapid and extensive distribution into the liver, ii) binding to the
asialoglycoprotein receptor (ASGPR) that is expressed abundantly and exclusively on hepatocytes, ii)
internalization via ASGPR-mediated endocytosis, iii) endosomal transport and receptor recycling, iv)
escape from the endosomal compartment into cytoplasm (via mechanisms not fully understood yet), v)
binding to cytoplasmic RISC, vi) stimulation of the natural degradation rate of the targeted mRNA by the
siRNA-loaded RISC, and vii) consequent inhibition of production of the targeted protein (Wei et al.,
2011; Gilleron et al., 2013; Nair et al., 2017; Humphreys et al., 2019; Brown et al., 2020; Debacker et al.,
2020). An interesting feature of the PK/PD relationship for siRNA is an apparent disconnect between
their transient plasma exposure and prolonged pharmacologic effects. This unique PK/PD characteristic
enables monthly, quarterly, or even twice-yearly dosing schedules to maintain therapeutic efficacy.

Although there has been a general understanding of a RISC-dependent mechanism for siRNA
(Wei et al., 2011; Nair et al., 2017), quantitative information on determinants influencing the extent and
duration of GalNAc-siRNA activity remains incomplete. As such, systematic, and quantitative model-
based interspecies assessments of GalNAc-siRNA disposition and gene silencing activity are warranted.
Mechanism-based characterization of PK-PD relationships of siRNA present some challenges and unique
opportunities due to the complex, cascading, and potentially nonlinear processes involved in their
disposition and pharmacology. This necessitates the use of integrative and multiscale modeling
approaches to delineate and quantify specific determinants controlling their dose-exposure-response
relationships in vivo. Beyond enabling the separation of drug- and system-specific determinants, properly
assembled translational, physiologically based PK-PD (PBPK-PD) models provide a mechanistic
platform to extrapolate and predict drug effects from in vitro and in vivo preclinical systems to clinical
responses for new and existing therapies (Mager and Jusko, 2008; Shah et al., 2012; Singh et al., 2020).
Based on a recent review article originating from the U.S. Food and Drug Administration (Fairman et al.,
2021), and to our knowledge, a PBPK-PD framework for characterizing RNAi mediated by GalNAc-
siRNA has not been reported thus far.
Here, we employed a stepwise approach to: i) develop a minimal PBPK-PD (mPBPK-PD) model to comprehensively characterize the whole body-to-intracellular PK-PD actions of GalNAc-siRNA, ii) physiologically scale the platform model across three preclinical species (mouse, rat, and monkey), iii) characterize clinical response in a first-in-human (FIH) study for a specific GalNAc-siRNA, and iv) demonstrate the utility of translational systems modeling to quantitate the PK-PD properties of this novel therapeutic platform. The model was developed by leveraging published data comprising in vitro experiments, comprehensive in vivo PK-PD studies conducted in preclinical species, and reported clinical Phase-I data for fitusiran (ALN-AT3), an investigational GalNAc-siRNA conjugate targeting endogenous antithrombin (AT) to treat hemophilia A and B (Pasi et al., 2017). Global sensitivity analysis (GSA) was further performed to understand the impact of selected model parameters on gene silencing. The proposed model provides a mechanistic framework that further assesses the preclinical and clinical PK-PD behavior of GalNAc-siRNA therapeutics.
METHODS

Minimal PBPK-PD model

Based upon the relevant physiological and pharmacological mechanisms of GalNAc-siRNA disposition and actions described in the ‘Introduction’ and found in the literature (Sarin, 2010; Bon et al., 2017; Nair et al., 2017; Humphreys et al., 2019; Debacker et al., 2020), a multiscale PK-PD model was developed (Fig. 1). The model was assembled in a stepwise manner. The definitions for all model parameters are provided within Table 2 and Table S2 in Appendix S2. Definitions for state variables in the model are listed in Table S1 in Appendix S2. The system of ordinary differential equations describing the model are provided in Appendix S3.

mPBPK model to characterize PK and biodistribution of GalNAc-siRNA. The whole-body disposition of GalNAc-siRNA was characterized using a mPBPK modeling approach (Cao and Jusko, 2012) (Fig. 1a). The model incorporated a distinct kidney compartment subdivided into vascular and tissue spaces. A bound depot was included to account for drug sequestration in the extracellular and/or tissue space(s). Renal filtration (i.e., elimination) of the free (unbound) drug was assumed to occur from the kidney vascular space. The main organ of pharmacologic relevance, liver, comprised a vascular space, an extracellular compartment, a bound pool, and a cellular space accounting for drug entry into hepatocytes via specific, ASGPR-mediated intracellular uptake.

Liver mPBPK-PD model to characterize target gene silencing by GalNAc-siRNA. Free (unbound) GalNAc-siRNA in the plasma entered from the liver vasculature into the extracellular space, controlled by the liver uptake clearance. Unbound siRNA either bound within a pool or interacted specifically with the ASGPR on hepatocytes. Tris-GalNAc binding to the ASGPR caused internalization of GalNAc-siRNA into endosomes followed by rapid cleavage of the GalNAc moiety, along with rapid and extensive recycling of a majority of free ASGPR (90%) back to the cell surface (Schwartz et al., 1982; Weigel and Oka, 1983). Further, it was assumed that a very small fraction of siRNA (1%) underwent endosomal...
escape into the cell cytoplasm (Gilleron et al., 2013), bound to free cytoplasmic RISC, and consequently caused gene silencing by stimulating the natural degradation rate of target mRNA (Debacker et al., 2020).

**Cellular model of target-mediated uptake and intracellular delivery of GalNAc-siRNA.** The kinetics of drug binding and uptake via the ASGPR was described by adapting the general framework of cell-level target-mediated drug disposition (TMDD) (Krzyzanski et al., 2016). The model incorporated the turnover of ASGPR on the cell surface (de novo synthesis and degradation), kinetics of tris-GalNac siRNA binding with ASGPR, internalization of the bound complex into endosome, subsequent GalNAc cleavage from conjugate, endosomal degradation and/or escape of siRNA, and rapid recycling of free ASGPR from the endosome to the cell surface. Upon binding to ASGPR, GalNAc-siRNA was assumed to distribute within the total volume of hepatocytes, which comprise 80% of the cellular volume of liver (Zhou et al., 2016). Since adult hepatocytes display a long average life span of 200 - 300 days (Duncan et al., 2009), constant numbers of hepatocytes were assumed over time.

Each ‘scale’ described within the mPBPK-PD model is related mathematically based on the underlying cellular and organ physiology. Physiological parameter values describing organ volumes, plasma flows, filtration rates, liver fractional volumes, and hepatocellularity in mice, rats, monkeys, and humans were obtained from the literature (Davies and Morris, 1993; Sohlenius-Sternbeck, 2006; Duncan et al., 2009; Delanaye et al., 2012; Shah and Betts, 2012; Iwama et al., 2014; Sasaki et al., 2014). Liver concentrations of ASGPR in each species was calculated by accounting for species-specific cellular expression, hepatocellularity, and liver weight (Appendix S4).

**Model assumptions**

Certain additional assumptions were made during the modeling. These include: i) negligible elimination of GalNAc-siRNA in plasma, which is supported by in vitro stability assessments (Nair et al., 2017), ii) presence of functional ASGPR (i.e. expression of protein subunits 1 and 2) is confined to the hepatocytes (https://www.proteinatlas.org/ENSG00000141505-ASGR1/celltype, https://www.proteinatlas.org/ENSG00000161944-ASGR2/celltype), iii) drug distribution is confined
within the vascular and extracellular volumes of ‘remainder’ organs (Dowdy, 2017), iv) only ‘free’ ASGPR (cleaved from conjugate) recycle to the cell surface, v) only free siRNA escaping the endosome can interact with the RISC in the cytoplasm (Sen and Blau, 2005; Gilleron et al., 2013), vi) once bound, dissociation of siRNA from RISC is negligible (Bartlett and Davis, 2006), vii) RISC-loaded siRNA concentrations stimulate the natural degradation rate of target mRNA, and viii) knockdown of mRNA is translated to decreased protein expression.

Datasets and a stepwise modeling approach

All data utilized in this study were obtained from the literature. The PK datasets (plasma, total liver, RISC-bound siRNA in liver, and kidney concentration-time profiles) examined in this analysis include GalNAc siRNA targeting AT, transthyretin (TTR), and delta-aminolevulinate synthase 1 (ALAS1) that were collected across four species (mice, rats, cynomolgus monkeys, and humans), whereas mRNA and protein time-courses were examined only for ALN-AT3, an investigational GalNAc siRNA targeting AT, in mice, monkeys, and humans (Akinc et al., 2012; Gilleron et al., 2013; Sehgal et al., 2015; Pasi et al., 2017). Relevant information (including target, dose, species, measurements) regarding each preclinical and clinical study obtained from the literature are summarized in Table 1, and briefly stated below. A stepwise approach was applied to develop and qualify the platform model using experimental data obtained from in vitro (hepatocyte) and in vivo preclinical studies as well as clinical responses in humans. Due to availability of rich data sets describing both PK and PD, mice, a relatively well-established preclinical animal to study GalNAc-siRNA pharmacology (Sehgal et al., 2015; Nair et al., 2017), was chosen as the starting species for model development and calibration. The stepwise model-building approach involved:

Step-1 (in vitro modeling of ASGPR-mediated GalNAc uptake): Two independent in vitro experiments examining kinetics of (GalNAc)$_3$-IL2 uptake in primary hepatocytes from mice (Sato et al., 2002) were modeled simultaneously using a basic model of receptor-mediated endocytosis (Fig. S1a in Appendix S1) to obtain select kinetic rate-constants for GalNAc endocytosis by ASGPR.
Step-2 (mPBPK model development in mice): A mPBPK model incorporating distinct kidney and liver compartments was developed to characterize the available plasma and tissue PK profiles in mice. For PK model development, plasma and total tissue (liver and kidney) concentrations of GalNAc-siRNAs targeting AT (ALN-AT3) or TTR (siTTR-2) in mice were obtained from the literature at doses ranging from 1 – 25 mg/kg IV or SC (Akinc et al., 2012; Sehgal et al., 2015; Nair et al., 2017) (Table 1). Specifically, concentration-time profiles of siTTR-2 in plasma and kidney after a 10 mg/kg IV and SC doses were obtained. In addition, both plasma and total liver concentration-time profiles for ALN-AT3 after 1, 2.5, 5, and 25 mg/kg single IV and/or SC doses were included.

Step-3 (mPBPK-PD model development and validation in mice): Based upon availability of multiple, comprehensive published PK-PD data sets for ALN-AT3 (fitusiran), this compound was chosen as a relevant case example for full PK-PD characterization. In mice, the time courses of RISC occupancy (liver Ago2-loaded siRNA concentrations), AT mRNA in liver, and serum AT protein following 1, 2.5, and 5 mg/kg SC were used for further model development and characterization of the PK-PD relationship (Sehgal et al., 2015; Nair et al., 2017). In addition, a dataset profiling the knockdown of serum AT protein following repeated 0.75, 1.5, and 3 mg/kg (QW × 5) SC dosing of ALN-AT3 in mice was used for model qualification (Sehgal et al., 2015).

Step-4 (mPBPK model-based scale up to rats): The proposed mPBPK-PD model was scaled based on PBPK principles (Kagan et al., 2011; Ayyar and Jusko, 2020) to predict the PK of GalNAc siRNA in higher-order species, including the rat. Model-based simulations in the rat, based on the model developed in steps 1-3, were compared with available data (total liver PK at 10 mg/kg SC and RISC-bound siRNA at 1 and 5 mg/kg SC; Table 1) for givosiran (Lee et al., 2019). The RISC association and turnover constants ($k_{on, RISC}$ and $k_{DR}$) were assumed as drug/sequence-specific and were therefore estimated. All other model parameters were either fixed or scaled based on the model developed in steps 1-3.

Step-5 (Modeling knockdown in NHP): Next, the platform model was applied to characterize the in vivo time profiles of plasma AT knockdown by ALN-AT3 in nonhuman primates. Relative AT activity in plasma after a single SC injection of ALN-AT3 at 1, 3, 10, and 30 mg/kg in cynomolgus monkeys were
obtained from Sehgal et al. (Sehgal et al., 2015). Two model parameters relating to PD, i.e. the *in vivo* drug potency ($SC_{50}$) and the AT protein degradation rate-constant ($k_{\text{deg,AT}}$), were estimated. All other model parameters were either fixed, scaled, or adjusted to species-specific values based on the model developed in steps 1-3.

**Step-6 (Modeling plasma PK and knockdown in humans):** Plasma PK and protein knockdown by fitusiran (ALN-AT3SC) were obtained from a published phase 1 dose-escalation study in 4 healthy subjects and 25 patients with moderate or severe hemophilia A or B (Pasi et al., 2017). Mean plasma concentrations of fitusiran over time after a single SC injection across a dose range of 0.015 – 1.8 mg/kg were investigated. In addition, the time-course of plasma AT knockdown was characterized at 0.03 mg/kg SC (single dose) in healthy subjects, and at 0.015 mg/kg QW x 3 SC, 0.225 Q4W x 3 SC, and 80 mg Q4W x 3 SC in subjects with hemophilia (Pasi et al., 2017). As a final model qualification step, AT knockdown profiles were simulated under 0.45 and 1.8 mg/kg Q4W x 3 SC regimens and compared with observed data. Two model parameters, namely the SC absorption rate constant ($k_a$) and $SC_{50}$ were estimated, whereas the AT protein degradation rate-constant ($k_{\text{deg,AT}}$) was fixed to a reported value (Menache et al., 1990). The structural model remained unchanged across species, and all other model parameters were either fixed or scaled based on the model developed in steps 1-3.

**Parameter global sensitivity analysis (GSA)**

Considering the complexity of the underlying system, a global sensitivity analysis (GSA) (Marino et al., 2008; Alden et al., 2013) was performed where multiple drug- and system-specific parameters describing the model (Fig. 1) were simultaneously varied over time to deduce the overall uncertainty described in the final model output, i.e., overall protein knockdown. Partial Rank Correlation Coefficient (PRCC) method was implemented in the R software environment (Singh et al., 2020), where a total of 30 model parameters were simultaneously perturbed with a sampling size of 5000. The limits for lower and upper bounds varied for each parameter and were based on physiologically plausible values. PRCC-based
sensitivity indexes were simulated as a time course for up to 40 days. PRCC describes the relative importance of a parameter along with its positive/negative correlation on the desirable model output.

**Data collection and model implementation**

The data utilized in this report were obtained from the literature. The data were extracted either as mean values or individual observations (where available) from published graphs by computer digitization (WebPlotDigitizer, version 4.1, [https://automeris.io/WebPlotDigitizer](https://automeris.io/WebPlotDigitizer)). Thus, estimated PK and PD parameters should be considered approximate and fit-for-purpose (i.e., ‘proof-of-concept’). The PK-PD analysis was performed in ADAPT 5 (D’Argenio et al., 2009). Data were pooled and fitted using the maximum likelihood algorithm. The residual error variance model was defined as:

\[ V_i = (\sigma_{\text{intercept}} + \sigma_{\text{slope}} \cdot Y_i)^2 \]

where \( V_i \) is the variance of the \( i \)th observation, \( Y_i \) is the \( i \)th model prediction, and \( \sigma_{\text{intercept}} \) and \( \sigma_{\text{slope}} \) are additive and proportional variance model parameters representing a linear relationship between the standard deviation of the model output and \( Y_i \). The goodness-of-fit was assessed by visual inspection of the fitted curves, improvement in the log-likelihood objective function (−2LL), examination of residual plots (observed versus predicted and predicted versus residual), and precision (CV%) of the estimated parameters.

R ([http://www.r-project.org](http://www.r-project.org)), R-studio, and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA) were used for GSA and graphical assessments, respectively.
RESULTS

Modeling in vitro kinetics of GalNAc endocytosis in mice hepatocytes (step-1)

A basic model of receptor-mediated endocytosis (Fig. S1a in Appendix S1) simultaneously captured observed in vitro cellular disposition data from two separate experiments in mouse primary hepatocytes (Sato et al., 2002) reasonably well (Figs. S1b and S1c in Appendix S1). The dissociation rate constant ($k_{off}$) and internalization rate constant ($k_{int}$) for ASGPR-mediated endocytosis were estimated as 0.022 min$^{-1}$ (23.9% CV) and 0.04 min$^{-1}$ (7.7% CV), which were subsequently fixed during the in vivo modeling (Table 2). To derive the association rate constant ($k_{on}$) for (GalNAc)$_3$ with ASGPR (Table 2), a reported value of 2.48 nM for the equilibrium dissociation constant ($K_D$) of this interaction was utilized (Kanasty et al., 2013). An inherent assumption in utilizing these data of tris GalNAc-IL-2 uptake within the current framework is that the entity conjugated to tris-GalNAc does not significantly influence the binding and internalization rates of the conjugate, which is instead driven primarily by the tris-GalNAc moiety itself.

MPBPK model development in mice (step-2)

The developed model (Fig. 1) was fitted simultaneously to multiple PK datasets obtained in mice, which included measurements of drug in plasma, liver (total and RISC-bound), and/or kidney for two compounds (ALN-AT3 and siTTR-2; Table 1). As depicted in Fig. 2 and in Figs. S2 and S3 in Appendix S1, the model characterized the concentration versus time profiles of both siRNA in plasma, liver, and kidney reasonably well across the broad dose range examined (1 mg/kg – 25 mg/kg IV/SC). Several model parameters were fixed apriori to physiologically tenable estimates, whereas selected parameters (e.g. liver uptake clearance, endosomal degradation constant, and siRNA-RISC association constant), which were unknown or best identified from in vivo profiles, were estimated with acceptable precision (Table 2). The model well-captured the initial steep, rapid drop in plasma siRNA concentrations following IV and SC dosing (Figs. S2a-c and S3a,b in Appendix S1), consistent with the short ‘apparent’ plasma half-life typical for this class of compounds (Fairman et al., 2021). In addition, the model fits
produced profiles which characterized a shallower, prolonged phase in plasma appearing at later times, which was evident particularly at the higher doses of 10 and 25 mg/kg IV/SC. This phase may be attributable to some exchange of drug between plasma and kidney, which displayed extensive uptake and prolonged concentration-time profiles (though to a lesser extent than liver). The model-estimated apparent SC bioavailability (F) in mice was ~33% (5.0 % CV) with a first-order SC absorption rate constant (\( k_a \)) value of 0.7 h\(^{-1} \) (6.5 % CV) (Table 2). The tissue:plasma partition coefficient for the remainder compartment (\( K_{P,\text{rest}} \)) was set as 0.1. This value was fixed because i) owing to their larger size and net negative charge, GalNAc-siRNA does not freely permeate into the cell space within non-ASGPR expressing tissues and hence are confined to extracellular fluid spaces, and ii) biodistribution studies indicate very low concentrations (relative to plasma exposure) in all tissues, except the liver and kidney (McDougall et al., 2021).

In general, liver concentration-time profiles of ALN-AT3 were captured reasonably well across 1 – 25 mg/kg SC doses (Figs. 2a and S2d in Appendix S1), with slight exception for the liver \( C_{\text{max}} \) at the highest dose. The rapid disappearance of drug in plasma (Figs. S2a-c and S3a,b in Appendix S1) was associated with efficient uptake into liver interstitium, with a net uptake clearance (\( CL_{\text{up,in}} \)) of 207 mL/h (8.5 % CV). As expected, based on the physicochemical properties of GalNAc-siRNA, the model-estimated value of \( CL_{\text{up,out}} \) was extremely small (0.0025 mL/h; 24 % CV), indicating minor to negligible drug re-entry into circulation after leaving the liver vasculature into tissue. Several parameters relating to ASGPR-mediated endocytosis of GalNAc-siRNA were fixed apriori based on published values or calculated (Fig. S1 in Appendix S1 and Table 1) (Schwartz et al., 1982; Sato et al., 2002; Prakash et al., 2014; Bon et al., 2017). Further, the rate-constant of cleavage of tris-GalNAc from the siRNA (\( k_{\text{cle}} \)) upon internalization was fixed as 1.32 h\(^{-1} \), calculated from a reported 30-min half-life associated with this step (Prakash et al., 2014). Of mechanistic relevance, simulation of the concentration-time profile of free surface ASGPR indicated that 1 mg/kg SC did not result in full occupancy of available ASGPR at any time, whereas 2.5 and 5 mg/kg SC doses produced a very transient ASGPR saturation in a dose-dependent manner (data not shown). On the other hand, a 25 mg/kg SC dose fully reduced free ASGPR
over time (> 48 hours after dosing), which may explain the less than dose-proportional increase in total liver \( C_{\text{max}} \) and AUC at this high dose (Fig. S2d in Appendix S1).

The prolonged half-life of drug in liver was well-captured, which was mostly governed by the degradation constant of siRNA in the endosomal compartment \( (k_{\text{deg,d}} = 0.01 \text{ h}^{-1}; 3.0 \% \text{ CV}) \), resulting in a prolonged profile for the escaped fraction of siRNA into the cytosol. To prevent overparameterization, two additional rate constants describing endosomal escape \( (k_{\text{esc}}) \) and cytoplasmic degradation of siRNA \( (k_{\text{deg,c}}) \) were fixed based on an imaging study tracking endosomal release of siRNA (Gilleron et al., 2013) and a local parameter sensitivity analysis, respectively (Table 2). As depicted in Fig. 2b, the concentration-time profile of RISC-bound ALN-AT3 in liver following 2.5 mg/kg SC (Nair et al., 2017) was well-described. Consistent with reports of a prolonged occupancy profile for RISC (Wang et al., 2009; Deerberg et al., 2013), the bound complex, which comprised only ~ 0.1 – 1% of total liver siRNA at any time, was characterized by a slow degradation rate constant \( (k_{\text{Dk}}; 0.005 \text{ h}^{-1}; 21 \% \text{ CV}) \).

Concentration-time profiles of drug in kidney were described using permeability-limited entry, incorporated a sequestered pool accounting for prolonged drug accumulation, and assumed renal filtration of free GalNAc-siRNA from the vascular space (Fig. 1a), which captured observed data well (Fig. S3c,d in Appendix S1). The model-estimated fraction unbound in kidney of 0.07 (15.7 \% CV) \( (f_{\text{u,k}}) \) was small in comparison to that in plasma \( (f_{\text{u,p}} = 0.15) \) (Humphreys et al., 2019), indicating greater distribution into the kidney. The permeability flow rate of free drug into kidney \( (PS \cdot f_{\text{u,p}}) \) was smaller than renal plasma flow, consistent with permeability rate-limited entry (Table 2).

**mPBPK-PD model development and validation in mice (step-3)**

In addition to GalNAc-siRNA PK and RISC occupancy in liver, gene knockdown by ALN-AT3, as assessed by liver AT mRNA and serum AT protein (Sehgal et al., 2015; Nair et al., 2017), were integrated within the model framework and were well characterized. Notably, modest onset delays (5-8 hours) in both mRNA and protein responses were accounted for by the mPBPK-PD model. Hysteresis among RISC-loaded siRNA, liver AT mRNA, serum AT were well-captured using tandem, precursor-like
indirect response models (Ayyar et al., 2018), which accounted for turnover of AT mRNA and protein
\[ k_{\text{deg,m}} = 0.06 \text{ h}^{-1} (41 \% \text{ CV}) ; k_{\text{deg,p}} = 0.05 \text{ h}^{-1} (25 \% \text{ CV}) ; \text{Table 2}. \] The \( S_{\text{max}} \) was calculated based on the maximal knockdown observed and fixed, whereas the \( SC_{50} \) was estimated as 2.4 ng/g (6.5 % CV; Table 2). Serum AT protein was reduced to a greater extent compared to its associated mRNA, which was captured empirically using an estimated \( \gamma \) value of 1.5 (5.6 % CV).

The developed mPBPK-PD model was used to simulate the time course of AT protein knockdown by ALN-AT3 after multiple weekly doses in mice (0.75, 1.5, and 3 mg/kg QW× 5). Based on the established mPBPK-PD relationship, the simulations predicted a dose-dependent knockdown in AT ranging from 70-90% relative to baseline (Fig. 1e). These simulations were qualified upon comparison with mean observations from a published study in mice (Sehgal et al., 2015), which assessed AT knockdown under the same dosing regimen (Fig. 1F). Both the extent of knockdown and time to achieve steady-state reductions were well-predicted, providing further confidence in the model predictiveness.

**Characterization of givosiran liver and RISC PK in rats (step-4)**

The developed mPBPK model was physiologically scaled from mice to rats. Available profiles of liver PK and RISC occupancy of givosiran in rats (Lee et al., 2019), were used for model evaluation. All parameters were either fixed or scaled (based on BW-based allometric exponents) based on step-2 (Tables S2 and S3 in Appendix S2), except the RISC association and turnover constants \( (k_{\text{on,RISC}} \text{ and } k_{\text{DR}}) \), which are considered drug/sequence-specific. As depicted in Fig. 3, the model successfully captured the concentration-time profile of givosiran liver PK at 10 mg/kg SC (Fig. 3a) as well as the bound siRNA-RISC profile after 1 and 5 mg/kg SC (Fig. 3b). Consistent with the RISC profile for ALN-AT3 in mice, the peak of RISC-bound siRNA for givosiran was delayed compared with the peak in total liver PK, and this profile was captured reasonably well by the model. The model-estimated value for \( k_{\text{on,RISC}} \) of givosiran \( (0.00016 \text{ nM}^{-1} \cdot \text{h}^{-1} ; 15.1 \% \text{ CV}) \) varied by 1.5-fold compared to that estimated for ALN-AT3 in mice (Table 2). This difference is plausible, and the results suggest that the current model may be extended to describe the kinetics of different GalNAc-siRNA compounds.
Characterization of serum AT dynamics in monkeys (step-5)

Next, the platform model was scaled to characterize pharmacodynamic effects of ALN-AT3 in nonhuman primates (cynomolgus monkeys). All model parameters related to plasma, tissue, and intracellular PK were either fixed or scaled based on BW-based allometric exponents (Tables S2 and S3 in Appendix S2). Two parameters related to PD, namely, the in vivo potency (SC\textsubscript{50}) and AT degradation rate-constant (k\textsubscript{deg,p}), were estimated. As depicted in Fig. 4, the model well-characterized the full reported time course and extent of reductions of approximately 50%, 70%, 80%, and >90% in AT protein levels following single SC doses of ALN-AT3 at 1, 3, 10, and 30 mg/kg (Sehgal et al., 2015). Of importance, the model inherently captured the delayed onset in response, evidenced at the first measured time-point ~48 h after dosing, which is likely attributable to the cascade of relevant PK and PD processes, including liver uptake, slow cytoplasmic release, RISC loading, and natural turnover of mRNA and protein. Notably, the in vivo SC\textsubscript{50} in cynomolgus monkeys was estimated to be reasonably similar compared to mice (i.e. ~ 2-fold, 2.4 vs. 5.3 ng/g). The k\textsubscript{deg,p} estimate was 5-fold lower in monkeys as compared in mice (see Fig. 7 and ‘Discussion’). Both parameters were estimated with good precision (Table S3 in Appendix S2).

Characterization of ALN-AT3 (Fitusiran) PK and PD in humans (step-6)

As a final step, published FIH study data for Fitusiran (Pasi et al., 2017) were evaluated using the model developed through preclinical data. Similar to the approach taken in step-4, nearly all model parameters related to plasma, tissue, and intracellular PK were either fixed or scaled (based on BW-based allometric exponents (Tables S2 and S3 in Appendix S2); only the k\textsubscript{a} for SC absorption was estimated based on the observed plasma profiles. Furthermore, the SC\textsubscript{50} parameter relating to PD effect was estimated. As shown in Fig. 5a, the model was able to characterize the observed plasma PK of fitusiran across a broad range (0.015 – 1.8 mg/kg SC). In addition, selected PD datasets of serum AT reduction in healthy volunteers or in subjects with Hemophilia at 0.03 mg/kg SC (single dose), 0.015 mg/kg QW x 3 SC, 0.225 Q4W x 3 SC, and 80 mg Q4W x 3 SC were characterized reasonably well by model (Fig. 5b).
Notably, there was some bias in capturing the exaggerated response at very low doses (reason currently unknown). To qualify the model in humans, AT knockdown profiles were simulated under 0.45 and 1.8 mg/kg Q4W x 3 SC regimens of fitusiran and compared with observed data (Pasi et al., 2017). The model simulations successfully predicted the mean AT knockdown response under these clinically relevant dosing regimens and over an extended duration (months) (Fig. 5c). Of translational relevance, estimated drug potencies were reasonably similar (i.e., within a 2-fold range) across mice, monkeys, and humans (Table S3 in Appendix S2).

**Parameter global sensitivity analysis (GSA)**

Fig. 6 describes a time-variant global sensitivity analysis (GSA) on the mPBPK-PD model developed for ALN-AT3 (Fig. 1, Table 2) on % protein knockdown. At early time points (e.g., < 5 days), it was observed that parameters related to drug absorption, liver uptake, and endosomal degradation were sensitive to initial protein knockdown. As expected, parameters such as apparent SC bioavailability and liver uptake clearance were positively correlated with protein knockdown, while rate constants for intracellular degradation in liver, including endosomal and bound RISC, were negatively correlated. Further, the GSA indicated that steps controlling siRNA degradation in liver (after entry into hepatocytes via ASGPR) remained sensitive at early times before gradually decreasing (e.g., endosomal degradation). This gradual decrease corresponded with a time-dependent increase in model sensitivity to the degradation rate constant for loaded RISC. This parameter, along with PD effect parameters (\(S_{\text{max}}, SC_{50}\), and \(\gamma\)), governed % protein knockdown over time (see \(SC_{50}\); others not shown). These findings are consistent with the mPBPK-PD modeling results (see Fig. 2).
DISCUSSION

Owing to their high selectivity for targeted RNA, siRNA-based therapeutics have witnessed an explosion of interest across academia and industry over the past decade. These compounds offer the ability to target previously undruggable human and viral genomes to selectively knock down disease-causing proteins (Dowdy, 2017). To date, three RNAi therapeutics, ONPATTRO®, GIVLAARi™, and OXLUMO™, the latter two being GalNAc-siRNAs, are approved for the treatment of hereditary amyloidogenic transthyretin amyloidosis, acute hepatic porphyria, and primary hyperoxaluria type 1, respectively. Another GalNAc-siRNA, inclisiran, has been submitted for new drug application to the FDA (Hu et al., 2020). As of 2020, seven siRNAs were undergoing phase 3 trials, and more candidates are under early development (Hu et al., 2020).

Despite tremendous successes, the quantitative impact of putative determinants of GalNAc-siRNA activity are only partially understood. Given their unique PK-PD characteristics in comparison with small molecule and protein-based therapeutics, it has been challenging to establish mechanistic PK-PD models for GalNAc-siRNA (Fairman et al., 2021), and there are, as yet, no established paradigms or defined guidelines that leverage preclinical data to predict safe and efficacious doses for GalNAc-siRNA in humans (Fairman et al., 2021). Transient plasma exposure accompanied by extensive uptake and prolonged residence within the liver is typically observed for this class. Rather than free (unbound) plasma concentrations, cytoplasmic concentrations of siRNA bound to RISC relate more closely with the time course of gene knockdown in target cells (Wei et al., 2011; Nair et al., 2017). As such, the development of mechanistic and translational systems PK-PD models could be a highly beneficial first step to integrate key factors associated with the whole-body-to-intracellular kinetics and activities of these agents.

Efforts in the mathematical modeling of RNAi have been published providing in-depth insights into the complicated interplay of different components (e.g. Dicer, Ago2), and better understanding of RISC assembly and molecular biology (Wang et al., 2009; Wang et al., 2012; Deerberg et al., 2013). The PK-
PD relationships of some antisense oligonucleotides (ASO) have been adequately characterized using simpler models. For instance, a mechanism-based PK-PD model for an ASO targeting hepatic apolipoprotein B mRNA in mice has been reported (Shimizu et al., 2015). Although the model well-characterized preclinical PK-PD profiles in mice, its scope for translation (viz. PK-PD prediction in humans) seems limited. Recently, a compartment-based allometric PK modeling approach employing rodent liver PK (liver and RISC-bound profiles) was used to predict human RISC PK (Lee et al., 2019).

Here, we describe a stepwise approach to develop a first-in-kind mechanistic platform model for GalNAc-siRNA, which demonstrated preclinical to clinical translatability. The first step of our modeling approach leveraged reported data characterizing the kinetics of IL-2-tris-GalNAc conjugate binding and uptake to ASGPR in primary hepatocytes from mice (Sato et al., 2002). By modeling these profiles with a simple, fit-for-purpose model (Fig. S1a in Appendix S1), estimates for the first-order dissociation and internalization rate constants were derived. These data did not provide information to distinctly identify both the association rate constant as well as ASGPR density. Therefore, upon transitioning to the second step (i.e., mPBPK model development in mice), published values for system-specific parameters related to ASGPR (e.g., receptors per cell, degradation half-life, and recycled fraction) derived from in vivo assessments in mice were obtained and fixed apriori (Table 2). This approach not only conferred mechanistic confidence in the cellular- and tissue-related processes described but also minimized the issue of over-parameterization frequently encountered with mechanistic systems models. In steps 2 and 3, the proposed model, in totality, well characterized multiple PK (plasma and tissue) and PD (mRNA and protein) datasets in mice (Fig. 2 and Figs. S2 and S3 in Appendix S1) and successfully predicted PD response under different ALN-AT3 dosing regimens (Fig. 2e,f). It is acknowledged that the model overpredicted plasma ALN-AT3 concentrations at two later time-points across the 1-5 mg/kg range.

In steps 4 and 5, the platform model was physiologically scaled from mice to rats and a higher-order preclinical species (i.e., cynomolgus monkeys; Tables S2 and S3 in Appendix S2). Key mechanistic aspects considered during interspecies extrapolation were physiological flows and volumes, hepatocellularity, ASGPR expression and kinetics, intracellular elimination/turnover constants
(endosomal, RISC, mRNA, and protein), intracellular binding constants, and the RISC-PD effect relationship ($S_{\text{max}}$ and $SC_{50}$; Tables S2 and S3 in Appendix S2). Since values for ASGPR expression were not found in rats and nonhuman primates, reported estimates in mice (1.8 million receptors/cell) (Bon et al., 2017) and in humans (1.1 million receptors/cell) (Miki et al., 2001) were employed for rats and monkeys, respectively. The affinity constants for (GalNAc)$_3$ binding to ASGPR were assumed to be unchanged across species. In addition, fraction unbound in plasma as well as binding constants in both kidney and liver were fixed across species (Humphreys et al., 2019). Due to unavailability of suitable published PK and PD data sets for ALN-AT3 in rats, reported liver PK and RISC occupancy profiles of givosiran, an approved GalNAc-siRNA therapeutic, were used. Using this approach, the model captured both the liver and RISC PK profiles reasonably well (Fig. 3). Further, the model well-characterized the time course and extent of serum AT reduction in monkeys across 1 – 30 mg/kg SC (Fig. 4).

Finally (step-6), the model was applied across a broad range of doses to describe observed plasma PK of fitusiran as well as associated serum AT reduction in humans. The model described the entire data set reasonably well (Fig. 5a,b). There was some bias (underprediction) of PD response at very low doses. The exact reason(s) for misprediction at very low doses is unclear. It is speculated that the efficiency for hepatocyte uptake and/or endosomal escape could be higher (than predicted) at very low doses, which needs further evaluation through experiments. This might also possibly be a consequence of underestimating the true SC bioavailability (discussed below). Nonetheless, as shown in Fig. 5c, simulations of AT reduction at pharmacologically relevant doses successfully predicted the observed clinical response. Of translational relevance, drug potency was found to be similar (within ~ 2-fold) across mice, monkey, and humans (Table 2 and Table S3 in Appendix S2).

Interspecies comparison of two parameters, which were estimated during model fitting, was performed. The analysis revealed BW-based allometric relationships in SC drug absorption ($k_a$) and serum AT removal constant ($k_{\text{deg,p}}$) across the species examined (Fig. 7a,b). The $k_a$ scaled with an exponent of -0.29 ($R^2 = 0.82$) and $k_{\text{deg,p}}$ with -0.22 ($R^2 = 0.95$). In both cases, these estimates scaled across body weight with exponents close to -0.25, consistent with allometric expectation (West and Brown,
Model-fitted estimates for $k_a$ in rat and cynomolgus monkey were obtained based on plasma PK for other GalNAc-siRNA (data not shown). A similar exponent value of -0.35 has been reported for the SC absorption constant of human recombinant erythropoietin, a peptide of ~30 kDa (Woo and Jusko, 2007). In addition, similar trends in plasma SC absorption profiles across rats, monkeys, and humans were shown for at least two other GalNAc-siRNA (McDougall et al., 2021). Whether interspecies differences in lymphatic physiology could explain the observed trends in $k_a$ following SC dosing requires further exploration.

Three mechanistic hypotheses have been proposed to explain the prolonged liver half-life of GalNAc-siRNA (Humphreys et al., 2019). These include i) existence of a ‘protein bound’ depot within liver, ii) that the ‘depot’ is a sub-cellular organelle such as the endosome, and iii) binding of siRNA to RISC in the cytosol and consequent ‘protection’ of free siRNA from intracellular degradation (Humphreys et al., 2019). The processes considered within our mPBPK-PD model generally reflect such mechanisms. For example, the ‘bound pool’ in liver, which is currently implemented in an empirical manner, may be rationalized by recent experimental findings (Brown et al., 2020), which show that acidic intracellular (endo-lysosomal) compartments can serve as a long-term depot for GalNAc-siRNA conjugates and contribute to the extended durability in tissue. Our model-based results and GSA suggest that intracellular (endosomal) degradation is a major determinant of liver half-life. Our findings also point to the importance of bound RISC kinetics, which also controls PD durability. However, since the RISC-bound siRNA comprises < 1% of total liver siRNA at any given time after dosing, it does not significantly influence the overall PK half-life of total siRNA in liver.

A few theoretical and experimental considerations on the PK of GalNAc-siRNA require further discussion within the context of the present mPBPK modeling. Experimental considerations and challenges relating to transient saturation of ASGPR following IV dosing have been described (McDougall et al., 2021), which can impact the estimation of the true SC bioavailability (F) of GalNAc-siRNA using conventional fitting to plasma PK following IV and SC. In this work, estimation of bioavailability relied on simultaneous fitting of multiple plasma PK datasets following dosing in mice.
and/or monkeys. Included IV data were following relatively high doses of siRNA (10 and 25 mg/kg) in mice. Therefore, it is likely that our reported ‘apparent’ F estimates are affected (i.e., reflect an underestimate of true SC bioavailability) resulting from an early, transient saturation of ASGPR following higher IV doses. Proper computation of the true absolute bioavailability may be achieved by conducting low-dose IV infusion studies, as an approach to reduce ASGPR saturation occurring with IV bolus dosing. Based on their size (~15 kDa) and hydrophilic nature, renal clearance of GalNAc-siRNA was assumed to occur as a function of the plasma fraction unbound ($f_{up}$), GFR, and concentrations within the kidney vascular space ($C_{k,vas}$). Under these assumptions, the current model predicts that renal clearance, at pharmacologic doses, accounts a relatively minor pathway of elimination at ~11% of total clearance of GalNAc-siRNA in humans. Interestingly, the rates of renal clearance of inclisiran and lumasiran was found to approximate the total GFR without accounting for $f_{up}$ (Wright et al., 2020). These findings possibly suggest that nonspecific binding of GalNAc-siRNA to plasma proteins may not effectively restrict glomerular filtration; however, more studies are needed.

Despite such considerations, the developed mPBPK model which integrated in vitro biomeasures, preclinical PK-PD data in animals, and clinical responses, successfully characterized the unique PK-PD properties of GalNAc-siRNA therapeutics. It is particularly encouraging that our mathematical model-based demonstration of feasibility in translating the PK-PD properties of GalNAc-siRNA across species (based on allometric/PBPK principles) was in general agreement with extensive absorption, distribution, metabolism, and excretion (ADME) and PK-PD experiments which showed good predictability in preclinical to clinical translatability for this drug class (McDougall et al., 2021). Although the current model offers a mechanistic framework for understanding the clinical behavior of GalNAc-siRNA, additional mechanistic studies (Humphreys et al., 2019; Agarwal et al., 2021) are needed to fully decipher the binding and cellular disposition properties of this drug class, which contribute to their unique PK-PD properties. In vitro quantification of the cellular and subcellular disposition kinetics of GalNAc-siRNA (of varying chemistries) in viable experimental cell systems will enable further in vitro-in vivo bridging using more evolved mathematical models. Similarly, there remains considerable opportunity to employ in
vitro stability and gene knockdown assays in hepatocytes from different species to inform in vivo potency apriori towards prediction of activity in humans (Basiri et al., 2020).

In summary, the approaches taken in this work demonstrate how several basic, well-established processes governing PK (e.g., species physiology, binding, transport, TMDD), target engagement, and PD (e.g., mechanism of action, biomarkers, and turnover processes) can be assembled (Ayyar and Jusko, 2020) to establish integrated PK-PD models for novel therapeutics such as GalNAc-siRNA. The proposed framework is expected to promote mechanistic and quantitative reasoning to guide experimental designs during the preclinical and early clinical development of GalNAc-siRNA. This mechanistic systems model may also form the basis for characterizing the PK-PD properties of other ligand-conjugated oligonucleotide therapeutics.
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Authorship contributions

Conceptualized and designed research: Ayyar and Heald

Conducted experiments: Ayyar, Song, Carpenter

Contributed new reagents or analytic tools: Ayyar, Song, Zheng, Carpenter, Heald

Performed data analysis: Ayyar, Song, Carpenter, Zheng

Wrote or contributed to the writing of the manuscript: Ayyar, Song, Zheng, Carpenter, Heald
REFERENCES


Footnotes

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Conflict of interest statement

All authors are past or current employees of Johnson and Johnson.
FIGURE LEGENDS

Figure 1. Schematic of the mPBPK-PD model for GalNAc-siRNA mediated target gene knockdown. The scheme shown in (a) is a mPBPK model depicting the whole-body disposition of GalNAc-siRNA. All organs are represented by a rectangular compartment and connected in an anatomical manner with blood flow (solid arrows). Arrows represent the direction of the flow. Each tissue compartment within this model, except plasma and remainder, is divided into sub-compartments. In (b), the structure of the organ-level mPBPK-PD model for GalNAc-siRNA disposition and pharmacodynamics is shown. The liver tissue is divided into vascular, extracellular, and cellular sub-compartments. Cellular (hepatocyte) uptake processes as relevant to ASGPR dynamics, GalNAc-binding, internalization, endosomal degradation, and cytoplasmic entry are shown (enlarged within dashed box). For a detailed description of the symbols and processes please refer to the “Minimal PBPK-PD Model” sub-section in the “Methods”.

Figure 2. Model-based characterization of the concentration-time profiles of total liver PK (a) and RISC-loaded siRNA in liver (b) as well as time courses of liver AT mRNA (% baseline) (c) and serum AT (% baseline) (d) following a single dose of ALN-AT3 in mice at 1, 2.5, or 5 mg/kg SC. Model simulation predicting serum AT following ALN-AT3 once-weekly (QW)× 5 at 0.75, 1 and 3 mg/kg SC injections are shown (e) and observed data (Sehgal et al., 2015) are overlaid (f). Solid lines represent model fitted or simulated profiles whereas symbols indicate observed data points. Use of unique colors and symbols are described within the figure.

Figure 3. Model-based characterization of the concentration-time profiles of total liver PK at 10 mg/kg SC (a) and RISC-loaded siRNA in liver at 1 and 5 mg/kg SC (b) givosiran in rats. Solid lines represent model fitted or simulated profiles whereas symbols indicate observed data points. Use of unique colors and symbols are described within the figure.

Figure 4. Model-based characterization of plasma AT (% baseline) following single doses of ALN-AT3 in cynomolgus monkeys at 1, 3, 10, and 30 mg/kg SC. Solid lines represent model fitted profiles whereas
symbols indicate observed data (Sehgal et al., 2015). Use of unique colors and symbols are described within the figure.

**Figure 5.** Model-based characterization of the plasma concentration-time profiles of fitusiran (a) and the time course of plasma AT (% baseline) following various SC dosing regimens in humans. Model simulations predicting plasma AT (% baseline) following fitusiran given once-monthly (Q4W) × 3 at 0.45 and 1.8 mg/kg SC, along with observed data (Pasi et al., 2017) overlaid, are shown (c). Use of unique colors and symbols are described within the figure.

**Figure 6.** Results from the global sensitivity analysis (GSA) on the developed mPBPK-PD model for ALN-AT3: PRCC-based sensitivity indexes of ‘Liver uptake clearance’, ‘SC bioavailability’, ‘SC_{50} – bound RISC potency’, ‘endosomal degradation’, ‘bound RISC degradation’, and ‘target protein degradation’ on target protein knockdown. PRCC, Partial Rank Correlation Coefficient

**Figure 7.** Interspecies comparisons of parameter values for SC absorption (k_{a}) and plasma AT degradation constant (k_{deg,p}).
Table 1. Summary of published preclinical and clinical *in vivo* datasets used to develop the proposed translational mPBPK-PD model.

<table>
<thead>
<tr>
<th>Target</th>
<th>Dose (mg/kg)</th>
<th>Species</th>
<th>Measurement(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>1 - 5 (SC, SD)</td>
<td>Mice</td>
<td>Plasma, liver, and RISC PK, AT mRNA, plasma AT</td>
<td>Sehgal et al., 2015; Nair et al., 2017</td>
</tr>
<tr>
<td>AT</td>
<td>25 (IV and SC, SD)</td>
<td>Mice</td>
<td>Plasma PK, Liver PK (SC)</td>
<td>Akinc et al., 2012</td>
</tr>
<tr>
<td>AT</td>
<td>0.75 - 3 (SC, MD)</td>
<td>Mice</td>
<td>Plasma AT</td>
<td>Sehgal et al., 2015</td>
</tr>
<tr>
<td>TTR</td>
<td>10 (IV and SC, SD)</td>
<td>Mice</td>
<td>Plasma, kidney PK</td>
<td>Nair et al., 2017</td>
</tr>
<tr>
<td>ALAS1</td>
<td>1 – 10 (SC, SD)</td>
<td>Rat</td>
<td>Liver, RISC PK</td>
<td>Lee et al., 2019</td>
</tr>
<tr>
<td>AT</td>
<td>1 – 30 (SC, SD)</td>
<td>NHP</td>
<td>Plasma AT</td>
<td>Sehgal et al., 2015</td>
</tr>
<tr>
<td>AT</td>
<td>0.015 - 1.8 (SC, SD, MD)</td>
<td>Human</td>
<td>Plasma PK, plasma AT</td>
<td>Pasi et al., 2017</td>
</tr>
</tbody>
</table>

SC: subcutaneous; IV: intravenous; SD: single dose; MD: multiple dosing; NHP: nonhuman primate. Target gene symbols defined in the text.
Table 2. mPBPK-PD model parameter values in mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Estimate</th>
<th>CV% or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{tot}$ (nM)</td>
<td>Baseline ASGPR density in mouse liver</td>
<td>633.4</td>
<td>Bon et al., 2017</td>
</tr>
<tr>
<td>$k_{deg}$ (h$^{-1}$)</td>
<td>Degradation rate constant for ASGPR</td>
<td>0.04</td>
<td>Bon et al., 2017</td>
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<tr>
<td>$k_{syn}$ (nM/h)</td>
<td>Synthesis rate for ASGPR</td>
<td>27.9</td>
<td>Calculated ($k_{syn} = R_{tot} \cdot k_{deg}$)</td>
</tr>
<tr>
<td>$k_{off}$ (h$^{-1}$)</td>
<td>Dissociation rate constant for GalNAc to ASGPR</td>
<td>1.32</td>
<td>Estimated (Sato et al., 2002)</td>
</tr>
<tr>
<td>$k_{on}$ (nM$^{-1}$·h$^{-1}$)</td>
<td>Association rate constant for GalNAc to ASGPR</td>
<td>0.53</td>
<td>Calculated* ($k_{on} = k_{eff}/K_D$)</td>
</tr>
<tr>
<td>$k_{int}$ (h$^{-1}$)</td>
<td>Internalization rate constant for bound ASGPR</td>
<td>2.4</td>
<td>Estimated (Sato et al., 2002)</td>
</tr>
<tr>
<td>$k_{rec}$ (h$^{-1}$)</td>
<td>ASGPR recycling constant</td>
<td>13.8</td>
<td>Schwartz et al., 1981</td>
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<tr>
<td>$k_{deg,R}$ (h$^{-1}$)</td>
<td>Degradation rate constant for endosomal ASGPR</td>
<td>1.53</td>
<td>Schwartz et al., 1981</td>
</tr>
<tr>
<td>$k_{cle}$ (h$^{-1}$)</td>
<td>Rate constant for GalNAc cleavage from siRNA</td>
<td>1.32</td>
<td>Prakash et al., 2014</td>
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<tr>
<td>$k_a$ (h$^{-1}$)</td>
<td>SC absorption rate constant</td>
<td>0.7</td>
<td>6.5</td>
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<tr>
<td>$F$ (%)</td>
<td>Apparent SC bioavailability</td>
<td>33</td>
<td>5.0</td>
</tr>
<tr>
<td>$f_{\mu,p}$</td>
<td>Plasma fraction unbound</td>
<td>0.15</td>
<td>Humphreys et al., 2019</td>
</tr>
<tr>
<td>$PS$ (mL/h)</td>
<td>Kidney permeability flow rate</td>
<td>49.6</td>
<td>31.9</td>
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<tr>
<td>$f_{a,k}$</td>
<td>Kidney fraction unbound</td>
<td>0.07</td>
<td>15.6</td>
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<tr>
<td>$k_{ass,kid}$ (h$^{-1}$)</td>
<td>Kidney association rate constant for binding</td>
<td>9.9</td>
<td>14.2</td>
</tr>
<tr>
<td>$k_{dis,kid}$ (h$^{-1}$)</td>
<td>Kidney dissociation rate constant for binding</td>
<td>0.026</td>
<td>13.8</td>
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<tr>
<td>$CL_{up,liv_{in}}$ (mL/h)</td>
<td>Liver uptake clearance</td>
<td>207.2</td>
<td>8.5</td>
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<tr>
<td>$CL_{up,liv_{eff}}$ (mL/h)</td>
<td>Liver release clearance</td>
<td>0.0025</td>
<td>23.9</td>
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<td>$k_{ass,liv}$ (h$^{-1}$)</td>
<td>Liver association rate constant for binding</td>
<td>0.0032</td>
<td>63.2</td>
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<td>$k_{dis,liv}$ (h$^{-1}$)</td>
<td>Liver dissociation rate constant for binding</td>
<td>0.0034</td>
<td>24.4</td>
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<tr>
<td>$K_{P,rest}$</td>
<td>Partition coefficient for remaining organs</td>
<td>0.1</td>
<td>Fixed</td>
</tr>
<tr>
<td>$f_{esc}$</td>
<td>Fraction siRNA in endosome escaping into cytosol</td>
<td>0.01</td>
<td>Gilleron et al., 2013</td>
</tr>
<tr>
<td>$k_{deg,d}$ (h$^{-1}$)</td>
<td>Rate constant for endosomal siRNA degradation</td>
<td>0.012</td>
<td>3.0</td>
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<tr>
<td>$k_{on,RISC}$ (nM$^{-1}$·h$^{-1}$)</td>
<td>Association rate constant of siRNA to RISC</td>
<td>0.00023</td>
<td>22.6</td>
</tr>
<tr>
<td>$k_{off,RISC}$ (h$^{-1}$)</td>
<td>Dissociation rate constant of siRNA from RISC</td>
<td>$10^{-7}$</td>
<td>Fixed (Bartlett et al., 2006)</td>
</tr>
<tr>
<td>$RISC_{tot}$ (nM)</td>
<td>Total RISC concentration in hepatocytes</td>
<td>30</td>
<td>Wang et al., 2012</td>
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<tr>
<td>$k_{DR}$ (h$^{-1}$)</td>
<td>Degradation rate constant for loaded RISC</td>
<td>0.005</td>
<td>21.0</td>
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<tr>
<td>$k_{deg,c}$ (h$^{-1}$)</td>
<td>Degradation rate constant for free cytosolic siRNA</td>
<td>0.1</td>
<td>Fixed (sensitivity analysis)</td>
</tr>
<tr>
<td>$k_{deg,m}$ (h$^{-1}$)</td>
<td>Degradation rate constant for AT mRNA</td>
<td>0.06</td>
<td>41.3</td>
</tr>
<tr>
<td>$k_{deg,p}$ (h$^{-1}$)</td>
<td>Degradation rate constant for AT protein</td>
<td>0.05</td>
<td>25.0</td>
</tr>
<tr>
<td>$S_{max}$</td>
<td>Maximal stimulation of target mRNA degradation</td>
<td>14.2</td>
<td>Fixed (calculated)</td>
</tr>
<tr>
<td>$SC_{50}$ (ng/g)</td>
<td>Bound RISC at half maximal effect</td>
<td>2.4</td>
<td>6.5</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Gamma coefficient for mRNA-protein translation</td>
<td>1.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>
$K_D$ value set as 2.48 nM (Kanasty et al., 2013)
Figures

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

(a) SC absorption rate-constant $k_a$ (m$^2$/s).

$\log_{10} k_a = 0.327 \cdot \log_{10} \text{BW} - 0.29$

$R^2 = 0.820$

(b) Serum AT degradation rate-constant $k_{\text{deg}}$ (h$^{-1}$).

$\log_{10} k_{\text{deg}} = 0.021 \cdot \log_{10} \text{BW} - 0.22$

$R^2 = 0.946$