Supplementary Material

Impact of enhanced metabolic stability on pharmacokinetics and pharmacodynamics of GalNAc-siRNA conjugates

*Jayaprakash K. Nair, Husain Attarwala, Alfica Sehgal, Qianfan Wang, Krishna Aluri, Xuemei Zhang, Minggeng Gao, Ju Liu, Ramesh Indrakanti, Sally Schofield, Philip Kretschmer, Christopher R. Brown, Swati Gupta, Jennifer L.S. Willoughby, Julie A. Boshar, Vasant Jadhav, Klaus Charisse, Tracy Zimmermann, Kevin Fitzgerald, Muthiah Manoharan, Kallanthottathil G. Rajeev, Akin Akinc, Renta Hutabarat, Martin A. Maier**

Alnylam Pharmaceuticals, Cambridge, MA 02142, USA

**To whom correspondence should be addressed. Email: mmaier@alnylam.com*

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at each time point (open symbols) as well as the mean (dotted lines) normalized to *Gapdh.*

General experimental information

Supplementary Table 1. Lower limits of siRNA quantification in mouse plasma and tissues with Atto-probe and SL-RT qPCR assays.

Biol. Matrix	Atto-Probe	SL-RT-qPCR
Mouse Plasma	8 ng/mL	4 pg/mL
Mouse Liver	80 ng/g	0.4 ng/g
Mouse Kidney	160 ng/g	0.4 ng/g

Supplementary Table 2. Atto-probes for analysis of siTTR and siAT siRNAs and SL-qPCR primers and probes for analysis of siAT siRNAs.

Reagent	Sequence (5′-3′)
Atto-probe for siTTR	(Atto425)aaaacaguguucuugcucuauaa
Atto-probe for siAT	(Atto425)cugguuaacaccauuuacuucaa
Stem-loop Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGTT
Forward Primer	GCCCTTGAAGTAAATGGTGT
Reverse Primer	GTGCAGGGTCCGAGGT
Taqman Probe	TGGATACGACCTGGTT

(Atto425) = Atto425 fluorescence label, lower case indicates 2′-*O*-methyl

PK and PD comparison of siAT-1 and siAT-2 in mice

Experimental procedures. All procedures using mice were conducted by certified laboratory personnel using protocols consistent with local, state, and federal regulations. Experimental protocols were approved by the Institutional Animal Care and Use Committee and, the Association for Assessment and Accreditation of Laboratory Animal Care International (accreditation number: 001345). C57BL/6 male mice, aged 6-8 weeks, acquired from Charles River Laboratories were administered siAT-1 and siAT-2 conjugates subcutaneously with a volume of 10 μL per gram of body weight (n=2 per time point) at a dose of 25 mg/kg. The study design is depicted in Supplementary Table 3. Two animals from each study group were sacrificed at desired time points to harvest plasma, liver and kidney samples for analysis. Mice were perfused with saline following blood collection and prior to organ harvest.

All samples were analyzed by an HPLC-based Atto-probe assay using fluorescent probes for detection of antisense strand. The lower limit of quantification (LLOQ) for plasma and liver were 8 ng/mL and 80 ng/g respectively. The concentration of the conjugates in plasma samples were detected by annealing at 95°C a fluorescent RNA probe (Atto-probe) complementary to the antisense strand of the siAT-1 and siAT-2.

The samples were then quickly cooled to 20ºC in the presence of a complementary anti-probe, which anneals to the sense strand and analyzed by ion exchange (IEX) HPLC with fluorescence detection, as described below. The gradient allows for separation of the Atto-probe: Antisense duplex from the anti-probe containing duplexes. The anti-probe containing duplexes elute during the wash portion of the HPLC gradient. The intensity of the fluorescent signal of the Atto-probe: Antisense duplex was measured against a calibration curve generated in the same matrix to determine the concentration of the antisense strand in a samples. The fluorescence intensity was directly proportional to the amount of conjugate present in the calibration standards and study samples. The standard curve range was from 8 to 1000 ng/mL duplex for the plasma. Tissue samples and blank tissues were ground to powders, and powders were digested in a Proteinase K lysis buffer at 50 or 100 mg/mL. PBS or a portion of the blank matrix was spiked with siAT-2 to generate the standard curves and QC samples. Study samples were analyzed by IEX-HPLC on a Shimadzu HPLC LC-20AD pump with a Shimadzu fluorescence detector RF-20Axs and a Dionex DNAPac PA-200 analytical column, 4.6x250 mm.

Supplementary Table 3. Study outline for PK and PD comparison of siAT-1 and siAT-2 in mice post a single 25 mg/kg dose.

Group	Test Article	Dose (mg/kg)	Route	No.of animals	Blood and Tissues Collection Time Points
1	siAT-1	25	SC	28	0.083, 0.25, 0.5, 1, 2, 4, 8, 24, 48, 96, 168 and 336 h post-dose
2	siAT-2	25	SC	28	0.083, 0.25, 0.5, 1, 2, 4, 8, 24, 48, 96, 168 and 336 h post-dose
3	PBS	-	SC	2	24 h post-dose

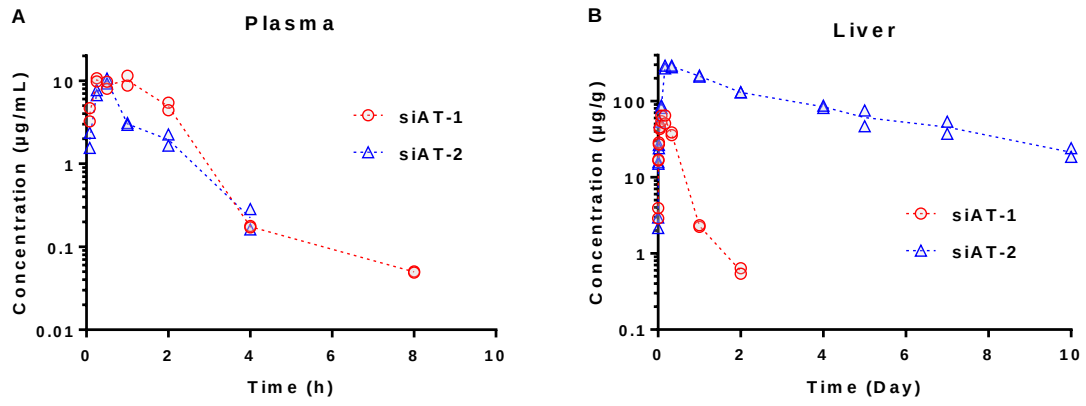

Figure S1. Concentration-time profiles of siAT-1 and siAT-2 in (A) plasma (P = 0.006) and (B) liver (P<0.0001) after single SC administration of 25 mg/kg in mice; shown are the individual data points (n=2 animals) at each time point as well as their mean (dotted lines).

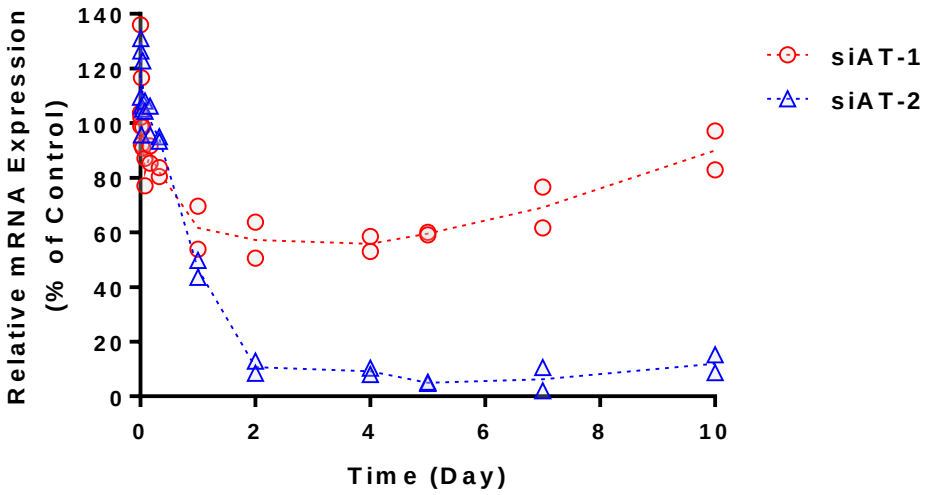

Figure S2. Antithrombin (AT) mRNA knockdown as a function of time in mice treated with 25 mg/kg of siAT-1 and siAT-2 relative to PBS-treated animals and normalized to G*apdh*; shown are the individual data points (n=2 animals) at each time point as well as their mean (dotted lines).

PK/PD relationship study: Antithrombin (AT) mRNA knockdown

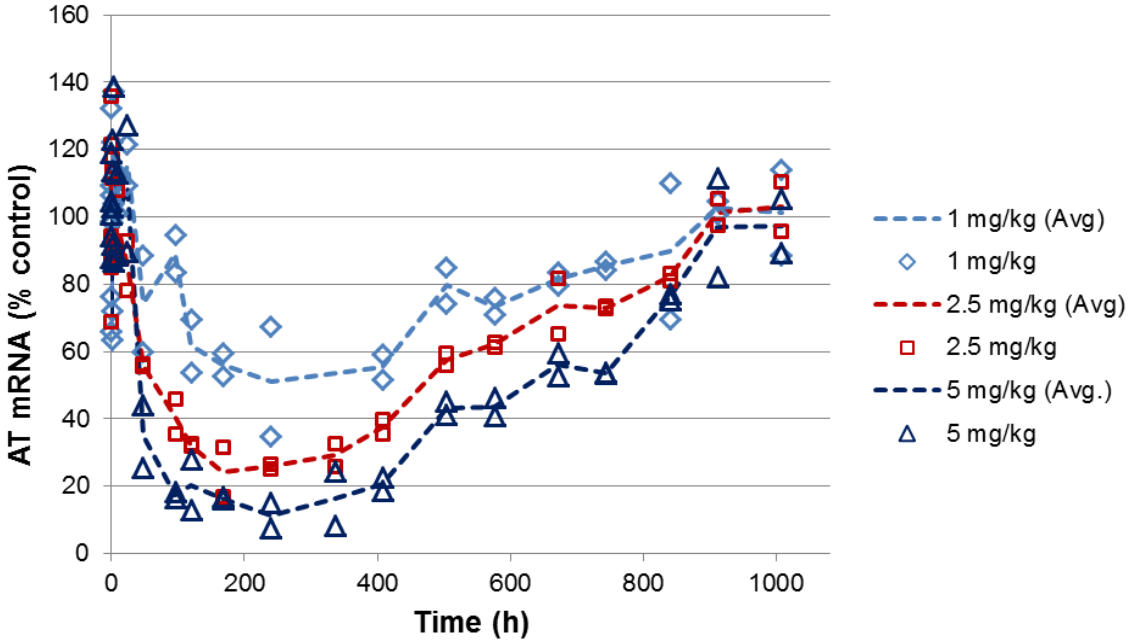

Figure S3. Antithrombin (AT) mRNA knockdown as a function of time in mice treated with 1, 2.5 or 5 mg/kg of siAT-2. Time points from 5 minutes to 8 hours post-dose were used to generate the baseline (100%) for calculating the percent AT mRNA reduction for subsequent time points; plotted are the individual data points from 2 animals at each time point (open symbols) as well as the mean (dotted lines) normalized to *Gapdh.*