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# The Nonclinical Disposition and PK/PD Properties of GalNAc-conjugated siRNA Are Highly Predictable and Build Confidence in Translation to Man

Author list: Robin McDougall*, Diane Ramsden*, Sagar Agarwal, Saket Agarwal, Krishna Aluri, Michael Arciprete, Christopher Brown, Elena Castellanos-Rizaldos, Klaus Charisse, Saeho Chong, Joseph Cichocki, Kevin Fitzgerald, Varun Goel, Yongli Gu, Dale Guenther, Bahru Habtemariam, Vasant Jadhav, Maja Janas, Muthusamy Jayaraman, Jeffrey Kurz, Jing Li, Ju Liu, Xiumin Liu, Steven Liou, Chris Maclauchlin, Martin Maier, Muthiah Manoharan, Jayaprakash K. Nair, Gabriel Robbie, Karyn Schmidt, Peter Smith, Christopher Theile, Akshay Vaishnaw, Scott Waldron, Yuanxin Yu, Xuemei Zhang, Ivan Zlatev, and Jing-Tao Wu

## SUPPLEMENTAL INFORMATION

**Supplemental Material**

## Synthesis of tritiated siRNA

RNA oligonucleotides were synthesized using modified synthesis cycles, based on those provided with the instrument. A solution of 0.6 M 5-(*S*-ethylthio)-1*H*-tetrazole in acetonitrile was used as the activator. The phosphoramidite solutions were 0.15 M in anhydrous acetonitrile with 15% dimethylformamide (DMF) as a co-solvent for 2′-OMe uridine and cytidine. The oxidizing reagent was 0.02 M $I_2$ in tetrahydrofuran (THF)/pyridine/water.*N*,*N*-Dimethyl-*N′*-(3-thioxo-3*H*-1,2,4-dithiazol-5-yl)methanimidamide (DDTT), 0.09 M in pyridine, was used as the sulfurizing reagent. The detritylation reagent was 3% dichloroacetic acid (DCA) in dichloromethane (DCM). Tritium-labeled oligonucleotides were synthesized at Alnylam following the above standard procedures modified to incorporate a single 5'-*C*-[$^3$H]-labeled nucleotide within the oligonucleotide sequence of the siRNA antisense strands. After completion of the solid-phase syntheses, the CPG solid support was washed with 5% (v/v) piperidine in anhydrous acetonitrile three times with 5-minute holds after each flow. The support was then washed with anhydrous acetonitrile and dried with argon. The oligonucleotides were then incubated with 28-30% (w/v) $NH_4OH$, at 35 °C for 20 h. The solvent was collected by filtration and the support was rinsed with water. Crude oligonucleotides were purified using strong anion exchange HPLC with phosphate buffers (pH = 8.5 or 11) containing 1 M sodium bromide, the appropriate fractions were pooled, and finally desalted. The identities and purities of all oligonucleotides were confirmed using electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS) and ion exchange high-performance liquid chromatography (IEX-HPLC). Oligonucleotide

solutions of ~ 1 $OD_{260}$ Units/mL were used for analysis of the crudes. ESI-LC-MS was performed on an Agilent 6130 single quadrupole LC-MS system using an XBridge C8 column (2.1 × 50 mm, 2.5 µm). Buffer A consisted of 200 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 16.3 mM triethylamine (TEA) in water, and buffer B was 100% methanol. A gradient from 0% to 40% of buffer B over 10 min followed by washing and recalibration at a flow rate of 0.70 mL/min. The column temperature was 75 °C. IEX-HPLC was performed on an Agilent 1200 HPLC system using an DNA Pac column (9 × 250 mm, 13 µm). Buffer A consisted of 20 mM sodium phosphate in water, pH 11, and buffer B was buffer A plus 1 M sodium bromide. A gradient from 0% to 65% of buffer B over 20 min followed by washing and recalibration at a flow rate of 1 mL/min was applied and the column temperature was 35 °C. Radiopurity of tritium-labeled oligonucleotides was established with the IEX-HPLC method using a Lablogic Systems beta-RAM 5 radio flow detector, coupled to the HPLC. All oligonucleotides were purified to an HPLC purity of >90% and desalted, then further annealed by mixing equimolar amounts of complementary sense and antisense strands to form the siRNAs as previously described (Foster et al., 2018).

**Supplemental Tables:**

Supplemental Table 1: In vitro evaluation of single and duplex stability in the presence of enzymes known to cleave RNA and DNA

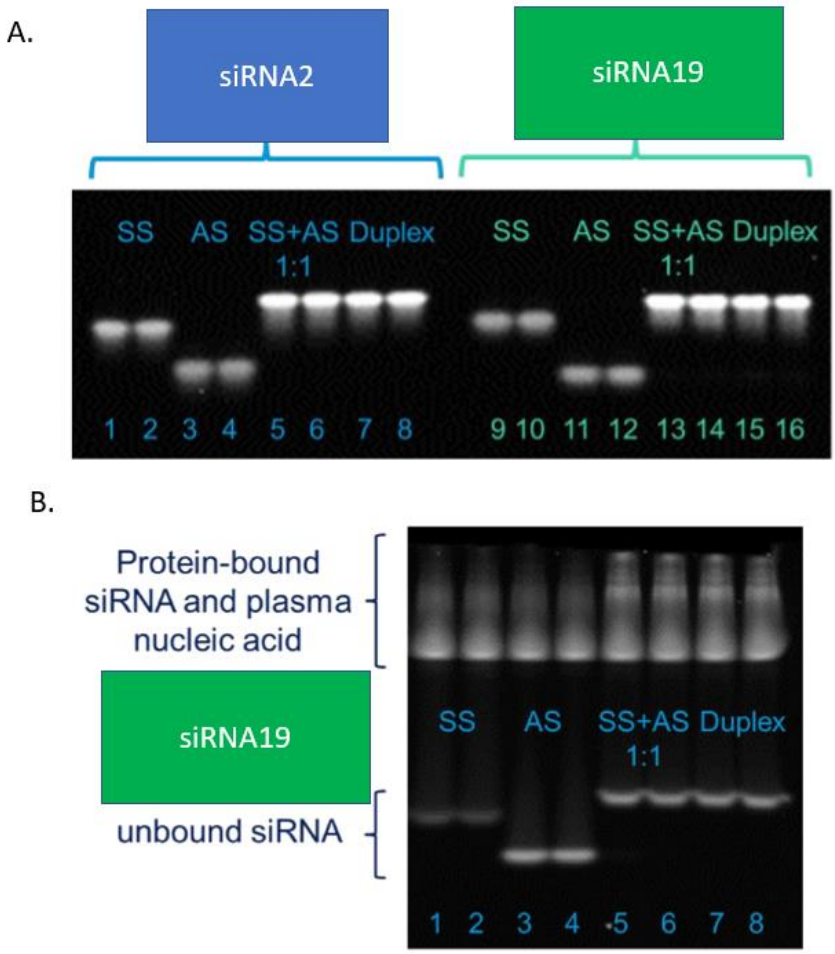
| Enzyme | Enzyme Concentration (units/rxn) | GalNAc-siRNA | Percent remaining relative to matrix | | | |
|---|---|---|---|---|---|---|
| | | | Duplex | | AS Alone | SS Alone |
| | | | AS | SS | | |
| Nuclease P1 60 min | 4 | siRNA2 | 125 | 125 | 91 | 84 |
| | 40 | siRNA2 | 130 | 120 | 12 | 28 |
| | 400 | siRNA2 | 122 | 130 | 0 | 1 |

Supplemental Table 2: Cross species metabolite profiles from plated hepatocytes and nonclinical liver samples

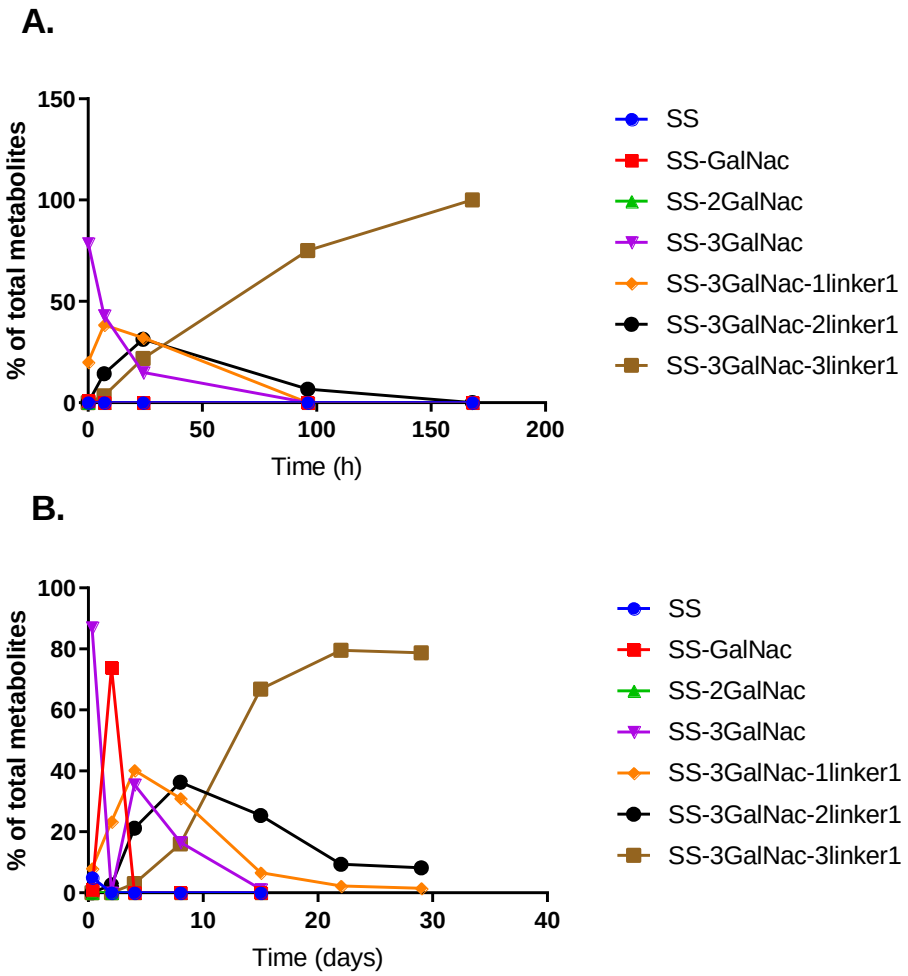
| Analyte | Major Metabolite | Rat | | Monkey | | Human in vitro |
|---|---|---|---|---|---|---|
| | | In vitro | In vivo | In vitro | In vivo | |
| siRNA6 | 3'N-1 | ✓ | ✓ | ✓ | ✓ | ✓ |
| | 3'N-3 | ✓ | ✓ | ✓ | ✓ | ✓ |
| siRNA2 | 3'N-1 | ✓ | ✓ | ✓ | ✓ | ✓ |
| siRNA5 | 3'N-1 | ✓ | ✓ | ✓ | ✓ | ✓ |
| siRNA9 | 3'N-1 | ✓ | ✓ | ✓ | ✓ | ✓ |

✓= observed

**Supplemental Figures:**




Supplemental Figure 1. Electrophoretic mobility shift assay (EMSA) results for siRNA2 and siRNA19 following a 1:1 mixture of sense strands (SS), antisense strands (AS) and duplex strands in PBS (Panel A). EMSA results for siRNA19, following a 1:1 mixture of sense strands, antisense strands, and duplex in human plasma. siRNA = small interfering RNA.




Supplemental Figure 2. Evaluation of sense strand metabolites in rat (Panel A) and monkey (Panel B) liver samples following SC administration of siRNA6. The data are displayed as the percent of the total metabolites recovered at each time point and are based on molecular mass for metabolite assignment. The designated metabolites (intact sense strand (SS), blue, SS missing 1 GalNAc moiety (red), SS missing 2 GalNAc moieties (green), SS missing 3 GalNAc moieties (purple), SS missing all GalNAc + 1 linker1 (orange), SS missing all GalNAc + 2 linker1 (black) and SS missing all GalNAc + 3 linker1 (brown) are compared with the total recovered metabolite quanitity to derive % of total metabolites (y-axis) over time (x-axis).