The Influence of GalNAc Valency on the Pharmacokinetic and Pharmacodynamic Parameters of siRNA in Rats

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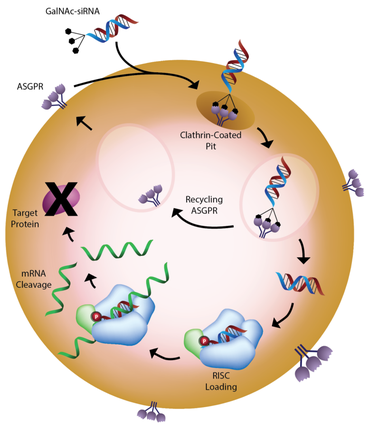
Abstract

Conjugating triantennary N-acetylgalactosamine (GalNAc) ligand to nucleic acid therapeutics such as small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs) has facilitated drug development through targeted, asialoglycoprotein receptor (ASGR) mediated uptake into hepatocytes.[1,2] Through this receptor-mediated approach, the drug is quickly cleared from circulation where it is taken up by the liver to produce efficient target knockdown with therapeutically relevant doses of compound.[3,4] To better understand this uptake mechanism, we investigated the impact of reducing the valency of GalNAc sugars presented to the ASGR. Sprague Dawley rats were dosed with a single subcutaneous injection of 2 mg/kg of siRNA with Tri-, Bi-, Mono- or No GalNAc. Multiple bioanalytical assays were used to compare the conjugates to determine the pharmacokinetics and pharmacodynamics including LC-MS, qPCR, RISC loading and protein binding. Reducing the number of GalNAc sugars to two had no impact on uptake, distribution, knockdown or RISC loading. A contrasting PK/PD profile with extended time in the plasma, reduced liver uptake, significant presence in the kidney and minimal RISC loading could be seen when a single GalNAc or unconjugated siRNA was tested. Pre-treatment with a siRNA targeting ASGR, with subsequent dosing of either Tri- or No GalNAc siRNA dramatically shifted the PK parameters of the Tri-GalNAc siRNA to match that of the siRNA lacking GalNAc. Collectively, this data further defines the influence of GalNAc valency on the distribution, pharmacodynamics and pharmacokinetic properties of siRNAs.

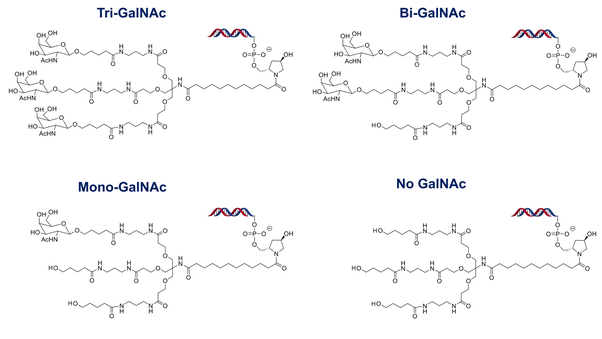
Figure 1: SC-Administered Platform for Targeted Delivery of GalNAc-siRNA Conjugates to Hepatocytes



Asialoglycoprotein Receptor (ASGPR) binds to the GalNAc ligand

- Highly expressed in hepatocytes (0.5-1 million copies per cell)
- Low to no expression in other tissues
- High rate of uptake
- Recycling time ~15 minutes
- Conserved across species

Figure 2: Schematics of siRNA Conjugates with Reduced GalNAc Valency



Chemical structure of No, Mono-, Bi-, and Tri- GalNAc sugars used on the siRNA duplexes in the study.

Figure 3: 8 Fold Higher Plasma AUC Observed with Mono- and No GalNAc siRNA Dosed at 2 mg/kg SC in Sprague Dawley Rats

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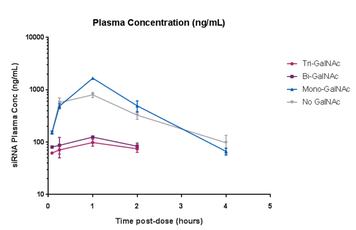

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Compound	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	t_{max}(h)	t_{last} (h)	$t_{1/2}$ (h)
Tri-GalNAc	98.6	164	1.0	2.0	NR
Bi-GalNAc	125	201	1.0	2.0	NR
Mono-GalNAc	1660	2510	1.0	4.0	NR
No GalNAc	801	1570	1.0	4.0	NR

(A) Total siRNA was measured by LC/MS in plasma collected up to 672 hours. All samples after 2 hours for Tri- and Bi- GalNAc compounds or after 4 hours for Mono- and No GalNAc compounds were below the limit of quantification and not shown on the graph. Additionally, one sample for Mono-GalNAc at 1 hour was below the limit of quantification and not shown on the graph. The data are represented as mean ± SD (n=2, n=1 for Mono-GalNAc 1 hour).
(B) PK Parameters were calculated using WinNonlin using non-compartmental analysis. Abbreviations: C_{max}= Maximum observed concentration occurring at t_{max}; AUC_{last}= area under the concentration-time curve from the time of dosing to the last measurable concentration; t_{max}= time to reach maximal concentration; t_{last}= time to last measurable concentration; $t_{½}$= elimination half life; NR= not reportable; insufficient values to calculate parameter.

Figure 4: 6 Fold Higher Liver AUC Observed with Tri- and Bi- GalNAc-siRNA

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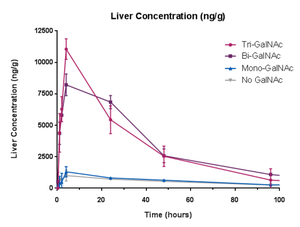

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Compound	C_{max} (µg/g)	AUC_{0-96h} (h*µg/g)	t_{max}(h)	t_{last} (h)	$t_{1/2}$ (h)
Tri-GalNAc	11.1	378	4.0	96.0	36.3
Bi-GalNAc	8.22	373	4.0	96.0	28.3
Mono-GalNAc	1.3	70.7	4.0	504	NR
No GalNAc	1.0	60.7	4.0	504	NR

(A) Total siRNA was measured by LC/MS in liver lysate collected up to 672 hours post-dose and graphed up to 96 hours post-dose. Concentration was reported as nanograms siRNA per gram tissue (ng/g) and values below the limit of quantitation reported as 0 ng/g. The data are represented as mean ± SD (n=2).
(B) PK Parameters were calculated using WinNonlin using non-compartmental analysis. Abbreviations: C_{max}= Maximum observed concentration occurring at t_{max}; AUC_{last}= area under the concentration-time curve from the time of dosing to the last measurable concentration; t_{max}= time to reach maximal concentration; t_{last}= time to last measurable concentration; $t_{½}$= elimination half life; NR= not reportable; insufficient values to calculate parameter.

Figure 5: Higher Kidney Absorption Observed with Mono- and No GalNAc-siRNA

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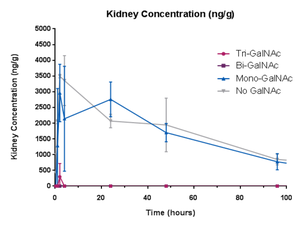

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Compound	C_{max} (µg/g)	AUC_{0-96h} (h*µg/g)	t_{max} (h)	T_{last} (h)	$t_{1/2}$ (h)
Tri-GalNAc	0.598	NR	2.0	2.0	NR
Bi-GalNAc	NR	NR	NR	NR	NR
Mono-GalNAc	2.96	171	2.0	96.0	39.7
No GalNAc	3.49	180	2.0	672	602

(A) Total siRNA was measured by LC/MS in kidney lysate collected up to 672 hours post-dose and graphed up to 96 hours post-dose. Concentration was reported as nanograms siRNA in gram tissue (ng/g) and values below the limit of quantitation reported as 0 ng/g. The data are represented as mean ± SD (n=2).
(B) PK Parameters were calculated using WinNonlin using non-compartmental analysis. Abbreviations: C_{max}= Maximum observed concentration occurring at t_{max}; AUC_{last}= area under the concentration-time curve from the time of dosing to the last measurable concentration; t_{max}= time to reach maximal concentration; t_{last}= time to last measurable concentration; $t_{½}$= elimination half life; NR= not reportable; insufficient values to calculate parameter.

Figure 6: Liver AT3 mRNA and Plasma AT3 Protein Knockdown Correlate with Number of GalNAc Sugars

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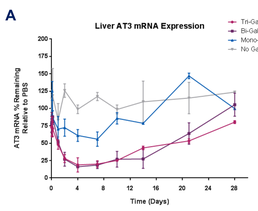

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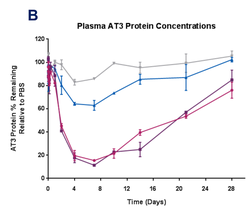

(A) Relative mRNA knockdown versus a saline control at Day 1 was calculated in liver lysates. Liver AT3 mRNA expression was quantified by RT-qPCR and normalized to Gapdh expression. The data are represented as mean ± SD (n=2, n=1 for Mono- and No GalNAc at 28 days).
(B) Relative AT3 protein knockdown versus a saline control at Day 1 was calculated by ELISA in plasma. The data are represented as mean ± SD (n=2, n=1 for Bi-GalNAc at 10 days).

Figure 7: mRNA Knockdown and Plasma AT3 Protein Concentrations Correlate to Ago Loading

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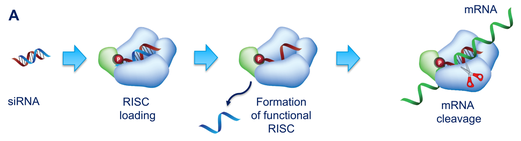

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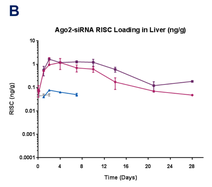

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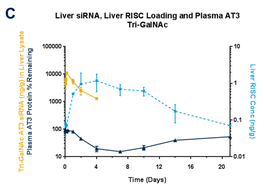
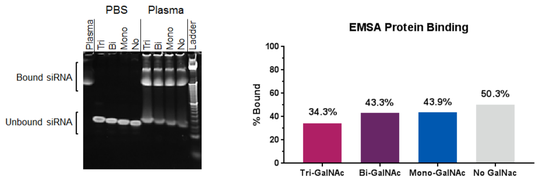


(A) Cartoon depicting RISC loading to mRNA cleavage.
(B) Ago2 immunoprecipitation was performed in liver lysates. The levels of Ago2-loaded antisense strand was quantified by stem-loop qPCR. Concentrations were reported as nanogram of antisense strand per gram of liver (ng/g) and values below the limit of quantitation not shown. The data are represented as mean ± SD (n=2, n=1 for Tri-GalNAc at 0.104 and 0.167 days; Bi-GalNAc at 28 days; Mono-GalNAc at 1 day; No GalNAc at 0.0104 and 0.167 days).
(C) Correlation of siRNA concentrations in liver lysate, RISC loading in liver lysate, and relative plasma AT3 protein concentrations in Tri-GalNAc siRNA samples. siRNA concentrations and RISC loading in liver lysate below the limit of quantitation are not shown. The data are represented as mean ± SD (n=2, n=1 for siRNA concentration at 4 days; RISC loading at 0.104 and 0.167 days).

Figure 8: Plasma Protein Binding by EMSA is Minimally Impacted by GalNAc Valency



Gel picture on left of Electrophoretic Mobility Shift Assay (EMSA) to determine siRNA protein binding. 5 µg/mL of siRNA in PBS or 99% Sprague Dawley rat plasma (n=1) was incubated for 1 hour and run on a 10% TBE gel, followed by a nucleic acid stain. The intensity of the unbound siRNA band in the plasma samples was compared to the respective unbound band intensity from the PBS sample (representing 100% free siRNA) to determine the % free siRNA. The graph on right represents the % bound siRNA as calculated from the gel (100 - % free siRNA = % bound siRNA).

Figure 9: Change Observed in Tri-GalNAc siRNA Plasma PK Following ASGR Knockdown by siRNA

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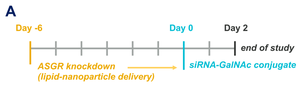

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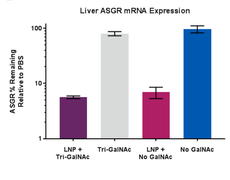

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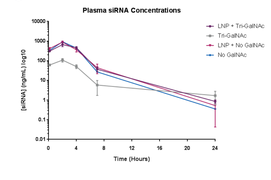
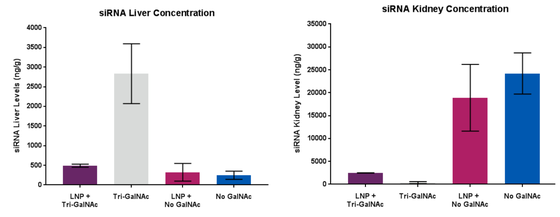


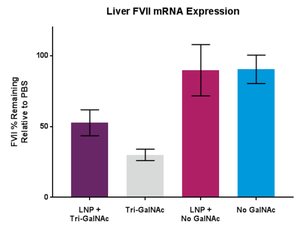
(A) Design for an ASGR Knockdown study. Rats (n=3) were dosed intravenously with 0.5 mg/kg ASGR unconjugated siRNA in an LNP formulation on Day -6. On Day 0, rats were dosed subcutaneously with 2.0 mg/kg of Factor VII siRNA with Tri-GalNAc or No GalNAc.
(B) Relative mRNA knockdown versus a saline control at Day 1 was calculated by qPCR in liver lysates. Liver ASGR mRNA expression was quantified by RT-qPCR and normalized to Gapdh expression. The data are represented as mean ± SD (n=3).
(C) Total FVII siRNA concentrations were quantified by stem-loop qPCR. The data are represented as mean ± SD (n=3).

Figure 10: ASGR Knockdown Impacts Tri-GalNAc Liver Distribution but not Kidney Distribution



Total FVII siRNA concentrations were quantified by stem-loop qPCR in liver lysate (left) and kidney lysate (right). The data are represented as mean ± SD (n=3).

Figure 11: Pre-treatment with ASGR siRNA Reduced mRNA Knockdown by Tri-GalNAc siRNA



Relative mRNA knockdown versus a saline control at Day 1 was calculated by qPCR in liver lysates. Liver FVII mRNA expression was quantified by RT-qPCR and normalized to Gapdh expression. The data are represented as mean ± SD (n=3).

Summary

- The valency of GalNAc directly impacts plasma and liver distribution confirming ASGR mediated liver uptake
  - Kidney uptake of siRNA appears independent of ASGR but is enhanced with Mono- or No GalNAc
- Both Tri- and Bi-GalNAc conjugates showed similar, robust AT3 liver mRNA and plasma protein knockdown
- RISC loading was comparable for both Tri- and Bi-GalNAc conjugates
  - RISC loading reveals a lag time after peak liver concentration is reached
- Degree of plasma AT3 protein knockdown correlates with RISC loading with Tri-GalNAc
- Plasma protein binding of siRNA is minimally impacted by GalNAc valency
- Pre-treatment with LNP siRNA for ASGR knockdown dramatically impacted Tri-GalNAc plasma PK and liver uptake, shifting the profile to that of an unconjugated siRNA lacking GalNAc

References

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