Mechanisms of Rat Hepatotoxicity of GalNAc-siRNA Conjugates

Saket Agarwal, Lauren Moran, Sara Woldemariam, Antoinette Hayes, Kellie D'Angelo, Kristina Perry, Alexandra Srnka, Samantha Chigas, Sean Dennin, Kirk Brown, Brenda Carito, Paul Gedman, Guo He, Muthusamy Jayaraman, Rohan Degaonkar, Xiumin Liu, Ju Liu, Arlin Rogers, Wendell Davis, Maja M. Janas

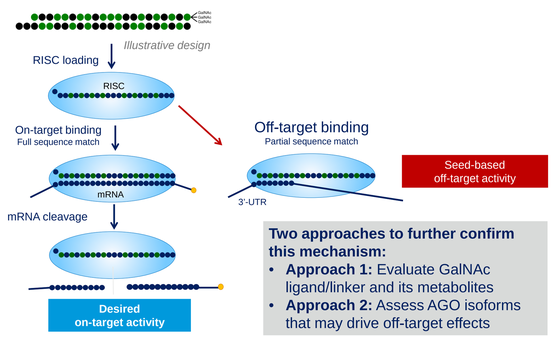
Alnylam Pharmaceuticals, Cambridge, MA, USA

Abstract

Nonclinical safety screening of short interfering RNAs (siRNAs) conjugated to a trivalent N-acetylgalactosamine (GalNAc) ligand is typically carried out in short-term repeat-dose rat toxicity studies at exaggerated exposures. In our previous work, we showed that seed hybridization-based off-target effects are important drivers of hepatotoxicity in rodent toxicity screens, and that GalNAc-siRNA chemistry or competition for RISC loading are not major contributors. Here we provide further mechanistic evidence that undesired off-target effects are driven by RNAi-like activity.

To this end, we evaluated two potential contributors to rat hepatotoxicity: 1) GalNAc ligand/linker and its metabolites, and 2) AGO isoforms that may drive off-target effects. In order to de-risk GalNAc ligand/linker, we used lipid nanoparticles (LNPs) to deliver siRNAs which were either conjugated or unconjugated to GalNAc and compared their safety. To identify AGO isoforms that drive off-target activity, we selectively knocked down AGO isoforms and assessed the activity on siRNA hepatotoxicity. Results presented here indicate that GalNAc/linker and its metabolites are not major contributors to rat hepatotoxicity, and that AGO2-siRNA RISC, and not AGO1 or AGO4, is driving off-target activity that underlies these liver effects.

Figure 1: Seed-Based Off-Target Activity is an Important Mechanism of Rat Hepatotoxicity of GalNAc-siRNA Conjugates

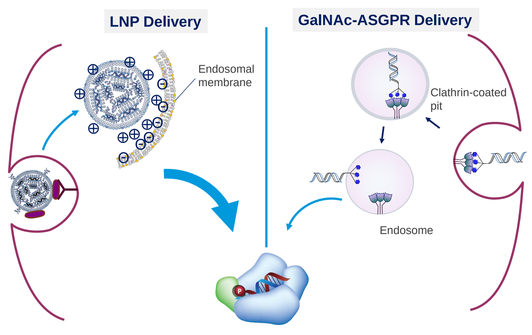


Two approaches to further confirm this mechanism:

- **Approach 1:** Evaluate GalNAc ligand/linker and its metabolites
- **Approach 2:** Assess AGO isoforms that may drive off-target effects

Janas, M.M., Schlegel, M.K., et al., 2018

Figure 2: Proposed Mechanisms of siRNA Uptake Delivered by LNP vs. GalNAc-ASGPR



Left panel: After intravenous administration, LNPs containing the siRNA cargo bind ApoE and are endocytosed via the ApoE receptor. The fusion of the LNP with the endolysosomal membrane leads to the release of siRNA into the cytosol. siRNA can then load into RISC for downstream knockdown of the target mRNA.
Right panel: After subcutaneous administration, GalNAc-siRNAs are endocytosed via the ASGPR receptor. The siRNA can then "escape" from the endolysosome by poorly understood mechanism(s) that are less efficient than the LNP-mediated endolysosomal release.

Approach 1: Assessing Rat Hepatotoxicity of siRNA Delivered via LNP vs. GalNAc-ASGPR

Group	Test/Control Article	Dose (mg/kg)	Animal Numbers (Male)	Route and Regimen	End of Study
1	0.9% NaCl	0	1001 - 1004	IV bolus (Day 1, 8, 15)	Day 16
2	-GalNAc bad actor siRNA	0.3	2001 - 2004		
3		1	3001 - 3004		
4		3	4001 - 4004		
5	+GalNAc bad actor siRNA	0.3	5001 - 5004		
6		1	6001 - 6004		
7		3	7001 - 7004		
8	+GalNAc bad actor siRNA	3	8001 - 8004	SC (Day 1, 8, 15)	
9		10	9001 - 9004		
10		30	10001 - 10004		

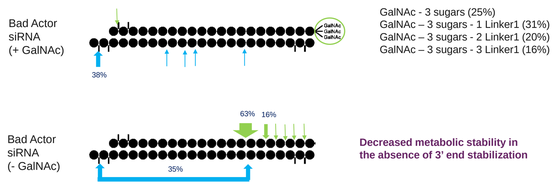
Objectives:

- De-risk GalNAc ligand: compare safety of siRNA +/- GalNAc (in LNP)
- De-risk the ASGPR trafficking: is bad actor siRNA still a bad actor siRNA if delivered in LNP?

Endpoints:

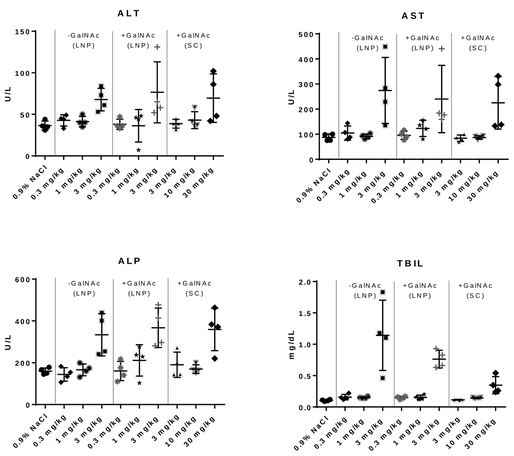
- Metabolic profiling
- Serum chemistry
- Anatomic pathology

Figure 3: Metabolic Profiling Shows Similar Metabolism of GalNAc Ligand/Linker Delivered by LNP or ASGPR Pathway



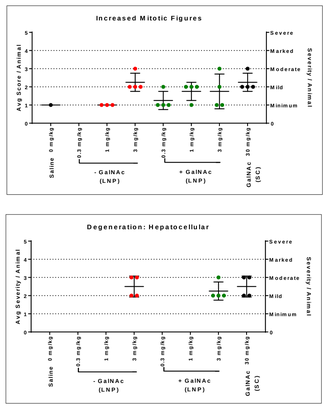
Rat liver metabolic profiling after intravenous administration of 3 mg/kg siRNA in LNP on Days 1, 8 and 15, assessed by mass spectrometry on Day 16. Green and blue arrows indicate sense strand and antisense strand cleavage sites, respectively. 0.3 and 1 mg/kg dose groups had similar metabolite profiles and aren't shown. Black circles = 2'OMe; bars = PS linkage.

Figure 4: Little Impact of GalNAc Ligand or Delivery Method on Bad Actor siRNA Liver Function Tests (LFTs)



Rat serum chemistry evaluation for liver injury biomarkers (Alanine Aminotransferase, ALT; Aspartate Aminotransferase, AST; Alkaline Phosphatase, ALP; and Total Bilirubin, TBIL) 24 hours post last dose. Rats were dosed IV with 0.3, 1, and 3 mg/kg siRNA with or without GalNac in LNP on Days 1, 8 and 15. GalNAc-siRNA was administered subcutaneously at 3, 10, and 30 mg/kg on Days 1, 8 and 15. Individual animal data is shown; error bars present standard deviation of the mean.

Figure 5: Little Impact of GalNAc Ligand or Delivery Method on Bad Actor siRNA Histopathology Findings

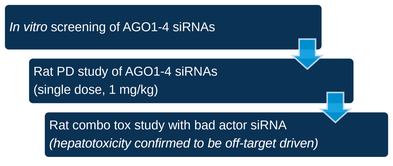


Rat liver microscopic findings 24 hours post last dose. Rats were dosed IV with 0.3, 1, and 3 mg/kg siRNA with or without GalNac in LNP on Days 1, 8 and 15. GalNAc-siRNA was administered subcutaneously at 3, 10, and 30 mg/kg on Days 1, 8 and 15. Each finding was graded (minimal to severe) and scored (1-5) based on severity as follows: minimal (1), mild (2), moderate (3), marked (4), and severe (5). Individual animal data is shown; error bars present standard deviation of the mean.

Approach 2: Strategy for Assessing which AGO Isoform(s) Drives Rat Hepatotoxicity of Bad Actor siRNA

There are four AGO isoforms in mammals (AGO1-4). AGO2 is the only* isoform that can mediate catalytic on-target mRNA cleavage, but all four AGO isoforms can potentially mediate microRNA-like off-target effects

1. Which AGOs are required for rat hepatotoxicity of bad actor siRNAs, if any?
2. Are off-target effects preferentially driven by certain AGO isoforms?



*New evidence suggests human AGO3 has slicer activity but that this activity depends on the guide RNA (Park et al., NAR, 2017)

Rat Tox Study Design to Evaluate Role of AGO Isoforms

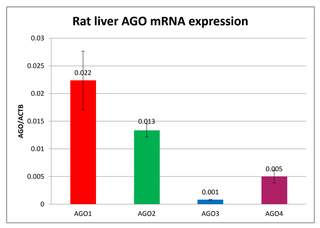
Treatment 1: AGO siRNAs

Group	First Test/Control Article	Dose (mg/kg)	Route and Regimen	Animal Numbers (Male)	End of Study
1	0.9% NaCl	0	SC (Days 1, 12)	1001 - 1004	Day 23
2	AGO1 siRNA	10		2001 - 2004	
3				3001 - 3004	
4	AGO2 siRNA			4001 - 4004	
5				5001 - 5004	
6	AGO4 siRNA			6001 - 6004	
7				7001 - 7004	

Treatment 2: Bad Actor

Group	First Test/Control Article	Dose (mg/kg)	Route and Regimen	Animal Numbers (Male)	End of Study
1	0.9% NaCl	0	SC (Days 8, 15, 22)	1001 - 1004	Day 23
2	0.9% NaCl	0		2001 - 2004	
3	Bad Actor siRNA	30		3001 - 3004	
4	0.9% NaCl	0		4001 - 4004	
5	Bad Actor siRNA	30		5001 - 5004	
6	0.9% NaCl	0		6001 - 6004	
7	Bad Actor siRNA	30		7001 - 7004	

Figure 6: Relative AGO mRNA Expression Levels in Rat Liver

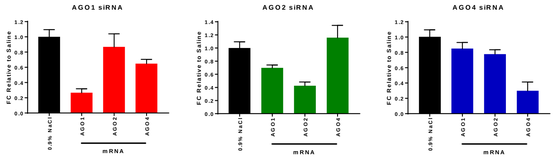


Relative to AGO2

AGO1	1.68	2-fold higher
AGO2	1.00	
AGO3	0.06	16-fold lower
AGO4	0.37	3-fold lower

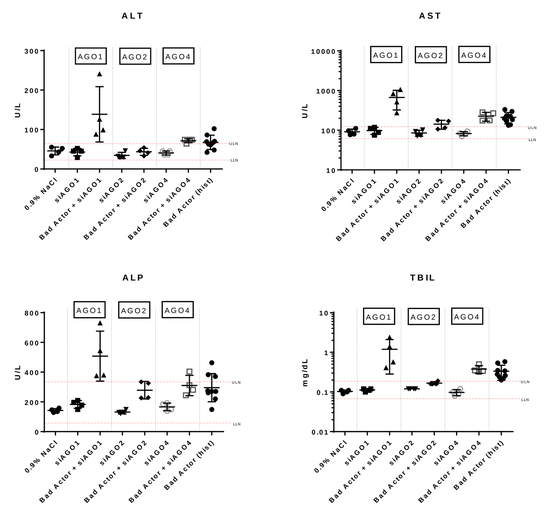
RNA was extracted from control male rat livers (N = 3), 8 weeks at the time of dosing, and AGO1-4 mRNA levels were assessed by qPCR relative to Actin β. Bars represent group means; error bars present standard deviations.

Figure 7: siAGO1 and siAGO4 are ~Equipotent and siAGO4 is Most Specific



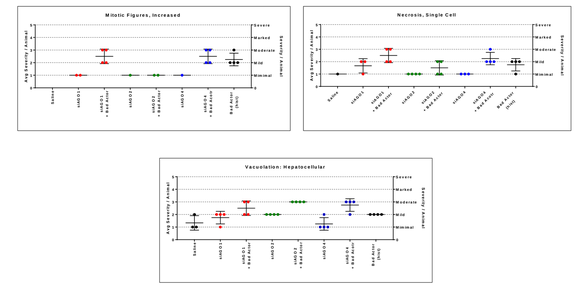
RNA was extracted on Day 23 from rat livers (n=4 per group) administered 10 mg/kg siRNA against AGO1, AGO2, or AGO4 on Days 1 and 12. mRNA expression level for ACTB, AGO1, AGO2, and AGO4 were measured using qPCR. The results were normalized to ACTB and reported as fold change relative to the saline treated control groups. Bars represent group means; error bars present standard deviations.

Figure 8: AGO1 Knockdown Tends to Exacerbate Bad Actor siRNA Liver Function Tests (LFTs) and AGO2 Knockdown Tends to Improve Bad Actor siRNA LFTs



Rat serum chemistry evaluation for liver injury biomarkers (Alanine Aminotransferase, ALT; Aspartate Aminotransferase, AST; Alkaline Phosphatase, ALP; and Total Bilirubin, TBIL) 24 hours post last dose of bad actor siRNA with or without AGO knockdown. Dosing regimen is described in rat tox study design. Individual animal data is shown; error bars present standard deviation of the mean.

Figure 9: AGO2 Knockdown Tends to Improve Bad Actor siRNA Histopathology Findings



Rat liver microscopic findings 24 hours post last dose of bad actor siRNA with or without AGO knockdown. Dosing regimen described in rat tox study design. Each finding was graded and scored based on severity as follows: minimal (1), mild (2), moderate (3), marked (4), and severe (5). Individual animal data is shown; error bars present standard deviation of the mean.

Figure 10: Re-Distribution of Bad Actor siRNA into AGO2 After AGO1 Knockdown

Rat cohorts (n=4 per group) were subcutaneously administered with 10 mg/kg siRNA against AGO1, AGO2, or AGO4, and with 30 mg/kg bad actor siRNA (dosing regimen described in rat tox study design). AGO1 and AGO2 RISC IPs followed by stem-loop qPCR for the bad actor siRNA was performed on Day 23 on rat liver lysates. Bars represent group means; error bars present standard deviations.

Summary

LNP Study

- Because LNP-delivered GalNAc-conjugated or unconjugated siRNAs had similar safety profiles in the rat, GalNAc, linker, or their metabolites are likely not contributing to hepatotoxicity of GalNAc-siRNAs.
- Because LNP-delivered or GalNAc-delivered siRNA had similar safety profiles in the rat, utilization of ASGPR-mediated uptake and endolysosomal trafficking are likely not contributing to hepatotoxicity of GalNAc-siRNAs.
- As shown previously (Janas, M., Schlegel, M. et al., 2018), hepatotoxicity is largely driven by sequence-based off-target effects.

AGO Study

- AGO2-siRNA RISC is driving off-target effects.
- AGO1-siRNA RISC is likely not a major driver of off-target effects.
- siRNA is not efficiently loading into AGO4, or AGO4-siRNA RISC is not a major driver of off-target effects.

References

1. Janas, M.M., Schlegel, M.K., et al., Selection of GalNAc-conjugated siRNAs with limited off-target-driven rat hepatotoxicity. Nat Commun, 2018, 9:723.
2. Park, M.S., et al., Human Argonaute3 has slicer activity. Nucleic Acids Res, 2017, 45(20): 11867–11877.

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