Selection of Well-Tolerated GalNAc-siRNAs by Screening for RNAi-Mediated Off-Target Effects in Rodent Toxicity Studies

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Agenda

- Nonclinical safety profile of GalNAc-siRNAs
- Mechanisms of GalNAc-siRNA hepatotoxicity in rodents
GalNAc-siRNA Conjugates
SC-Administered Platform for Targeted Delivery to Hepatocytes

**siRNA**
- Metabolic stability
- Duration of effect
- Intrinsic potency
- Safety
- CMC

**Ligand**
- Receptor affinity & specificity
- Metabolic stability
- Safety
- CMC

**Asialoglycoprotein Receptor (ASGPR)**
- Highly expressed in hepatocytes
- High turnover (recycling time ~15 min)
- Conserved across species
GalNAc-siRNA Conjugates
Nonclinical Considerations

- Highly specific distribution to liver via ASGPR
- Limited uptake/distribution in other organs
- Liver to kidney ratio >30 at PD doses
- Short plasma half-life; long tissue half-life; long PD effects
- Metabolized largely by intracellular exo- and endo-nucleases
- Maximum liver drug concentration limits relevant toxicological doses
  - Loss of dose-proportionality, approaching saturation of liver uptake via ASGPR
  - Cannot achieve MTDs
    - Excess circulating siRNA levels not taken up by liver; excreted through kidneys
GalNAc-siRNA Conjugates
Platform-Wide Responses at Toxicological Doses

Rat

○ Liver
  – Hepatocellular vacuolation: increased number and size of normal rat hepatocellular vacuoles, most contain lipid
  – Increased single cell necrosis
  – Increased mitosis and regeneration

○ Kidney
  – Basophilic granules in proximal tubular epithelium; represents drug accumulation

NHP

○ Liver
  – Basophilic granules in Kupffer cells and hepatocytes; represents drug accumulation

○ Lymph nodes
  – Vacuolated macrophages (with basophilic stippling); represents phagocytosis of drug

Primarily dose-dependent and the result of drug accumulation in tissue
Path Towards a Development Candidate (DC)

*In silico* prediction & *In vitro* efficacy

*In vitro* screen for predicted off-targets

Rodent Knockdown

Rat Tox @ >100x PD dose

NHP Knockdown

**DC**

Good Actors (60%): No hepatotoxicity

Bad Actors (40%): Show hepatotoxicity

Single cell necrosis and/or hepatocellular degeneration with ↑LFT 2x upper limit of normal
Potential Causes of Hepatotoxicity in Rodent Toxicity Studies

1. Non-RNAi drug effects
e.g. protein binding

2. Competition for Ago binding with miRNAs

3. RNAi-mediated off-target activity

Illustrative design

- RISC loading
- On-target binding: Full sequence match
  - mRNA cleavage
  - Desired on-target activity
- Off-target binding: Partial sequence match
  - 3'-UTR
5’ Modifications on the Antisense Strand Can Block RISC Loading With No Impact on Liver Exposure

Dose: 30 mg/kg
Regimen: q2d x 6
Necropsy: Day 15
Blocking Antisense RISC Loading Without Altering the 2’F/2’OMe/PS Content Mitigates Hepatotoxicity

Dose: 30 mg/kg
Regimen: q2d x 6
Necropsy: Day 15
Changing siRNA Chemical Modification Pattern Does Not Reduce Liver Exposure or RISC Loading

Dose: 100 mg/kg
Regimen: qw x 9
Necropsy: Day 58

ESC

Advanced ESC

Illustrative design

Altered modification pattern for improved metabolic stability

Rat Liver Exposure

Rat RISC Loading

ug AS / g liver

ng AS / g liver

ESC

Advanced ESC

Alnylam PHARMACEUTICALS
Changing siRNA Chemical Modification Pattern Does Not Mitigate Hepatotoxicity

Dose: 100 mg/kg
Regimen: qw x 9
Necropsy: Day 58
Potential Causes of Hepatotoxicity in Rodent Toxicity Studies

1. Non-RNAi drug effects
   e.g. protein binding

2. Competition for Ago binding with miRNAs

3. RNAi-mediated off-target activity

Illustrative design

On-target binding
Full sequence match

Off-target binding
Partial sequence match

Desired on-target activity

RISC loading

RISC

mRNA

mRNA cleavage

3’-UTR
Reversir™ Platform Achieves Tailored Control of RNAi Pharmacology

![Diagram showing mRNA target cleavage and RISC activation]

**Graph:**
- **Y-axis:** Relative Target Protein Level
- **X-axis:** Time (Days)
- **Data Points:**
  - 3 mg/kg GalNAc-siRNA
  - 0.1 mg/kg GalNAc-Reversir

**Legend:**
- **Reversir**
- **No Reversir**
Blocking RISC-Loaded Antisense Strand with Reversir Does Not Reduce Liver Exposure or RISC Loading

Prevention

Treatment

Reversir siRNA

3-10 mg/kg
30 mg/kg

Reversir siRNA

3-10 mg/kg
30 mg/kg
Reversing Activity of the RISC-Loaded Antisense Strand Mitigates Hepatotoxicity
Swapping Seed Regions Does Not Reduce Liver Exposure or RISC Loading

Dose: 30 mg/kg
Regimen: q2d x 6
Necropsy: Day 15

Rat Liver Exposure

Rat RISC Loading
Swapping Seed Regions Mitigates Hepatotoxicity

Dose: 30 mg/kg
Regimen: q2d x 6
Necropsy: Day 15

**Rat ALT**
GalNAc-siRNA Conjugates Can Cause Seed-Mediated Off-Targets at High Doses

RNAseq in Rat Hepatocytes (24 hrs, 10 nM)

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<th>Downregulated genes</th>
<th>Upregulated genes</th>
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<tr>
<td></td>
<td>Antisense seed</td>
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RNAi Activity is Driving Gene Dysregulation *In Vivo* at High Doses
RNAseq in Rat Liver (24 hrs, 50 mg/kg)

### RISC loading block

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Conclusions

- Antisense strand-driven RNAi-mediated hybridization-based off-target effects, not chemical modifications, are a major driver of hepatotoxicity of a subset of GalNAc-siRNA conjugates that fail in rodent toxicity studies at >100x PD dose.

- Careful selection of "good actor" siRNAs is an important part of identifying optimal siRNA molecules for clinical development.
**Thank You!**

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<tr>
<th>Nonclinical Safety and Bioanalysis</th>
<th>Research</th>
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