Recent Advancements for the Delivery of siRNAs to the Central Nervous System
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Advancements in Conjugate-Based Delivery Serve as a Blueprint for Extrahepatic Applications

Evolution of conjugate design with improved potency and specificity

- Potency:
  - > 10 GalNAc-siRNAs with human PoC;
  - 3 approved so far

- Specificity:

![Graph showing ED50 in mouse (SD) over time for different conjugates: Partially modified, STC, ESC, Advanced ESC, ESC+]

Investigational RNAi therapeutics for CNS
Investigational RNAi Therapeutics for CNS Diseases
Devastating diseases with enormous burden and unmet need

Many dominantly inherited neurodegenerative diseases:

- Alzheimer’s disease
- Amyotrophic lateral sclerosis (ALS)
- Cerebral amyloid angiopathy
- Frontotemporal dementia
- Huntington’s disease
- Multi-system atrophy
- Parkinson’s disease
- Spinocerebellar ataxia

A large number of genetically validated targets are known but few disease modifying therapies for these devastating, life threatening disorders

RNAi therapeutics directed to disease-causing, CNS-expressed genes represent an opportunity to address diseases with some of the greatest unmet need
Conjugation of 2'-O-palmityl (C16) to siRNAs Enables Robust and Durable Target Knockdown in the Rat CNS

Optimization of siRNA lipophile, position and design chemistry for CNS delivery

2'-O-palmityl Uridine: All nucleobases amenable to C16 modification

Single nt walk with C16: Impact on inherent RNAi activity

Optimal position for C16 benefit in vivo

Comparison of lipophiles
Greatest Potency Across Rat CNS with siRNA Combining Both 2'-C16 and 5'-VP: a Stable Phosphate Mimic

Single 0.9 mg Rat IT Dose (Day 28): Optimal C16 Position

% SOD1 mRNA Remaining Relative to aCSF

- Lumbar Spinal Cord
- Thoracic Spinal Cord
- Cervical Spinal Cord
- Brainstem
- Cerebellum
- Hippocampus
- Temporal Cortex
- Frontal Cortex
- Liver
- Kidney

Bioorg Med Chem Lett.2016; 26:2817
Potency benefit of 2'-C16 and 5'-VP Translates to Higher Species

Single 60 mg IT Dose (Day 84) in Cynomolgus Monkeys

% APP mRNA Remaining Relative to aCSF

Lumbar Spinal Cord
Thoracic Spinal Cord
Cervical Spinal Cord
Brainstem
Cerebellum
Hippocampus
Temporal Cortex
Frontal Cortex
Striatum
Liver
Kidney
Potency and Durability of Intrathecal Dosed Optimized siRNA Conjugates

Single and multi-dose response in rat

siRNAs targeting SOD1 in single dose or dose response
- Single siRNA conjugate doses of 0.9 mg, 0.3 mg, 0.07 mg
- Multidose arm - 0.3 mg monthly × 5
- Time points through 6 months for SOD1

Tissues: Spinal cord: Lumbar, thoracic and cervical
- Brain: prefrontal cortex, cerebellum and remaining brain
- Fluids: CSF and plasma
- Assays: mRNA, tissue siRNA levels, Histology
Robust and Durable Silencing Demonstrated Following a Single IT Dose

Silencing of SOD1 following a single or multiple IT doses

![Graphs showing silencing of SOD1 in different regions after a single or multiple IT doses.](image-url)
Robust Silencing of SOD1 Throughout the Brain Post Single IT-Dose

Intrathecal delivery of siRNA provides durable knockdown throughout CNS

Consistent lowering across animals in most regions of the brain
Highly Durable Amyloid Precursor Protein (APP) Knockdown in NHP

Single Intrathecal Dose of ALN-APP Supports Bi-Annual or Less Frequent Regimen

**CSF sAPPα and sAPPβ Protein Knockdown**  
(Single Intrathecal Dose in NHPs)

- **NHP1-sAPPα**
- **NHP1-sAPPβ**
- **NHP2-sAPPα**
- **NHP2-sAPPβ**
- **NHP3-sAPPα**
- **NHP3-sAPPβ**
Using Radio Imaging to Assess Distribution of siRNA in the CNS and Periphery
Rat CNS Distribution of Radiolabeled Conjugate-siRNA

Study Groups
Three groups:
Lumbar IT catheter
N = 2 animals/siRNA
3 toolkit siRNAs

Dose Formulation

Unlabeled siRNA

\[^{111}\text{Indium}]\text{-DTPA-conjugated siRNA\]

Dosing (per animal)

80 µCi
900 µg

\[^{111}\text{In} \ t_{1/2} = 2.8 \text{ days}\]

SPECT/CT Imaging
0-1.5 (dynamic), 4, 24 and 48 h

Sampling
Whole-organ and blood gamma counting, and immunohistochemistry for siRNA

Radiological quantification
\[^{111}\text{In} \ \text{whole-body uptake per timepoint, and uptake by 13 brain regions}\]
Biodistribution of IT-Dosed $^{111}$In-siRNA in Rodents

Co-registered SPECT/CT images facilitate anatomical orientation

ID 83, BW: 292 g
78.8 µCi, IT

H0-0.25  H0.25-0.5  H0.5-1  H1-1.5  H4  H24  H48

MIP: Maximum intensity projections derived from projecting the voxel with the highest value on every view throughout the volume onto a 2D image.
Biodistribution of IT-Dosed $^{111}$In-siRNA in Rodents

SPECT reveals rapid movement through CSF to brain (<1 h) followed by drainage to systemic circulation.

ID 83, BW: 292 g
78.8 µCi, IT

MIP: Maximum intensity projections derived from projecting the voxel with the highest value on every view throughout the volume onto a 2D image.
NHP CNS Distribution of Radiolabeled siRNAs: PET Imaging

Objective: Use higher resolution PET imaging to study the distribution of siRNAs in cynomolgus monkeys

[\(^{124}\text{I}\)]-SIB conjugation to amine

\[ \begin{array}{c}
\text{[}^{124}\text{I}\text{-SIB]} \\
\text{[}^{124}\text{I}\text{-SIB]} \\
\text{[}^{124}\text{I}\text{-siRNA]} \\
\text{[}^{124}\text{I}\text{-siRNA]} \\
\end{array} \]

\[ \begin{array}{c}
\text{succinimidyl} \\
\text{[}^{124}\text{I}\text{-iodobenzoate} \\
\end{array} \]

Iodine-124 (\(^{124}\text{I}\))

- High energy, high specific-activity positron emitter
- \( t_{1/2} = 4.18 \text{ days} \); \(^{125}\text{I}\)-siRNA stability in CSF = 14 days

\[ \text{Dosing (per animal):} \]

- \(^{124}\text{I}\)-conjugated siRNA
- Unlabeled siRNA
- 1-2 mCi
- 60 mg
- 2 mL

Dose Formulation

PET Imaging

- 0.5-1.5, 6, 24, 48, 96 h, 7 & 14 days

\(^{2}Z\text{alutsky 1988 Cancer Res 15, 1446-50; Chen 2014 Pharm Res 31, 2810-21.} \]
Representative Images Following IT Dosing

Co-registered PET/CT images facilitate anatomical orientation

Subject Information: ID 8502, Female, 5.4 kg
Injected Activity: 1.59 mCi
Representative Images Following IT Dosing

PET reveals rapid movement through CSF to brain (<1h) followed by drainage to systemic circulation

Subject Information: ID 8502, Female, 5.4 kg
Injected Activity: 1.59 mCi
Representative Images Following IT Dosing

PET at higher-sensitivity scaling shows wide distribution across the body, yet long retention within the CNS

Subject Information: ID 8502, Female, 5.4 kg
Injected Activity: 1.59 mCi

Same images with adjusted scale to facilitate viewing of brain exposure
GEMINI (Bis-RNAi™) Platform in the CNS
**GEMINI Platform**

**Objective**

- Effectively combine conjugate siRNAs for the simultaneous silencing of two transcripts or same (e.g. for viruses) using single chemical entity

*Three-strand 2xC16 CNS design*
## CNS Gemini Study 1: Mouse ICV

Objective: to evaluate the efficacy of multiple bis siRNA with varying nucleotide linkers after a single intracerebroventricular dose administration in C57BL/6 mice

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatment</th>
<th>Linker</th>
<th>n</th>
<th>Time Point</th>
<th>ICV Dose (ug) in 5ul</th>
<th>Readouts</th>
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<tr>
<td>1</td>
<td>aCSF</td>
<td>-</td>
<td>4</td>
<td>D21</td>
<td>--</td>
<td>qPCR: right hemisphere, left hemisphere, cerebellum, brainstem</td>
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<tr>
<td>2</td>
<td>Duplex Mixture</td>
<td>-</td>
<td></td>
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<td>50ug + 50ug</td>
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<tr>
<td>3</td>
<td>Multiplex 1</td>
<td>dTdTdT (DNA)</td>
<td></td>
<td>D21</td>
<td>100ug</td>
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<tr>
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<td>Multiplex 2</td>
<td>uuu (2’ OMe)</td>
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<tr>
<td>5</td>
<td>Multiplex 3</td>
<td>UUU (RNA)</td>
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<td>6</td>
<td>Multiplex 4</td>
<td>UFUUF (2’ F)</td>
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</tbody>
</table>

- Predicted metabolic very stable (stable in plasma, liver cytosol and tritosome)
- Predicted metabolic medium stable (2’ F linker cytosol cleaved in liver, dTdTdT cleaved in cytosol and tritosome)
- Predicted metabolic unstable (rapidly cleaved in plasma)
Comparison of Linker Chemistry Following ICV Administration

Best activity seen with the DNA (dTdTdT) linker

- DNA (dTdTdT) linker performed best
- 50%+ KD of mSOD1 and mCTNNB1
- DNA>Mix>2’F>2’OMe>RNA

![Graph showing SOD1 and CTNNB1 remaining relative to aCSF (GAPDH) across different hemispheres and brain regions.](image)
Summary

• Advancements in siRNA chemistry together with improvements in mechanistic understanding have been the predominant drivers behind the evolution of the conjugate platform technology

• Conjugation of 2’-O-palmityl (C16) to siRNAs along with 5’-VP enables safe, robust and durable target knockdown in the CNS of preclinical species

• Alnylam has developed an understanding of siRNA delivery, distribution and activity throughout the CNS across preclinical species.
  ◦ siRNA conjugates are active across CNS regions
  ◦ Radiolabeled studies show distribution of the siRNA throughout the CNS following IT administration through the primary CSF flow routes within 30 minutes
  ◦ Dose clears quickly, likely due to systemic drainage, with less than 5% remaining in the CNS at 48 hrs
  ◦ Rapid and substantial tissue peripheral distribution (highest concentration in liver)

• GEMINI platform can be used to target two separate transcripts in the CNS with a single drug entity