Developing life-saving oligonucleotide-based therapies for H-ABC Leukodystrophy

evotec | SynaptixBio

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Evotec's Integrated Discovery and Development Platform

- *in silico* design
- *in vitro* scale Synthesis
- Primary screen
- Hit Validation
- Toxicity prediction *in vitro*
- *in vivo* scale synthesis
- *in vivo* tolerability (ICV)
- PK, Pathology
- Lead selection
- Off-target analysis (Transcriptomics)
- Immunogenicity prediction (hPBMCs)
- *in vivo* scale synthesis
- Limited Rat PK (non-GLP)
- NHP PK/PD (non-GLP)
- *Candidate nomination*
- *in vivo* scale synthesis (non-GMP)
- IND enabling Tox

Introduction

- When healthy toddlers stumble and fall, their gait and coordination improve with time as they learn. This is unfortunately not the case for children with H-ABC (Hypomyelination with Atrophy of Basal Ganglia and Cerebellum), who are faced with a progressive and tragic decline of recently gained motor skills. H-ABC results in a spectrum of neurological symptoms such as dystonia, progressive gait impairment, speech, and cognitive deficits. Abnormalities in the cerebellum and striatum with associated loss of neurons and oligodendrocyte function contribute to these defects. H-ABC is a leukodystrophy caused by sporadic, typically *de novo*, heterozygous mutations in the TUBB4A gene and currently, there are no curative therapies available.
- Approximately 50 mutations have been identified in the TUBB4A gene, which are associated with TUBB4A-related leukodystrophy. The four most extensively studied mutations are listed in Table 1.
- We designed antisense oligonucleotides (ASOs) with precise specificity for the TUBB4A gene, exhibiting perfect conservation in humans and primates, ensuring high specificity and efficacy. Importantly, they do not intersect with mutations linked to leukodystrophies, thereby broadening the potential patient population.
- In this study, we used a combination of *in vitro*, *in vivo*, and bioinformatic-based approaches to reduce the number of acutely toxic molecules synthesized and tested in mice.

Mutation	Nucleotide change	Phenotype	Cell type Affected
R2G (often with S316F)	c.4C>G	Whispering Dysphonia	Neurons
D249N	c.745G>A	Classical H-ABC	Neurons and Oligodendrocytes
R282P	c.845G>C	Isolated hypomyelination	Oligodendrocytes
R391H	c.1172G>A	Hypomyelination	Oligodendrocytes

Table 1: Top four known mutations in TUBB4A gene.

Figure 1: The TUBB4A ASOs do not intersect with mutations linked to leukodystrophies.

In silico design

- ASO design conducted in-house utilizing optimized algorithms. The *in silico* process included thorough evaluation of target gene and existing patents, application of different filtering strategies balanced with conservation across species, EG: elimination of sequences containing potential toxic motifs, GGG repeats, and sequences with a high likelihood of forming homo- or hetero-dimers.

1) Detailed target analysis
2) Generate ASOs of specified sizes
3) Align ASOs using BWA to: Genome, Pre-mRNA, Mature mRNA
4) Process and organise the output, add ASO annotations
5) Future platform development : AI/ML based efficacy prediction

- **A few hundred sequences were selected for screen**
- **MOE gapmer-ASOs with full phosphorothioate (PTO) backbone**
- **Small and medium-scale ASO synthesis were conducted in-house at Evotec**

Primary screening

- Primary screening in transfected HeLa cells
  - Cells reverse-transfected in 384w format, ASOs spotted with Echo dispensing
  - Single conc 100 nM (4 biol reps), 24h timepoint
  - RT-qPCR using 1-step RT-qPCR protocol (multiplexed GOI and HSK)
  - Plate Acceptance criteria $RSD_{(\Delta Ct\ Ctrl-)} \leq 0.5$
- Selected hits (>70% GOI inhibition) were retested

- **Efficacious ASOs were subsequently chosen for hit validation.**

Figure 2: Primary screen in Hela cells. A) Comparable QC between screening plates. B) Analysis of HSK intraplate variation to spot anomalously high C_t values, which were flagged as possible toxic ASOs. C) Outcome of the primary screen represented as % TUBB4A expression, with hit threshold of 70% KD.

Hit validation

- **Confirmed hits were validated in iPSC-derived neurons** through gymnosis at concentrations of 4 and 20 µM.

Figure 3: Hit validation in iPSC-derived neuron. INR002: non-targeting ASO.

In vitro safety

To ensure the safety of ASOs before *in vivo* application, three assays were conducted. These assays included the prediction of hepatotoxicity, the prediction of acute neurotoxicity, and the assessment of potential immunogenicity.

Prediction of Hepatotoxicity

- The Caspase-Glo® 3/7 Assay (Promega) was employed to detect apoptotic activity, generating a luminescent signal via luciferase. The intensity of the luminescence is directly proportional to the level of caspase activity present.
- ASOs were evaluated in a 384-well format. Two control ASOs were included: LNA32, which is non-hepatotoxic, and LNA43, which is hepatotoxic. **A few ASOs were excluded from further analysis due to exhibiting high levels of apoptotic activity.**

Figure 4: Assessment of ASOs in a 384-well format for *in vitro* hepatotoxicity prediction. LNA43: control hepatotoxic ASO, LNA32: control non-hepatotoxic ASO.

- **Based on *in silico* data, potency in iPS-derived neurons, and hepatotoxicity prediction results, several ASOs were selected for *in vitro* neurotoxicity prediction assays.**

Prediction of neurotoxicity

Microelectrode array (MEA) can predict neurotoxicity and allow for the recording of extracellular local field potentials from cultured neurons, providing functional readouts that can assess the effects of ASOs on neuronal activity.

Figure 5: Microelectrode Array (MEA). A) Maestro system, 96-well plates with 8 electrodes/well from Axion BioSystem are used for the assay. B) Neuronal activities are recorded upon ASO treatment[4,5].

DIV 13: Mouse cortical primary neurons + Astrocytes → + ASO → Recording neuronal activity after 1, 2, 4, 24 hr

Figure 6: Prediction of neuronal toxicity using microelectrode array (MEA): Test ASOs were added on DIV13, their activities were recorded for 1h, 2h, 4h, and 24h post treatment.

- **Selected ASOs were qualified, non-toxic and undefined, for *in vivo* tolerability assay**

Immunogenicity

Immunogenicity prediction: CCL22 mRNA induction in Human B cells

- ASOs were classified as either highly immunogenic, with low immunogenicity, or undefined.
  - 384w format, 5-point CRC,
  - Clinically relevant controls were used

Figure 7: Induction of CCL22 mRNA in human B cells indicates the immunogenic potential of an ASO. Based on the results of the CCL22 assay, ASOs were classified into three categories: low-immunogenic, undefined, and highly immunogenic.

In vivo tolerability

Selected ASOs were administered to the murine brain through intracerebroventricular (ICV) injection.

We were interested in:

- Evaluating the tolerability of ASOs following central administration.
- Biodistribution of ASOs across different brain regions using relative quantification techniques.

Figure 8: Evaluation of ASO tolerability via ICV administration. 2-month-old male C57Bl/6J mice received either the test ASOs, vehicle (PBS), or non-targeting control ASO INR002 at 250 µg in total volume of 4 µl. The dose was reduced in half if not tolerated. Four mice per ASO. Animals were euthanized 2 weeks post injection.

In-life observations

- During the in-life phase, a neurobehavioral assessment test (toxicity scoring system), was utilized to evaluate six distinct features. Scores ranged from 1, indicating normal behavior (table 2, green), to 4, indicating maximum impairment (table 2, red).
  - Six distinct features were evaluated to assess the effects of ASOs: Hyperactivity, Decreased activity/consciousness, Motor function, Posture, Breathing, Involuntary movement (tremors, seizures)
- Body weight and food consumption were tracked.

Histopathology

- Microscopic examination was conducted only on cohorts that passed the neurobehavioral assessment.
- No microscopic abnormalities attributable to the administration of the ASOs were observed, suggesting a favorable safety profile.

Figure 9: Standard trimming of the adult rodent brain applied at Evotec.

ASO Biodistribution

- Tissue ASO concentrations were quantified utilizing a Triple Quad 6500+ LC-MS/MS system (Sciex, MA, USA) following a specifically developed internal protocol.

Tissue Homogenization & ASO Extraction → LC-MS/MS Analysis → Data Analysis → Evaluation of tissue exposure

Figure 10: Diagrammatic depiction of the methodology for quantifying ASOs exposure across specific tissues.

- The ASOs demonstrated consistent and comparable biodistribution across various brain regions, with concentrations ranging from approximately x µg/g to 4x µg/g tissue.

Summary

ASO ID	Target expression in HeLa assay	Target expression in iPSC neurons at 4 µM	Target expression in iPSC neurons at 20 µM	% Caspase hepatotoxicity, 30 nM	Immunogenicity	G content	MEA, Neurotoxicity	ICV at 250 µg	ICV at 125 µg
1						I			
2						I			
3						I			
4						I			
5						I			
6						I			
7						I			
8						I			
9						I			
10						I			
11						I			
12						I			
13						I			
14						I			
15						I			
								A B C D	E F G H
								Animal ID	

Table 2: Summary of the *in vitro* studies on the Evotec-designed ASOs targeting the TUBB4A gene, along with the *in vivo* tolerance of the selected ASOs following ICV administration. Candidate data removed, data for illustration purposes only.

Legend: G content; Efficacy; Well tolerated; Moderate symptoms with recovery; Severe reaction (not tolerated)

- By employing optimized algorithms and comprehensive filtering strategies, we effectively reduced the number of antisense oligonucleotide (ASO) candidates in the *in silico* design phase. This reduction significantly decreased the initial pipeline costs. With a success rate of approximately 30%, our results support previous studies that associate the number of guanine (G) nucleotides with toxicity.
- Our *in vitro* safety assays, which predict hepatotoxicity and neurotoxicity, enabled us to narrow down the number of ASOs for *in vivo* testing
- To date, we have narrowed down the selection to a few potent lead ASOs which show good tolerance following ICV administration, and comparable biodistribution within the brain.
- Further studies including early tox, PK and stability and off-target analyses using transcriptomics will be harnessed to refine the selection of a candidate.

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