Tools to Monitor Cell Type-Specific Gene Silencing and Functional siRNA Delivery

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1: siRNA-mediated gene silencing in the liver

Silencing a therapeutic target across multiple species

Besides recognizing the human target mRNA, it may be advantageous for a lead siRNA to recognize the target in other species. Not always can a single molecule fulfill those criteria and the development of surrogate siRNAs for certain species may become a requirement for simultaneous monitoring of pharmacological activity and mRNA/protein silencing as well as determination of early safety parameters in different animal models.

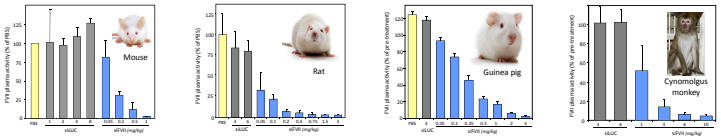


Fig.1: **Dose dependent and specific silencing coagulation factor 7 (FVII) across different species using LNP formulated siRNAs.** Surrogate siRNAs have been developed for mouse/rat and guinea pig, whereas the lead siRNA recognized FVII-mRNA in non-human primates and human. FVII protein activity was determined 48 hours post a single i.v. dose of the LNPs.

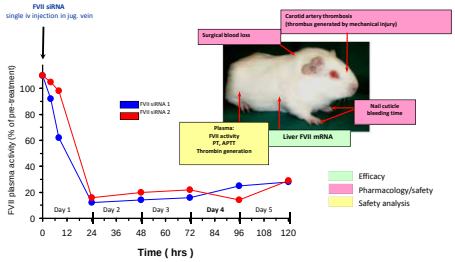


Fig.2: **siRNA-mediated FVII silencing in guinea pig.** A guinea pig specific FVII, LNP formulated siRNA was used to silence FVII. Besides the reduction of FVII activity in plasma and FVII mRNA in the liver, a number of pharmacological (thrombus formation, bleeding time and surgical blood loss) and safety parameters were monitored. In collaboration with Jacques Himbeer, Hoffman La Roche, Basel, Switzerland.

Liver cell type tropisms of siRNA formulations

Understanding cell type-specific siRNA delivery in the liver allows to select the ideal target/formulation combination for different siRNA-therapeutic programs.

In order to monitor cell type tropisms, we developed validated siRNAs for genes exclusively expressed in one of the 4 major liver cell types: Hepatocytes, stellate cells, Kupffer cells and endothelial cells. After i.v. administration of those marker-siRNAs in the test formulation, livers are harvested and marker mRNA silencing is determined by bDNA analysis. Formulations with different lipid composition show marked differences in liver cell type-specific delivery of siRNAs.

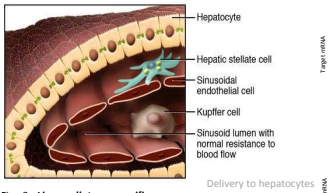
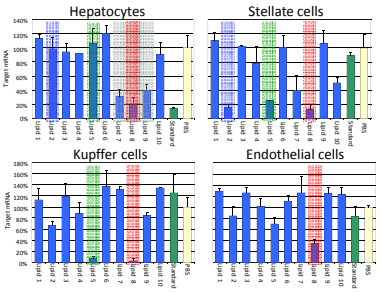
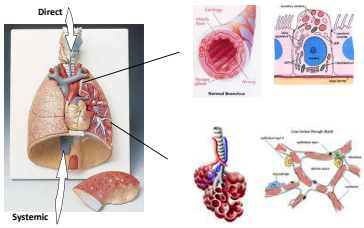


Fig. 3: **Liver cell type specific siRNA delivery.** LNPs containing different lipids show altered cell type tropisms.



2: Analysis of siRNA delivery to different lung cell types

Pursuing the concept of silencing of cell type specific marker genes as reporters for functional siRNA delivery, we identified potent, specific and chemically modified siRNAs against targets exclusively expressed in 8 different and therapeutically relevant cell types of the lung, including epithelial and endothelial cells as well as different alveolar cell types and macrophages. Activity of those siRNA in different formulations and administered either the pulmonary or the systemic route can be monitored by analysis of marker mRNA silencing in a total lung mRNA preparation.



Delivery to lung endothelial cells (in Collaboration with E-GEN)

The ability of a functionalized lipopolyamine to deliver siRNA to different lung cell types after systemic administration was assessed using the described marker siRNA approach (Fig. 4A). Silencing in endothelial cells was demonstrated for two different CD31 siRNAs and was transferred to another target (Tie-2) as second endothelial marker gene (Fig. 4B).

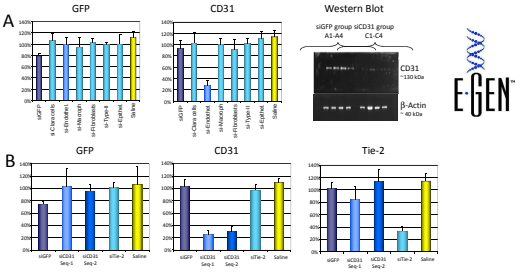


Fig. 4: **Marker-mRNA silencing with lipopolyamine formulated siRNA. A:**Mice were injected i.v. with 2 mg/kg formulated siRNAs on two consecutive days. Tissues were harvested 24 hours after the last dose and reporter gene expression was quantified using the bDNA assay.
B: To confirm specificity, animals were injected with a second siRNA targeting CD-31 and with an siRNA targeting Tie-2. GFP siRNA formulated in lipopolyamine served as control.

Switching from marker genes to therapeutically relevant targets, silencing of VEGF-R as well as silencing of EG5 in endothelial cells was demonstrated in a Lewis Lung Carcinoma tumor model. As all cells in the tumor express EG5, EG5 mRNA silencing was not as effective as observed for mRNAs exclusively expressed in endothelial cell. Dividing tumor cells show normal chromosome segregation, whereas dividing endothelial cells show monopolar spindles in tumors of animals treated with Eg5 siRNA.

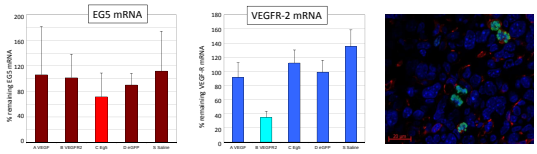


Fig. 5: **Lipopolyamine-mediated siRNA delivery to endothelial cells in a tumor model.** Tumor bearing mice were injected i.v. with 2 mg/kg formulated siRNAs on two consecutive days. Tumors were harvested 24 hours after the last dose and VEGF-R and EG5 gene expression was quantified. Chromosomes were visualized by phosphohistone H3 staining (blue) and endothelial cells were stained with a CD31 antibody (red).

3. Detection of nucleic acid based therapeutics in biologic samples

The use of a hybridization based fluorescence HPLC assay allows for a sensitive detection of oligonucleotides and their metabolites from biological samples. Axolabs' proprietary assay system is suitable for detection of single stranded as well as double stranded oligonucleotide therapeutics comprising of a wide range of chemical modifications. Extraction free sample preparation from plasma and tissue increases assay consistency and minimizes hands on time required. Lower limit of detection is currently 0.1ng ml^{-1} from blood and 0.2ng g^{-1} from tissue samples. Work is in progress to further improve the detection limit. The assay is compatible with subsequent metabolite identification by LC/MS.

Furthermore, in case of siRNA, the content of 5'-phosphorylated antisense strand can be quantified as marker for cytoplasmic delivery and thus enables establishment of a PD/biodistribution correlation.

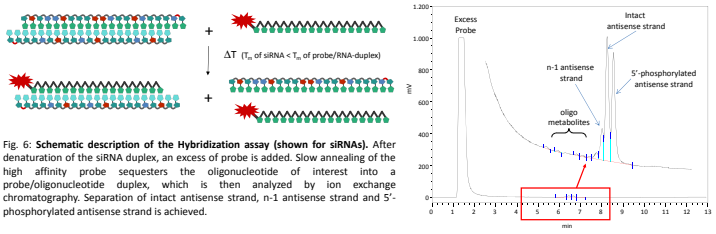


Fig. 6: **Schematic description of the Hybridization assay (shown for siRNAs).** After denaturation of the siRNA duplex, an excess of probe is added. Slow annealing of the high affinity probe sequesters the oligonucleotide of interest into a probe/oligonucleotide duplex, which is then analyzed by ion exchange chromatography. Separation of intact antisense strand, n-1 antisense strand and 5'-phosphorylated antisense strand is achieved.

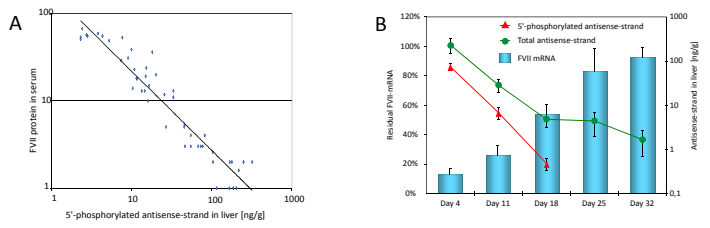


Fig. 7: **siRNA antisense strand 5'-phosphorylation correlates with siRNA activity and with duration of silencing.**
A: a total of 48 rats were dosed with either 0.2 or 3 mg/kg siRNA and 4 concentration of Dynamic Polyconjugates (1, 2, 5, and 10 mg/kg). 48 hours post dosing FVII activity was determined from serum samples and concentration of 5'-phosphorylated antisense strand in liver was determined using the hybridization-based fluorescence-HPLC assay. **B**: mice were dosed with siRNA and Dynamic Polyconjugates. mRNA silencing and liver concentration of 5'-phosphorylated siRNA were monitored over time by bDNA assay and hybridization-based fluorescence-HPLC , respectively

4. Early safety evaluation in rodents

Establishing a safety profile in rodents is an important step during the identification of functional siRNA delivery systems. Besides monitoring standard parameters like liver enzymes and clinical chemistry, specific focus should be directed towards analysis of innate immune system activation. Fig. 8 summarizes a number of parameters, which can be addressed in a safety screen. In combination with siRNA efficacy, those parameters allow to establish a therapeutic window for a given delivery system in rodents.

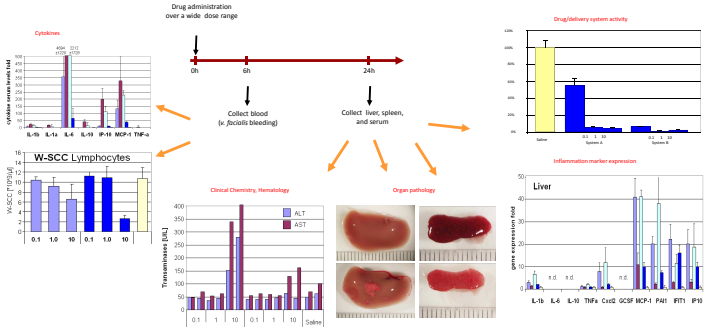


Fig. 8: **Comprehensive toxicological characterization of key safety parameters for oligonucleotide-based therapeutics and efficacious delivery systems**

5. Axolabs' service portfolio

Oligonucleotide Drug Pharmacology

Lead identification and optimization
Bioinformatics/*in silico* sequence pre-selection
Automated screening processes
In vitro specificity and off-target analysis
In vivo efficacy and early safety assessment of oligonucleotide-based therapeutics

Functional delivery *in vitro* and *in vivo*
In vivo models to monitor cell type-specific oligonucleotide delivery
Rational oligonucleotide design tailored for specific delivery systems
Confocal microscopy and flow cytometry facilities

Oligonucleotide Manufacturing

Synthesis
High-throughput synthesis for lead identification and lead optimization
Large scale synthesis up to multiple grams

Chemically modified oligonucleotides
Fluorescence labels
Small molecule, carbohydrate and peptide conjugates
Wide range of modified building blocks

Potency and stability optimization
Optimization of sequence, structure and chemistry

Manufacturing process optimization

Analytics and Bioanalytics

Analytics
Full characterization of single-stranded and double-stranded oligonucleotides by state-of-the-art mass spectrometry, HPLC and UPLC
Physicochemical and thermodynamic characterization of oligonucleotides (e.g. digital scanning calorimetry)
Established tech-transfer to CMOs

Bioanalytics
Oligonucleotide phamacokinetics and toxicokinetics
Characterization of Absorption, Distribution, Metabolism and Excretion (ADME) properties of oligonucleotide-based therapeutics