Improved *in vivo* potency for divalent GalNAc-siRNA conjugates over tri-antennary GalNAc conjugates.

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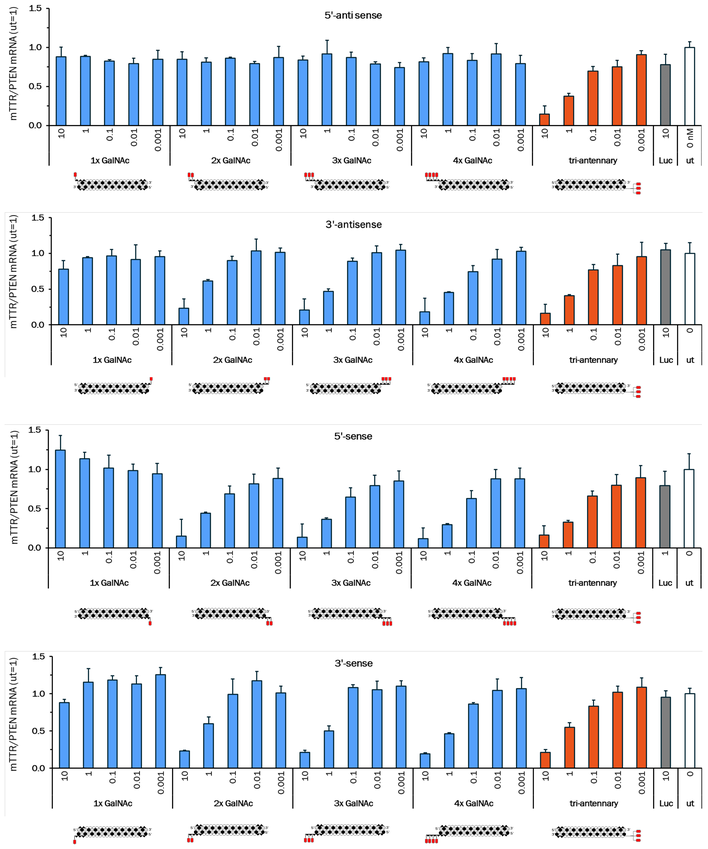
1. Introduction

Targeting the hepatic asialo-glycoprotein receptor (ASGPR) with GalNAc-conjugated oligonucleotides has been shown to be sufficient for both, hepatocyte targeting and functional delivery as required for RNAi. Here we focus on the serial conjugation of serinol-GalNAc units to siRNA to investigate the impact of position and carbohydrate valency to achieve target knockdown *in vitro* and *in vivo*. In a systematic manner we probed dose response of siRNA conjugates at all four termini with GalNAc-units (n = 1 to 4) in primary murine hepatocytes. Moreover, we investigated distance dependencies for conjugates with two single GalNAc units. Finally, we assessed siRNA stability in endosomal and lysosomal milieu and saw clear improvements that correlate with improved *in vivo* duration and dose response.

9. Summary and conclusions

- Simple design with two single serinol-GalNAc units positioned one at each terminus of the sense strand demonstrated 3-fold increased activity (ED_{50} ≈ 0.1 mg/kg) and duration of action compared to a tri-antennary GalNAc cluster, *in vivo*.
- Functional improvement by this design was confirmed in four out of four different sequences *in vivo* (data not shown).
- This simple design results in increased RNA stability at low pH in endosomal & lysosomal environment.
- Equal performance of two, three and four serial GalNAc units conjugated to siRNA, *in vitro* and *in vivo*.

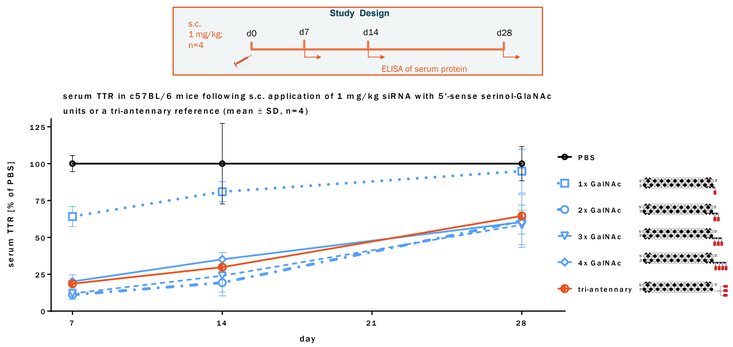
2. Design and *in vitro* activity of serial GalNAc units at terminal siRNA positions



- A single GalNAc unit at either of the four termini of the siRNA showed strongly reduced or no dose response in silencing of target messenger RNA 24h post treatment in primary murine hepatocytes. Similarly, no silencing was observed for 5'-antisense conjugates with serial GalNAc units. However, at the 5'-sense, the 3'-sense and 3'-antisense siRNA conjugates with two and more serial GalNAc-units showed equal dose response of silencing as compared to the tri-antennary reference.

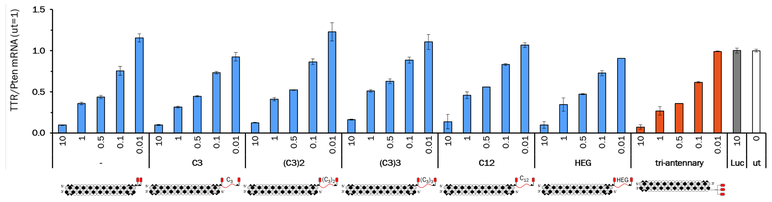
▲ PS ◠ PO ● 2´F ◎ 2´OMe serinol-GalNAc tri-antennary

3. *In vivo* duration of action of 5' sense conjugated serial GalNAc units



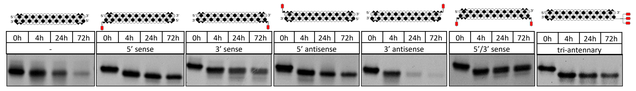
- *In vivo* serum TTR levels following s.c. administration of a single 1 mg/kg dose show equal target protein down regulation for two, three and four GalNAc units at the 5' sense as compared to the tri-antennary 5'-sense reference. A single GalNAc unit at that terminus shows strongly reduced knockdown which returns to base level at day 14 post treatment.

4. *In vitro* dose response to 2x GalNAc with different spacers



- As two single GalNAc units were sufficient for uptake and subsequent target transcript silencing, we tested whether the distance between the two GalNAc units had a major impact on the knockdown. We introduced spacers of different length between the two GalNAc units conjugated to the 3' end of the antisense strand of the siRNAs. The dose response was not altered by the increased distance between the GalNAc units.

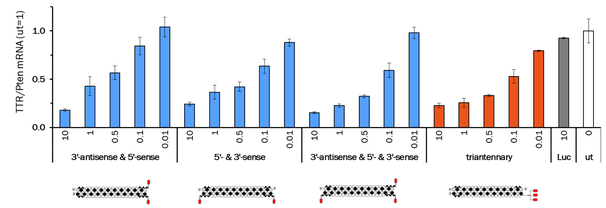
5. Position dependent stability in endosomal / lysosomal environment



- Conjugation of single GalNAc linker units to fully chemically modified siRNA resulted in altered RNA stability. Best stabilizing effect was achieved by conjugation of single units to both sense strand termini.

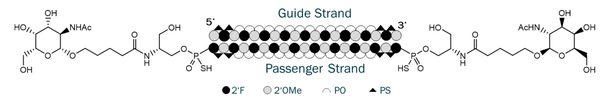
▲ PS ◠ PO ● 2´F ◎ 2´OMe serinol-GalNAc tri-antennary

6. Spacing of two GalNAc units can be as distant as on both termini of the sense strand of the siRNA

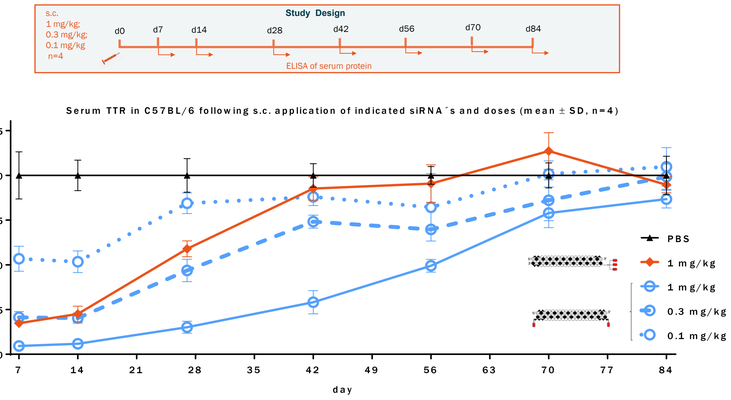


- As two single GalNAc units were sufficient and distance did not reduce the target transcript silencing, we tested whether combinations of different terminal single GalNAc units are accepted as well. All conjugates showed equal to slightly reduced dose response compared to the tri-antennary reference.

7. Improved divalent GalNAc-siRNA conjugate



8. Improved *in vivo* DR and DoA with terminal GalNAc units at the sense



- *In vivo* dose response and duration of action are improved approximately 3 fold by conjugation of single GalNAc units to termini of the sense strand as compared to the tri-antennary 5'-sense reference.

▲ PS ◠ PO ● 2´F ◎ 2´OMe serinol-GalNAc tri-antennary