Stereopure Oligonucleotides Incorporating Phosphoryl Guanidine Backbone Increase Durability of Gene Silencing by RNAi

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Summary

- We optimized phosphorothioate (PS) stereochemistry, the position and chirality of phosphoryl guanidine (PN) modifications, and the relationship between backbone modifications and 2'-sugar chemistry to improve the potency and durability of silencing for two different GalNAc-siRNA designs.[1,2]
- We performed an extensive analysis evaluating hundreds of stereopure GalNAc-siRNAs targeting mouse *Transthyretin* (*Ttr*) to optimize stereopure siRNA configurations.
- One of the most effective stereopure PS- and PN-modified GalNAc-siRNAs show enhanced potency and durability compared with the reference siRNAs, and this enhanced activity is driven at least in part by increased Ago2 loading.
- Stereopure PN/PS/PO siRNAs do not disrupt endogenous RNAi pathways.
- Application of our siRNA design principles to a clinically relevant human target,[3] *Hydroxysteroid 17-beta 13* (*HSD17B13*), yields 75%-80% silencing that persists for at least 3 months after a single injection in a transgenic mouse model.
- These preclinical data suggest that stereopure oligonucleotides incorporating PS/PN backbone increase durability of gene silencing by RNAi.

Introduction

- Chemically modified siRNAs promote degradation of mRNA via the RNAi pathway.
- We apply PRISM™, our discovery and drug development platform to generate stereopure oligonucleotides with controlled sequence, chemistry, and stereochemistry (**Figure 1**).
- We have developed chimeric siRNAs containing PO, PS and PN backbones (**Figure 1**). Herein, we investigate whether control of the position and configuration of these backbones can impact silencing by RNAi.

Figure 1. Introduction to PRISM and PN chemistry

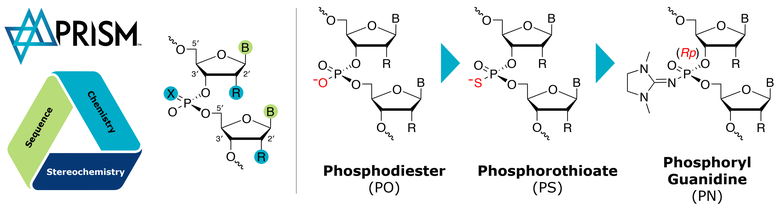
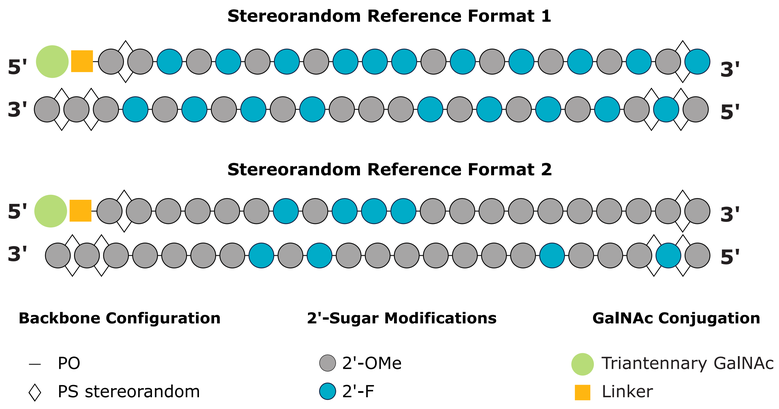


Figure 2. Stereorandom reference formats for structure-activity relationship with PRISM

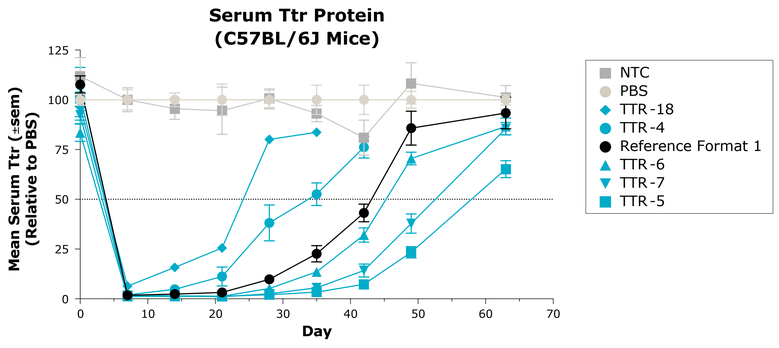


Structure-Activity Relationship Strategy

- We interrogated stereorandom reference siRNAs based on two previously published GalNAc-siRNA formats with enhanced stability (**Figure 2**).[1,2,4]
- To assess the impact of PS stereochemistry on stereorandom reference format 1, we evaluated all possible combinations of siRNAs with stereorandom PS linkages replaced by stereopure PS linkages. From this exercise, we identified configurations that yield robust silencing activity.
- To assess the impact of PN stereochemistry, we evaluated siRNAs containing a single stereopure PN linkage at each backbone position, as well as in multiple positions in a stereopure version of reference format 1.
- We then applied our learnings from reference format 1 to reference format 2, re-optimizing as needed for the differences in 2'-ribose modification patterns (**Figure 2**).

Results

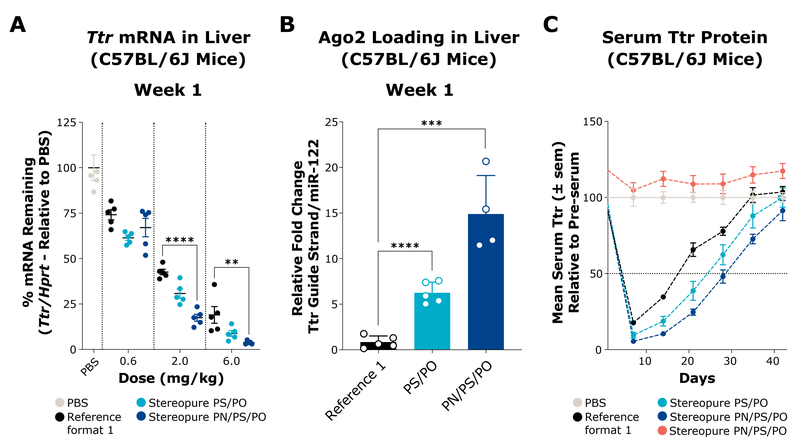
Figure 3. Stereochemistry of PS modifications impacts activity



C57BL/6J mice were treated with 6 mg/kg of the indicated siRNA or PBS by subcutaneous (SC) injection on day 0. Serum Ttr protein levels were assessed each week by ELISA through the end of the experiment.

- We treated mice with the indicated siRNAs. TTR-4, TTR-5, TTR-6, TTR-7 and TTR-18 are stereopure siRNAs based on stereorandom reference format 1.
- Reference format 1 led to ~98% decrease in Ttr serum protein levels by day 7 and recovered to levels comparable to mice treated with PBS by ~49 days. TTR-6 performed comparably. TTR-4 and TTR-18 were less durable. TTR-5 and TTR-7 were more durable.

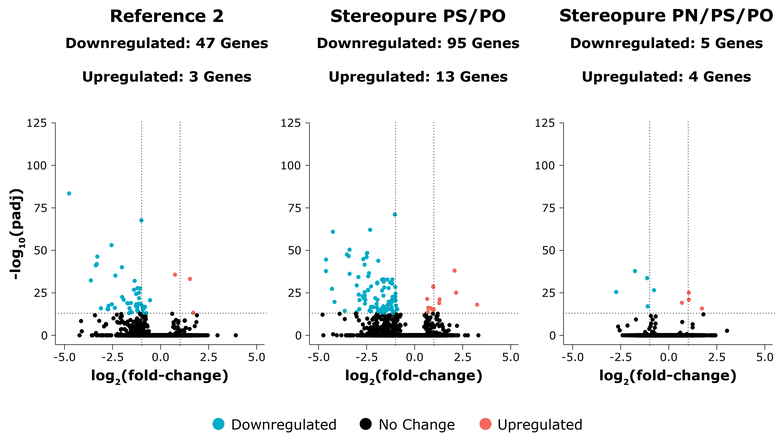
Figure 4. Incorporation of PN chemistry increases *Ttr* silencing by promoting Ago2 loading



(A) C57BL/6J mice were treated with 0.6, 2, or 6 mg/kg of the indicated siRNA or PBS by SC injection on day 0. Liver *Ttr* mRNA levels were quantified by RT-PCR 1-week later. Stats: 2-way ANOVA ** P<0.01, **** P<0.0001. (B) Ago2 loading was quantified by RT PCR from livers in panel A after 1 week of treatment with 2 mg/kg siRNA. Stats: Welch's 1-way ANOVA *** p<0.001, **** P<0.0001. (C) Mice were treated with 6 mg/kg of the indicated siRNA or PBS by SC injection, and serum Ttr protein levels were assessed each week by ELISA through the end of the experiment.

- After interrogating the impact of PN chemistry across Reference format 1, we compared the activity of a stereopure siRNA containing an optimal configuration of PS linkages (Stereopure PS/PO) to one with the addition of stereopure PN linkages (Stereopure PN/PS/PO) to one based on stereorandom reference format 1 in mice.
- One-week post-injection, all siRNAs led to dose-dependent decreases in *Ttr* mRNA in mouse liver (**Figure 4A**). The stereopure siRNAs were more potent than reference format 1, and the inclusion of stereopure PN linkages increased potency compared with both the stereopure PS/PO siRNA and the reference.
- We evaluated Ago2 loading 1-week post-injection. Stereopure PS/PO and PN/PS/PO siRNAs elicited more Ago2 loading than the reference, as measured by loading of the guide strand, and the PN-containing siRNA showed the largest Ago2 loading advantage (**Figure 4B**).
- In a longer experiment, the reference siRNA led to maximum decrease in serum Ttr protein levels (83%) ~1-week post-dose, with Ttr protein levels recovering until day 35 when they matched levels in mice treated with PBS. The stereopure siRNAs followed the same general pattern but with greater maximal silencing (PS/PO 91%; PN/PS/PO 95%) that remained significantly lower than the reference siRNA for most of the experiment ($p<0.01$, linear mixed effects model) (**Figure 4C**). In this experiment, we also included an siRNA with the opposite configuration of PN linkages that otherwise matched the chemistry of the PN/PS/PO siRNA. This siRNA had silencing activity comparable to PBS (**Figure 4C**).
- We applied stereochemistry and PN chemistry to siRNAs in Reference 2 format, and after optimization, we observed similar improvements.

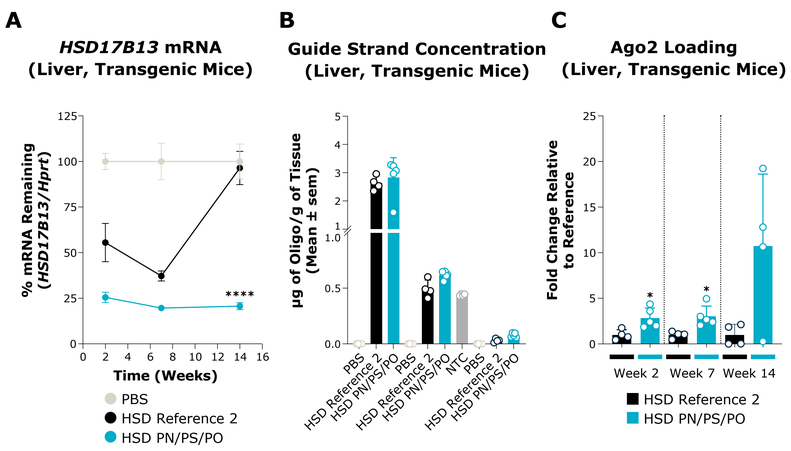
Figure 5. Incorporation of PN chemistry does not affect endogenous RNAi pathways in primary mouse hepatocytes



Primary mouse hepatocytes were treated with the indicated siRNAs. Gene expression was evaluated by RNA-seq. Genes that are upregulated, downregulated or unchanged in response to treatment are indicated.

- With increased Ago2 loading comes the risk that siRNAs could disrupt endogenous RNAi pathways. If they do, we expect to see an increase in gene expression after treatment, as this would decrease endogenous pools of Ago2-loaded microRNAs.
- We evaluated the impact of stereopure siRNAs on gene expression in primary mouse hepatocytes at doses 3-4X higher than the IC_{50} (**Figure 5**).
- We observed relatively minor changes in mRNA expression upon treatment with PN/PS/PO siRNAs, indicating that they do not disrupt endogenous RNAi pathways.

Figure 6. Durable *HSD17B13* silencing is driven in part by increase in Ago2 loading



Mice expressing a human *HSD17B13* transgene were treated with the indicated siRNA, liver mRNA, guide strand concentration, and Ago2 loading were quantified at the indicated times post-dose. Mice were dosed with 3 mg/kg siRNA or PBS by SC injection. Stats: Two-way ANOVA with post-hoc test * P<0.05, ****P<0.0001.

- We applied the same general stereopure PN/PS/PO GalNAc-siRNA design from Ttr to HSD17B13 and evaluated its activity in human *HSD17B13* transgenic mice compared with a siRNA with stereorandom counterpart.
- At 2-weeks post-dose, the control HSD siRNA (reference 2 format) exhibited a mean silencing of ~45%. HSD PN/PS/PO led to more mean silencing at the same time point (~75%). By 7-weeks post-dose, the control reached ~60% silencing, whereas HSD PN/PS/PO reached ~80% silencing. By 12 weeks (3 months) post-dose, these differences increased, with HSD17B13 mRNA levels recovering in samples treated with the control (mean silencing: ~5%) but remaining significantly suppressed in HSD PN/PS/PO-treated samples (mean silencing: ~80%; $p<0.0001$, linear mixed effects model) (**Figure 6A**).
- Differences in silencing were unlikely derived from tissue uptake and stability, as the concentration of reference 2 and HSD PN/PS/PO were comparable throughout the study (**Figure 6B**).
- Differences in silencing were due, at least in part, to an increase in Ago loading for HSD PN/PS/PO (**Figure 6C**).

References: 1. Nair, et al., 2017. *Nuc. Acids Res.* 45, 10969-10977; 2. Kandasamy, et al., 2022. *Nuc. Acids Res.* 50, 5401-5423; 3. Abul-Husn, et al., 2018. *New Engl. J. Med.* 378, 1096-1106; 4. Foster, et al., 2018. *Mol. Ther.* 26, 708-717. **Acknowledgments:** The authors are grateful to Amy Donner (Wave Life Sciences) and Eric Smith for editorial and graphical support, respectively. This work was funded by Wave Life Sciences.