Impact of Phosphoryl Guanidine (PN) Variants on siRNA Potency and Durability in Hepatic and Extrahepatic Tissues

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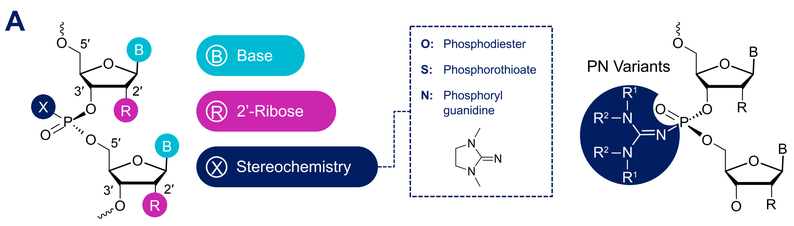
SUMMARY

- Using PRISM™, our discovery and drug development platform, we develop new siRNA chemistry design that improves durability and leverages the chemical flexibility of PN linkages to increase potency and enhance extra-hepatic delivery.
- In mouse liver, *N*-Acetylgalactosamine (GalNAc)-siRNA conjugate using the new stereopure design supports more durable knockdown compared to either a stereorandom reference GalNAc-siRNA or a GalNAc-siRNA with our previously published design.[1]
- The incorporation of PN linkages with variant chemistry (PN variants) in the new siRNA design enhanced potency and enabled knockdown in liver, white adipose, and brown adipose without ligand conjugation to the siRNA.
- An siRNA incorporating a PN variant supported potent, durable knockdown across central nervous system (CNS) tissues up to 16 weeks post single intracerebral ventricular (ICV) injection.
- Knockdown by PN variant siRNA corresponded with persistence of the siRNA in CNS tissues and sustained Ago2 loading up to 16 weeks posy-single injection.
- PN variants, which titrate siRNA lipophilicity, impact the delivery, potency, and durability of siRNAs in various tissues.

INTRODUCTION

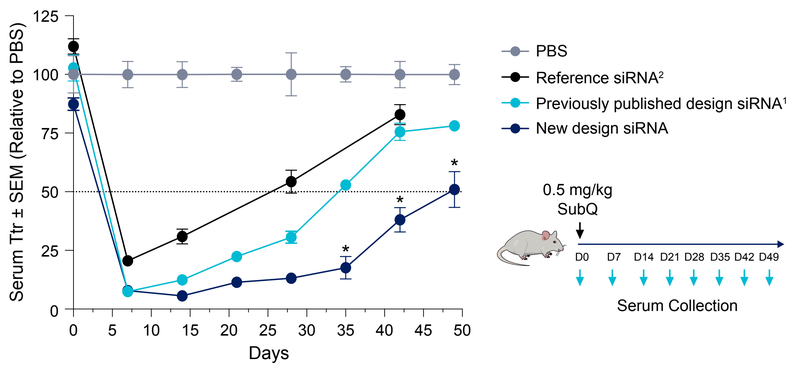
- Using PRISM™, we generate stereopure oligonucleotides consisting of chimeric phosphodiester (PO)/ phosphorothioate (PS)/PN backbone with controlled sequence, chemistry, and stereochemistry (**Figure 1A**).
- We previously demonstrated that judicious incorporation of stereopure PN linkages in siRNA backbones increases the potency and durability of knockdown in mouse liver *in vivo* compared to siRNAs containing phosphorothioate and phosphodiester linkages alone, in part through enhanced antisense strand loading on Ago2, a RISC enzyme involved in the silencing mechanism (**Figure 1B**).[1]
- A key feature of PN linkages is that they can be chemically modified, which enables an opportunity to explore PN variants that support altered pharmacological properties. We have previously shown that incorporating some PN variants in siRNAs can support similar knockdown *in vitro*.[1]
- We evaluate the impact of a novel chemical design and incorporation of PN variant linkages on the durability, potency, and tissue distribution of siRNAs in mice.

Figure 1. Introduction to PN chemistry and RNAi



RESULTS

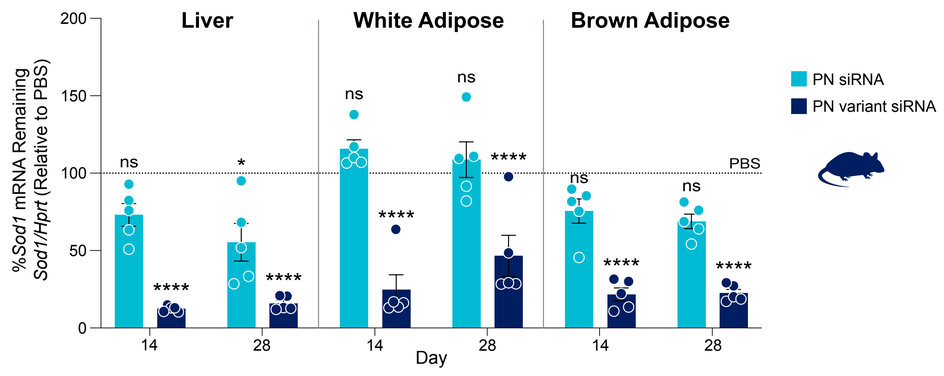
Figure 2. The new siRNA design improves durability of GalNAc-siRNA in mice



C57BL/6 mice were administered PBS or 0.5 mg/kg of siRNA directed against *Ttr* (subcutaneous) at day 0 and serum was collected at time points indicated. Serum Ttr protein levels were assessed by ELISA. Stats: mixed Two-way ANOVA followed by post hoc test comparing our previously published design siRNA vs. new design siRNA per day derived from linear mixed effects model. Data presented as mean ± SEM of *n* = 5/group, * P < 0.0001.

- We compared the activity of a stereopure PS/PO/PN GalNAc-siRNA incorporating our new siRNA design to a reference stereorandom PS/PO GalNAc-siRNA[2] and a GalNAc-siRNA incorporating our previously published PS/PO/PN design.[1]
- Both our previously published design siRNA and the new design siRNA led to similar maximum decreases in serum Ttr protein ~1 week post dose (~92%). The stereopure siRNAs resulted in greater maximal silencing than the stereorandom reference siRNA (~81%) (**Figure 2**).
- The new design siRNA resulted in more durable silencing compared to the other siRNAs. At 42 days post-dose, the new design siRNA resulted in 62% silencing, compared to 24% silencing with our previously published design siRNA and 17% silencing with the reference siRNA.

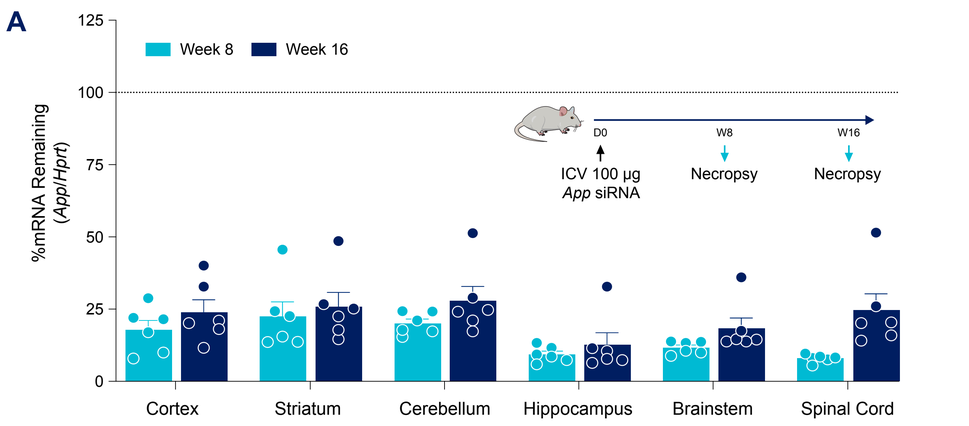
Figure 3. Incorporation of PN variant chemistry increases *Sod1* silencing by unconjugated siRNAs in mouse liver and adipose



C57BL/6 mice were administered PBS or 5 mg/kg of *Sod1* siRNA (unconjugated) via subcutaneous injection. Indicated tissues were collected at 14 or 28 days post-dose. Taqman qPCR assays used for RNA quantitation; relative fold changes of *Sod1* to *Hprt* mRNA were normalized to % of PBS group. Stats: Data expressed as mean ±SEM, n=5; Three-way ANOVA followed by Bonferroni-adjusted post hoc test comparing condition to PBS (represented by dashed line; data not shown) * P < 0.05, **** P < 0.0001, ns nonsignificant.

- We compared the activity of unconjugated stereopure siRNAs with either PN linkages or PN variant linkages in the liver, white adipose, and brown adipose tissues in mice.
- The PN siRNA (without GalNAc) supported modest significant *Sod1* knockdown in liver at 28 days (44%; P < 0.05) but not 14 days post-dose and did not support significant knockdown in adipose tissues at either time point (**Figure 3**).
- By contrast, the PN variant siRNA supported potent, significant *Sod1* silencing in adipose tissues by 14-days post-dose (white adipose, 75%; brown adipose, 78%; P < 0.0001), which was sustained to 28 days post-dose (white adipose 63%; brown adipose 77%) (**Figure 3**).
- Incorporation of PN variant linkages in non-GalNAc *Sod1* siRNA altered tissue delivery, enabling potent knockdown in adipose tissues and enhancing potency in liver compared to unconjugated siRNAs with PN linkages.

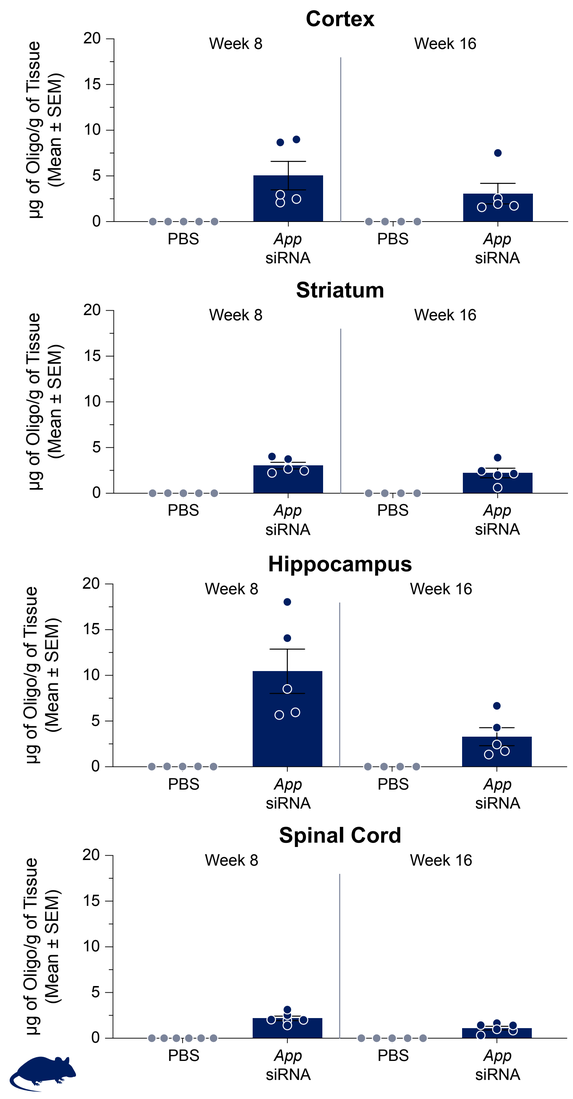
Figure 4. siRNA incorporating a PN variant supports durable knockdown across the mouse CNS



C57BL/6 mice were administered PBS or a single 100 µg intracerebral ventricular (ICV) injection of siRNA directed against mouse *App*. Mice were sacrificed at 8 and 16 weeks. (**A**) PCR assays for mRNA knockdown, relative fold changes of *App* to *Hprt* mRNA normalized to % of PBS. Stats: Three-way ANOVA followed by Bonferroni-adjusted post hoc test comparing condition to PBS (data not shown). Stats: n=7; Mean ±SEM shown; siRNA-treated mice significantly different (P < 0.0001) at both time points for all tissues. (**B**) Immunohistochemical analysis of fresh frozen paraffin-embedded mouse brain tissue labeling App protein (Brown). Nuclei were counterstained with Hematoxylin (Blue). Representative images are shown, magnification 100X.

- We evaluated the expression of App mRNA and protein in mouse CNS tissues (cortex, striatum, hippocampus, cerebellum, and brain stem) 8 or 16 weeks after a single ICV injection of PBS or PN variant *App* siRNA.
- By 8 weeks post-injection, App mRNA (**Figure 4A**) and protein (**Figure 4B**) was reduced in siRNA-treated mice across all CNS tissues examined when compared to PBS-treated mice. The reduction in App persisted to 16 weeks post-injection.

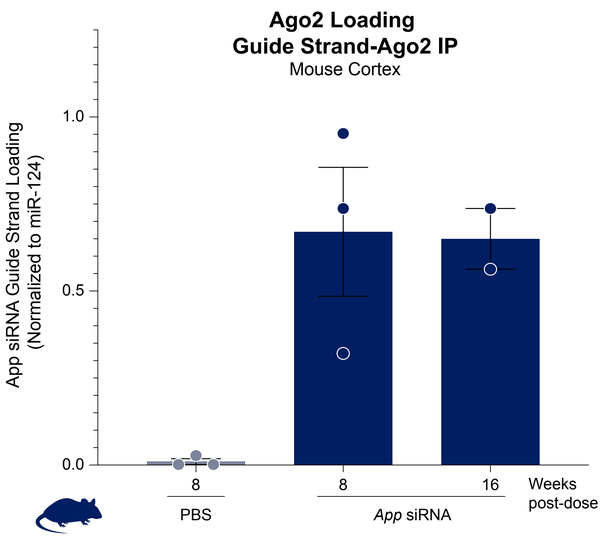
Figure 5. PN variant siRNA is present in the mouse CNS up to 16 weeks post-injection



C57BL/6 mice were administered PBS or a single 100 µg intracerebral ventricular (ICV) injection of siRNA directed against mouse App. The cortex, hippocampus, striatum, and spinal cord CNS tissues were lysed at time points indicated and siRNA antisense strand was quantified by hybridization ELISA.

- To further understand how the PN variant siRNA supported durable knockdown in the CNS, we evaluated the presence of siRNA antisense strand in mouse CNS tissues 8 or 16 weeks after a single ICV injection of PBS or PN variant *App* siRNA.
- *App* siRNA was detected across CNS tissues, including the cortex and striatum, from 8 weeks post-injection and siRNAs persisted to 16 weeks post-injection (**Figure 5**).

Figure 6. Ago2 loading of PN variant siRNA guide strand persists up to 16 weeks in the mouse cortex post single-injection



C57BL/6 mice were administered PBS or a single 100 µg ICV injection of siRNA directed against mouse *App*. Cortex tissue was lysed, Ago2 was immunoprecipitated via anti-Ago2 antibody, and Ago2-associated antisense strand or miRNA-124 (control) was quantified by RT-qPCR at the indicated times post-dose.

- We evaluated Ago2 loading of the siRNA guide strand in mouse cortex 8 or 16 weeks after a single ICV injection of PBS or PN variant *App* siRNA.
- Ago2 loading of the PN variant *App* siRNA guide strand is observable by 8 weeks and persists up to 16 weeks post-injection (**Figure 6**).
- These results are consistent with our observations that PN variant siRNA induced durable knockdown of *App* and is persistently detectable across CNS tissues for up to 16 weeks.

References: 1. Liu W et al., *Nucl Acids Res.* 2023 51(9); 2. Foster, DJ. et.al. *Mol Ther.* 2018, 26(3), 708. **Acknowledgments:** The authors are grateful to Nicole Neuman (Wave Life Sciences) and Eric Smith for editorial and graphical support, respectively. This work was funded by Wave Life Sciences.