Impact of Nitrogen-containing Backbone Linkages on Stereopure Antisense Oligonucleotides in the CNS

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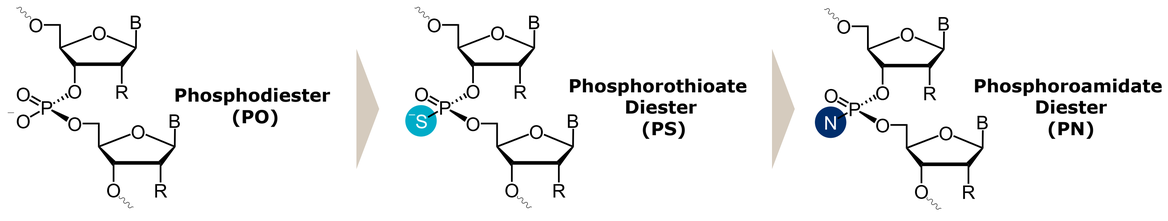
Summary

- We report the stereopure synthesis and the application of nitrogen-containing backbone linkages (PN linkages) to oligonucleotides acting through an RNase H-mediated mechanism, using *Malat1* and *C9orf72* as benchmarks
- Various types of nitrogen-containing moieties in the oligonucleotide backbone can increase potency in cultured neurons ~10-fold compared with comparably modified stereopure phosphorothioate (PS) and phosphodiester (PO)-based molecules
- One of these backbone types, called PN-1, yielded profound pharmacological and activity benefits *in vivo* throughout the mouse CNS
- The incorporation of PN linkages into stereopure oligonucleotides with chimeric backbone modifications has the potential to decrease the frequency of dosing and expand the scope of neurological indications amenable to oligonucleotide therapeutics

Introduction

- Antisense oligonucleotides can be designed to promote degradation of a targeted transcript through an RNase H-dependent mechanism.
- Achieving oligonucleotide distribution throughout the CNS, beyond the spinal cord and other brain tissues that are readily accessed upon intracerebroventricular (ICV) or intrathecal (IT) administration, remains challenging.
- We applied PN chemistry (**Figure 1**) to gapmer oligonucleotides acting through an RNase H-mediated mechanism to test whether it can impact their activity in the CNS.

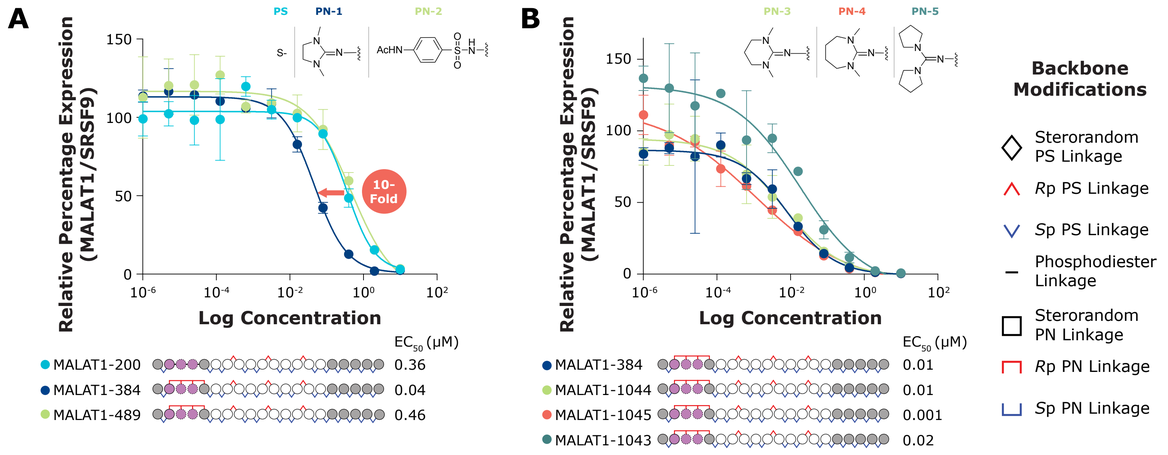
Figure 1. Evolution of phosphoroamidate diester (PN) chemistry



- We have developed a backbone chemistry called PN, where a nitrogen-containing moiety replaces a non-bridging oxygen in the phosphodiester bond (PO).
- Similar to the phosphorothioate (PS) backbone, this creates a chiral center.
- We control the configuration of these chiral backbones to generate stereopure oligonucleotides.

Results

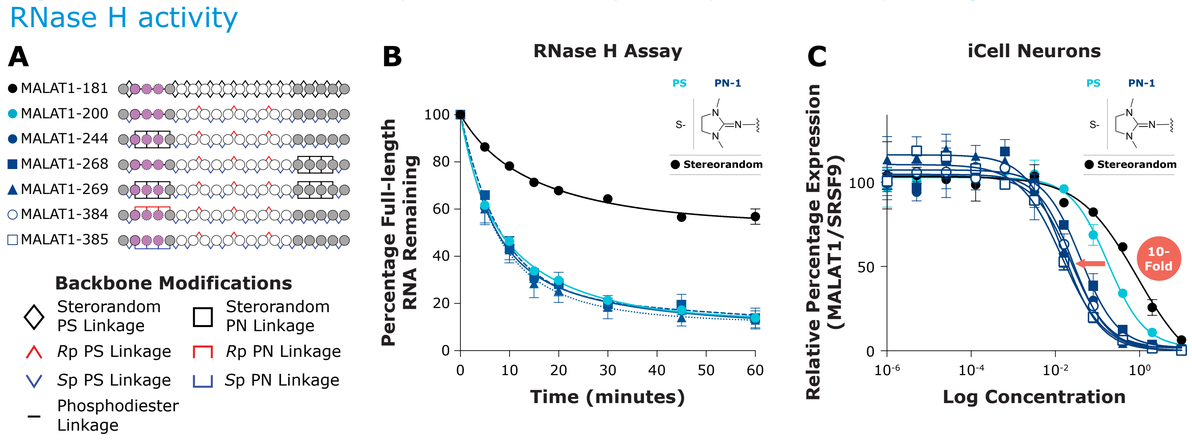
Figure 2. PN backbone modifications increase potency in cultured neurons



iCell neurons were treated with increasing concentrations of oligonucleotide. MALAT1 RNA was normalized to SRSF9. Mean ± sd are shown, n=3 per concentration. Half maximal effective concentrations (EC_{50}) are calculated with GraphPad Prism software.

- We applied multiple types of PN chemistry to oligonucleotides targeting MALAT1, a long noncoding RNA that is enriched in cellular nuclei (**Figure 2**).
- We first compared the activity of benchmark stereopure PS/PO backbone (MALAT1-200) to PN-1 and a sulfonamide-containing backbone PN-2. The PN-1 containing oligonucleotide (MALAT1-384) showed 10-fold potency improvement over MALAT1-200. MALAT1-489, containing the sulfonamide modification, showed no potency benefits (**Figure 2A**).
- We explored the impact of changing the size of the 5-membered imidazolidine ring in PN-1 by converting it to a 6-membered hexahydropyrimidine ring in PN-3 (MALAT1-1044) or a 7-membered 1,3-diazepane ring in PN-4 (MALAT1-1045). We also evaluated a backbone with exo-cyclic guanidine in PN-5 (MALAT1-1043). These PN variants performed comparably to PN-1, at least in this configuration (**Figure 2B**).
- Given this comparable activity *in vitro*, we opted to further evaluate oligonucleotides based on the PN-1 backbone.

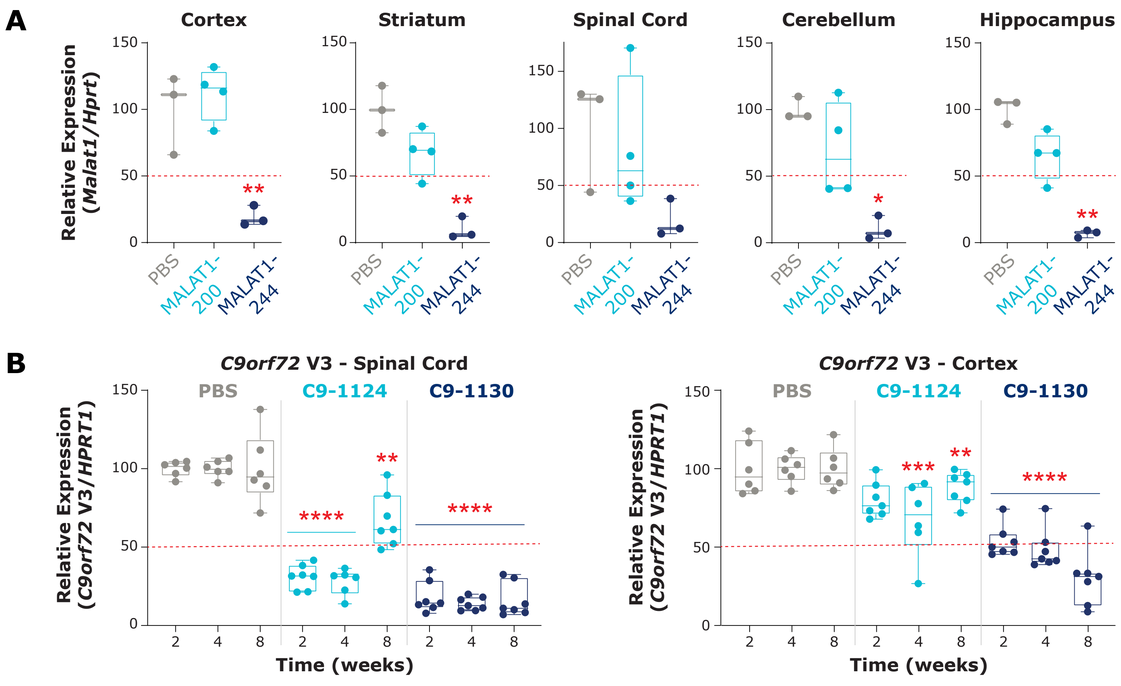
Figure 3. PN-1 backbone improves cellular potency without impacting RNase H activity



(B) Biochemical RNase H assays performed on MALAT1 RNA-oligonucleotide duplex. Mean ± sem are shown, n=3 per time point. (C) iCell neurons were treated with increasing concentrations of oligonucleotide. MALAT1 RNA was normalized to SRSF9. Mean ± sd are shown, n=2 per concentration. Half maximal effective concentrations (EC_{50}) are calculated with GraphPad Prism software.

- To assess the impact the position and configuration of the PN-1 backbone, we evaluated another series of MALAT1-targeting oligonucleotides (**Figure 3A**) in assays measuring relative RNase H activity and MALAT1 depletion in cultured human iCell neurons.
- As expected,[1] stereopure PS-modified MALAT1-200 was more active in biochemical RNase H assays than stereorandom MALAT1-181 (**Figure 3B**). The introduction of PN-1 linkages in the wings did not alter on this biochemical activity.
- Stereopure MALAT1-200 (EC_{50}=0.18 μM) was more potent in cellular RNase H assays performed in iCell neurons under gymnotic (i.e., free uptake) conditions than stereorandom MALAT1-181 (EC_{50}=1.4 μM) (**Figure 3C**). MALAT1-244 (EC_{50}=0.02 μM), containing stereorandom PN-1 linkages, was ~10-fold more potent than stereopure MALAT1-200 and ~70-fold more potent than MALAT1-181 (**Figure 3C**).
- All other PN-1-containing oligonucleotides in this experiment had potency comparable to MALAT1-244.

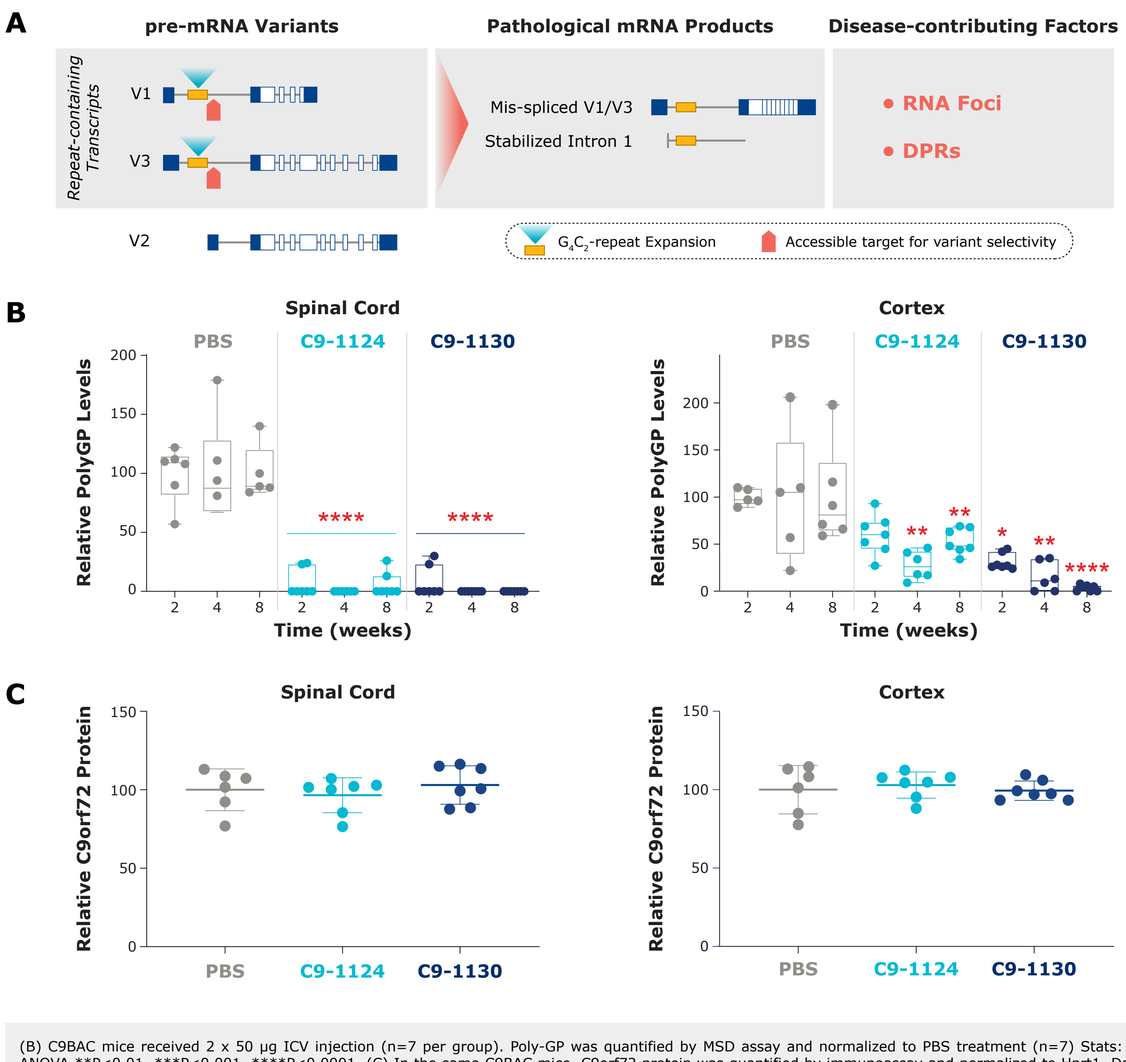
Figure 4. PN-1 increases potency and durability throughout CNS



(A) Mice received a single 100 μg ICV injection (n=3 per group). Relative percentage *Malat1* expression (normalized to *Hprt1*) is shown for the indicated tissues 10-weeks post-dose. Stats: 1-way ANOVA. (B) C9BAC mice received 2 x 50 μg ICV injection (n=7 per group). *C9orf72* V3 is normalized to *Hprt1*. Stats: 2-way ANOVA. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. PBS, phosphate buffered saline.

- We next tested whether activity benefits observed *in vitro* translated into pharmacology benefits *in vivo* in the mouse CNS (**Figure 4**).
- In wild-type mice, PN-1-containing MALAT1-244 was more potent and durable than PS-based MALAT1-200 throughout the CNS (**Figure 4A**).
- We also applied PN-1 chemistry to a more challenging target, *C9orf72*, where we designed oligonucleotides to selectively target transcriptional variants impacted by a hexanucleotide repeat expansion (**Figure 4B**).[2]
- In C9BAC mice,[3] a PN-1-containing oligonucleotide (C9orf72-1130) selectively decreased V3 transcripts in the spinal cord and cortex (**Figure 4B**), and this decreased expression persisted for longer than we observed for a comparable PS-based oligonucleotide (C9orf72-1124), especially in the cortex.
- In both cases, these potent and durable effects with PN-1-containing oligonucleotides were achieved with a low dose (100 μg).

Figure 5. PN chemistry preserves variant selectivity but improves pharmacology *in vivo*



(B) C9BAC mice received 2 x 50 μg ICV injection (n=7 per group). Poly-GP was quantified by MSD assay and normalized to PBS treatment (n=7) Stats: 2-way ANOVA **P<0.01, ***P<0.001, ****P<0.0001. (C) In the same C9BAC mice, C9orf72 protein was quantified by immunoassay and normalized to Hprt1. Data are mean ± sd, n=7. PBS, phosphate buffered saline.

- For *C9orf72*, we aim to selectively decrease expression of the transcripts impacted by a hexanucleotide repeat expansion (V1 and V3) that is the causative mutation for *C9orf72*-associated ALS and FTD.[4,5] This variant-selective approach is designed to deplete pathogenic RNA species and dipeptide repeat proteins (DPRs) that are derived from these transcripts but to preserve the expression of V2 and C9orf72 protein, which is predominately derived from V2 (**Figure 5A**).[2]
- We applied PN chemistry to oligonucleotides targeting a sequence that is accessible only in V1 and V3 transcripts.
- PN-1-containing C9orf72-1130 led to more potent and durable depletion of the DPR poly-GP (**Figure 5B**) than PS-modified C9orf72-1124, especially in the cortex.
- C9orf72-1124 had no detectable impact on the expression of C9orf72 protein over the same time period, confirming that PN-1-modified oligonucleotides preserve the desired variant-selective mechanism of action.

References: 1. Byrne, M. et al., 2021. Stereochemistry enhances potency, efficacy, and durability of Malat1 antisense oligonucleotides *in vitro* and *in vivo* in multiple species. *Transl. Vis. Sci. Technol.* 10(1):23. doi: 10.1167/tvst.10.1.23.; 2. Liu, Y. et al., 2021. Variant-selective stereopure oligonucleotides protect against pathologies associated with *C9orf72*-repeat expansion in preclinical models. *Nat Commun.* 12(1), 847. doi:10.1038/s41467-021-21112-8; 3. O'Rourke, J. G. et al., 2015. *C9orf72* BAC transgenic mice display typical pathologic features of ALS/FTD. *Neuron. 88*(5), 892-901. doi:10.1016/j.neuron.2015.10.027; 4. DeJesus-Hernandez, M. et al., 2011. Expanded GGGGCC hexanucleotide repeat in noncoding region of *C9orf72* causes chromosome 9p-linked FTD and ALS. *Neuron.* 72(2):245-56. doi: 10.1016/j.neuron.2011.09.011. 5. Renton, A.E. et al., 2011. A hexanucleotide repeat expansion in *C9orf72* is the cause of chromosome 9p21-linked ALS-FTD. *Neuron.* 72(2):257-68. doi: 10.1016/j.neuron.2011.09.010. **Acknowledgments:** Eric Smith and Amy Donner (Wave Life Sciences) provided graphical and editorial support for this poster, respectively.