Variant-selective Stereopure Oligonucleotides Protect Against Pathologies Associated with *C9orf72*-repeat Expansion in Preclinical Models

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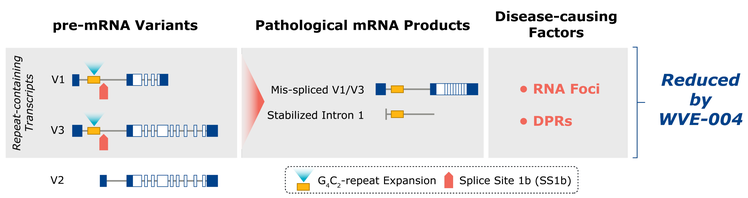
Summary

- We identified a novel targeting site that is only accessible in pathogenic C9orf72 transcripts (those containing the G_4C_2-repeat expansion). An antisense oligonucleotide targeting this site selectively decreases the expression of pathogenic C9orf72 transcripts and thereby address the genetic root cause of *C9orf72*-associated ALS or FTD[1]
- Evaluation in *C9orf72* BAC transgenic (C9BAC) mice demonstrates that WVE-004, a variant–selective stereopure oligonucleotide that contains Wave's novel backbone chemistry, has a more desirable activity profile *in vivo* than oligonucleotides containing more traditional phosphorothioate (PS) and phosphodiester (PO) backbones
- In the C9BAC mouse model, WVE-004 selectively decreases pathogenic transcripts and the pharmacodynamic biomarker poly-glycine-proline (poly-GP), preserves expression of C9orf72 protein, and leads to durable effects in the CNS
- These preclinical data support further development of WVE-004, which is now being tested in a phase 1b/2a clinical trial called FOCUS-C9 in patients with *C9orf72*-associated ALS or FTD

Introduction

- G_4C_2-repeat expansion in the first intronic region of *C9orf72* is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).[2]
  - Wild-type *C9orf72* alleles produce three mRNA transcripts: variant 1 (V1), V2, and V3. V2 expression is higher than V1 and V3 in the CNS.[2]
  - When expanded, the G_4C_2 repeats lead to pathological V1 and V3 transcript by-products that form disease-causing nuclear RNA foci and dipeptide repeat proteins (DPRs), including poly-GP[2] (**Figure 1**).
- The repeat expansion also decreases expression of C9orf72 protein, resulting in partial loss of function.[2]
- Antisense oligonucleotides that selectively decrease expression of transcripts containing the repeat expansion can mitigate disease-associated pathologies in preclinical models.[1,3]

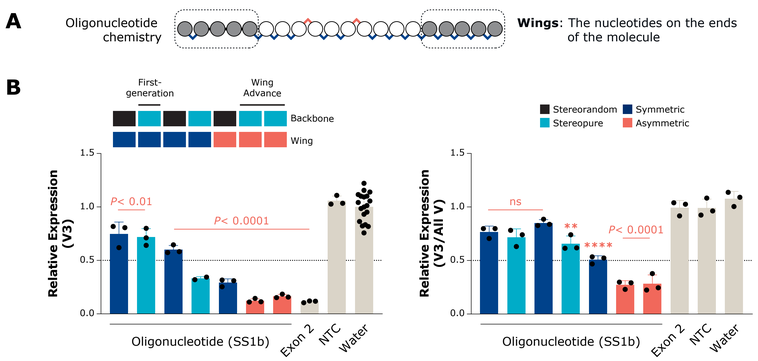
Figure 1. Variant-selective targeting strategy



- We aim to selectively decrease expression of the transcripts impacted by the repeat expansion (V1 and V3), and thereby deplete pathogenic RNA species and DPRs that are derived from them, as well as to preserve the expression of V2 and C9orf72 protein.
- We designed WVE-004 to target a sequence that is accessible only in V1 and V3 transcripts.

Results

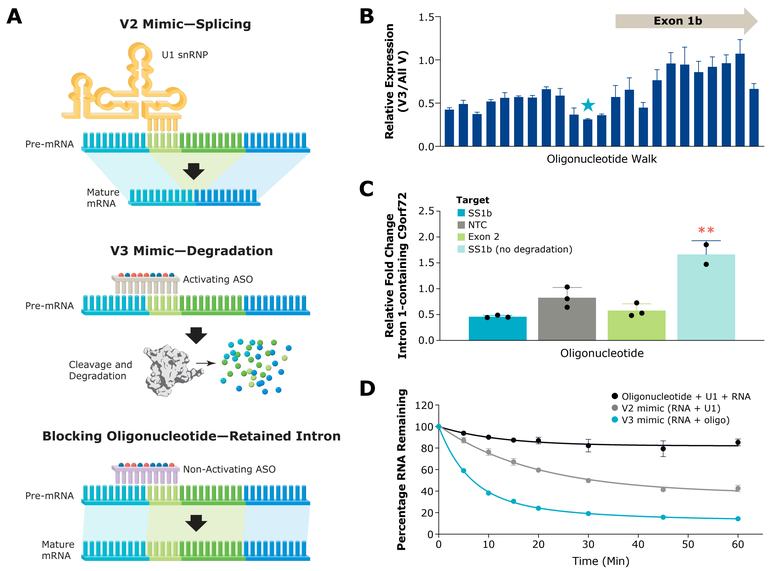
Figure 2. Discovery of splice site 1b (SS1b) and optimization of activity



- We screened antisense oligonucleotides (**Figure 2A**) complementary to a 1 kilobase region of C9orf72 RNA spanning intron 1 and identified a sequence near the exon 1b-intron 1 junction called SS1b (**Figure 1**) that yielded efficient and selective knockdown of repeat containing transcripts.[1]
- Through chemical modification, we optimized the oligonucleotide for selectivity and potency (**Figure 2B**). A combination of stereopure backbone chemistry and asymmetric wing chemistry, the incorporation of distinct 2'-ribose modification patterns in the 5'-and 3'-wings, yielded the best activity profiles (**Figure 2B**, red).
- Control oligonucleotides (targeting exon 2 or NTC) are also shown (**Figure 2B**).

Relative expression of V3 with respect to human HPRT transcripts in human ALS motor neurons derived from iPSCs after gymnotic treatment with 10 μM of chemically modified oligonucleotides targeting SS1b or controls. Data are presented as mean ± SD, n=3. Stats calculated by 1-way ANOVA.

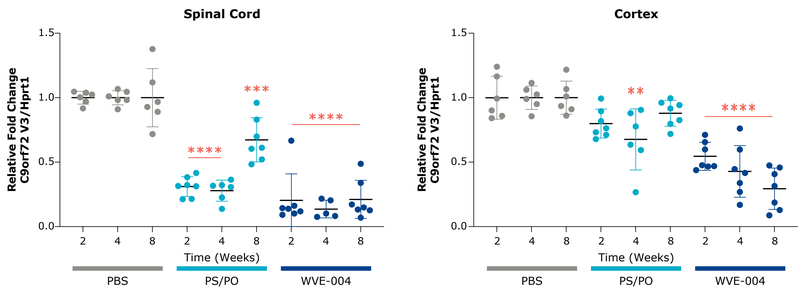
Figure 3. Mechanism of selectivity



- To investigate the mechanism for selectivity at SS1b, we tested the hypothesis that for V2 only, splicing machinery (including U1 snRNP) interacts with this sequence (**Figure 3A**, V2 mimic). For V1 and V3, SS1b is accessible, so antisense oligonucleotide leads to RNA degradation (**Figure 3A**, V3 mimic). In the presence of a blocking oligonucleotide, splicing would be disrupted and intron 1 would be retained (**Figure 3A**, Blocking oligonucleotide).
- We performed a microwalk with oligonucleotides complementary to this region (**Figure 3B**). Oligonucleotides binding at or near SS1b (blue star) show the most preference for V3 compared with all variants, whereas those binding sequences closer to exon 1b were less selective.
- Next, we tested a blocking oligonucleotide - an inactive oligonucleotide complementary to SS1b with very high thermal stability (**Figure 3C**, light blue). This oligonucleotide formed a stable duplex with C9orf72 RNA and disrupted splicing of intron 1.
- Finally, we tested the ability of a U1 RNA surrogate (U1) to protect C9orf72 RNA (RNA) from RNase H-mediated degradation in the presence of the oligonucleotide in a biochemical assay (**Figure 3D**). In one scenario (V3 mimic, light blue), we premixed RNA with oligonucleotide, and the RNA was readily degraded. In a second scenario (V2 mimic, gray), we premixed RNA with U1, and the RNA was protected from degradation. In a third scenario (black), we added all components at the same time, and observed protection of the RNA when RNase H was limiting (shown, black) but less protection as the RNase H concentration increased (not shown).[1]

B) Ratio of expression of V3 to all variants (All V) with respect to human HPRT transcripts in human ALS motor neurons after gymnotic treatment with 10 μM oligonucleotide, from a series of molecules complementary to the intron 1a-exon 1b boundary region that differ from each other by one nucleotide position. Data are presented as mean ± SD, n=3. C) Relative expression of intron-1-containing transcripts with respected to HRPT in human ALS motor neurons treated as in B. Data are presented as in B. D) Percentage of full-length RNA remaining after heteroduplex formation under conditions that mimic V2 or V3 in the presence of RNase H. Data are presented as mean ± SEM, n = 3.

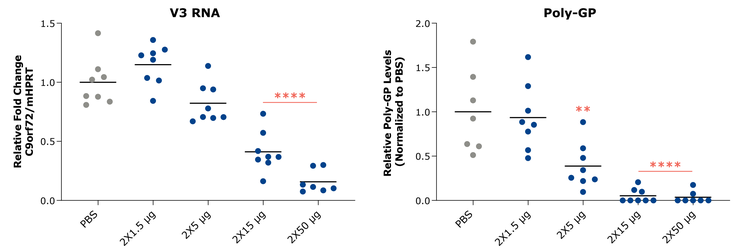
Figure 4. Application of PN chemistry increases activity and durability



- We have developed a backbone chemistry called PN, where a nitrogen-containing moiety replaces a non-bridging oxygen in the phosphodiester bond. Similar to the PS backbone, this creates a chiral center. We control the configuration of these backbones to generate stereopure oligonucleotides.
- In C9BAC mice, WVE-004, which contains PN chemistry and targets SS1b, has better activity and durability against V3 transcripts in the spinal cord (left) and cortex (right) than a control PS/PO molecule of the same sequence and 2'-ribose chemistry (**Figure 4**).

C9BAC mice were administered PBS or 50 μg of oligonucleotide by intracerebral (ICV) injection on days 0 and 7. Mice were evaluated up to 8 weeks after the first dose. The relative fold change of human V3 and all variants (All V) to mouse Hprt1 RNA were determined. Data are presented as mean values (n=7-8, $^{**}P<0.01$, ***, $P<0.001$, $^{****}P<0.0001$). *P* values calculated by 1-way ANOVA with comparisons to PBS.

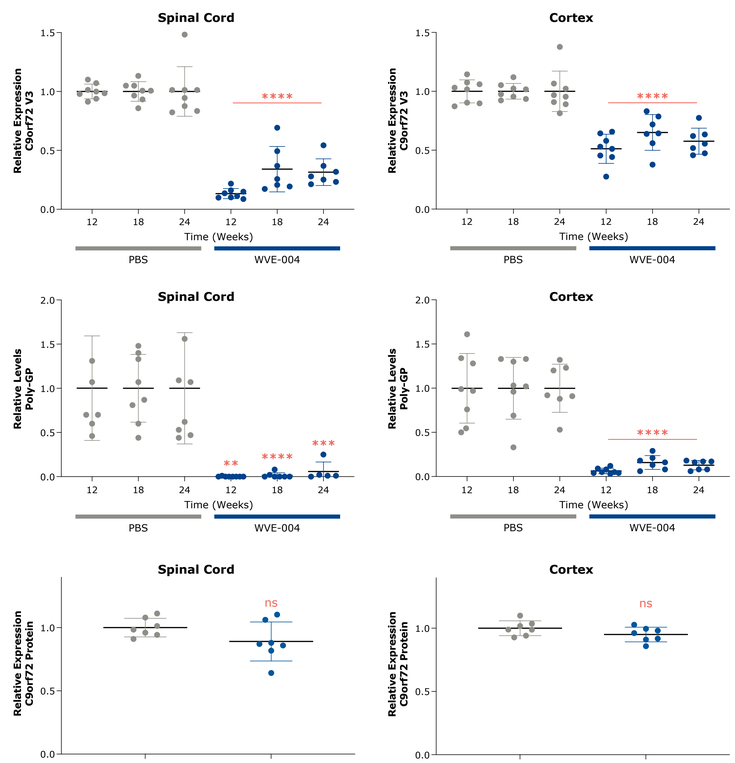
Figure 5. WVE-004 dose-dependently knocks down V3 RNA and poly-GP in C9BAC mice



- WVE-004 led to a dose-dependent decrease in V3 transcripts and poly-GP in spinal cord (**Figure 5**) and cortex (data not shown) of C9BAC mice.

C9BAC mice were administered PBS or the indicated dose of WVE-004 by ICV injection on days 0 and 7. Mice were evaluated 6 weeks after the first dose. The relative fold change of human V3 to mouse Hprt1 RNA (left) and the relative poly-GP levels normalized to PBS (right) were determined. Data are presented as mean values (n=7-8, $^{**}P<0.01$, $^{****}P<0.0001$). *P* values calculated by 1-way ANOVA with comparison to PBS.

Figure 6. The effects of WVE-004 *in vivo* persist for at least 6 months



- In C9BAC mice, WVE-004 selectively decreased V3 transcripts in the spinal cord and cortex, and this decrease in expression persisted for at least 24 weeks (**Figure 6A**).
- In the same experiment in C9BAC mice, WVE-004 also decreased levels of the pharmacodynamic biomarker poly-GP for at least 24 weeks (**Figure 6B**).
- Although V3 and poly-GP were decreased at 24 weeks, total C9orf72 protein was unaffected by WVE-004, further confirming the selectivity of WVE-004 for expansion-containing transcripts (**Figure 6C**).

C9BAC mice were treated as described in Figure 4. Mice were evaluated up to 24 weeks after the first dose. The relative fold change of human V3 to mouse Hprt1 RNA (left), the relative poly-GP levels normalized to PBS (right), and the relative fold change of human C9orf72 protein to mouse Hprt1 protein were determined. Data are presented as mean values (n=7-8, ns, not significant, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$). *P* values calculated by 1-way ANOVA with comparison to PBS.

References: 1. Liu, Y. et al. Variant-selective stereopure oligonucleotides protect against pathologies associated with *C9orf72*-repeat expansion in preclinical models. *Nature Com.* 12, 847, doi:10.1038/s41467-021-21112-8 (2021); 2. Balendra, R., Moens, T. G. & Isaacs, A. M. Specific biomarkers for *C9orf72* FTD/ALS could expedite the journey towards effective therapies. *EMBO Mol. Med.* 9, 853-855, doi:10.15252/emmm.201707848 (2017); 3. Jiang, J. et al. Gain of toxicity from ALS/FTD-linked repeat expansions in C9ORF72 is alleviated by antisense oligonucleotides targeting GGGGCC-containing RNAs. *Neuron* 90, 535-550, doi:10.1016/j.neuron.2016.04.006 (2016). **Acknowledgments:** Editorial and graphic support for this poster was provided by Amy Donner (Wave Life Sciences) and Eric Smith, respectively.