# Chemical Design of Oligonucleotides That Support Targeted RNA Editing in the CNS of Non-Human Primates

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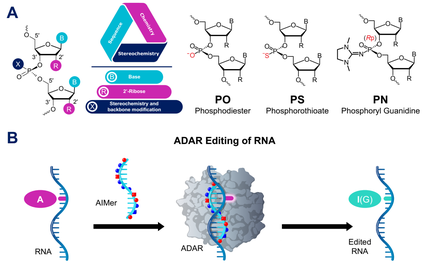
## SUMMARY

- Leveraging PRISM™, our discovery and drug development platform, we developed relatively short oligonucleotides that elicit A-to-I RNA editing with high efficiency using endogenous ADAR enzymes, which we call AIMers (**Figure 1A**).[1-3]
- Using N-Acetylgalactosamine (GalNAc)-modified AIMers with a stereopure chimeric phosphodiester (PO)/ phosphorothioate (PS)/ phosphoryl guanidine (PN) backbone pattern, we previously demonstrated up to 50% editing in nonhuman primate (NHP) liver.[2]
- Here, we have further optimized AIMer design by identifying base, sugar, and backbone modification patterns that improve editing across target and nearest neighbor sequences.
- Here, we show that AIMer RNA base editing technology is applicable in the central nervous system (CNS). AIMers support editing of housekeeping RNA in neurons and astrocytes *in vitro*, and unconjugated AIMers broadly direct durable RNA editing across the CNS of mice and NHPs.
- AIMers with optimized chemistry support editing of a disease-relevant transcript in neuronal cells. *MECP2* AIMers direct RNA editing to convert the Rett Syndrome mutation *MECP2*$^{R168X}$ into the missense codon *MECP2*$^{R168W}$ in human and mouse neuronal cell models.
- A-to-I(G) editing of RNA-encoding Mecp2$^{R168X}$ restores expression of full-length Mecp2$^{R168W}$ protein in neuronal cells, which correctly colocalizes with heterochromatin. MECP2$^{R168W}$ protein also associates with wild-type MECP2 binding partners, suggesting functionality.

## INTRODUCTION

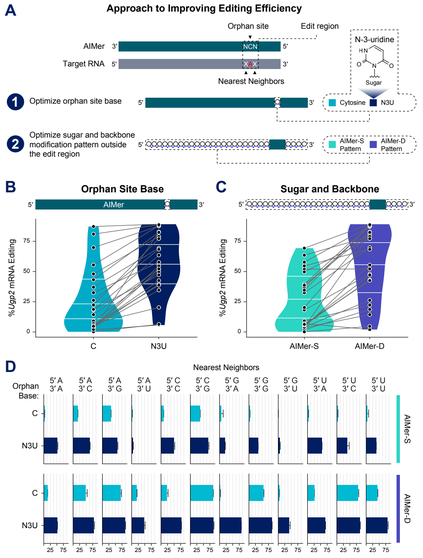
- PRISM™ generates stereopure oligonucleotides with controlled sequence, chemistry, and stereochemistry (**Figure 1A**).[1]
- PRISM™ can be applied to optimize AIMer design for editing efficiency, target sequence, and target tissue.

### Figure 1. Introduction to PRISM™, PN chemistry, and AIMers




## RESULTS

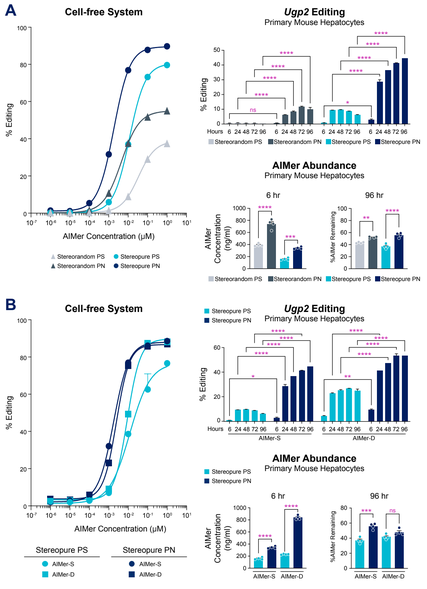
### Figure 2. AIMer base, sugar and backbone modifications enhance editing efficiency across nearest neighbor combinations in cells




(**A**) Schematic of approach to improving editing efficiency through AIMer backbone, sugar, and base chemistry. (**B**, **C**, **D**) Primary mouse hepatocytes from human *ADAR1-p110* hemizygous mice were treated with 3 μM AIMers (unconjugated), directed toward the *Ugp2* mRNA, with variable edit region sequence, chemistry pattern (AIMer-S or AIMer-D), and orphan base (C or N3U) for 72 hours. *Ugp2* RNA editing was quantified by Sanger sequencing. (B) Lines connect complexes (represented by circles) with identical 5'- and 3'-nearest neighbors and chemistry format. (C) Lines connect complexes (represented by circles) with identical 5'- and 3'-nearest neighbors and orphan base. Stats: mean of n=3; error bars represent SEM.

- AIMers with orphan site N3U supported higher mean percent RNA editing than AIMers with orphan site C for all nearest neighbor combinations tested, although the magnitude of increase varies (**Figure 2B**).
- The AIMer-D pattern conferred a higher mean percent RNA editing compared to the AIMer-S patternfor most sequences tested (**Figure 2C**).
- The impacts of orphan site N3U base modification and the AIMer-D pattern appear largely additive (**Figure 2D**).
- AIMers with orphan site N3U and the AIMer-D pattern support highly efficient editing for many nearest neighbor combinations in primary mouse hepatocytes.

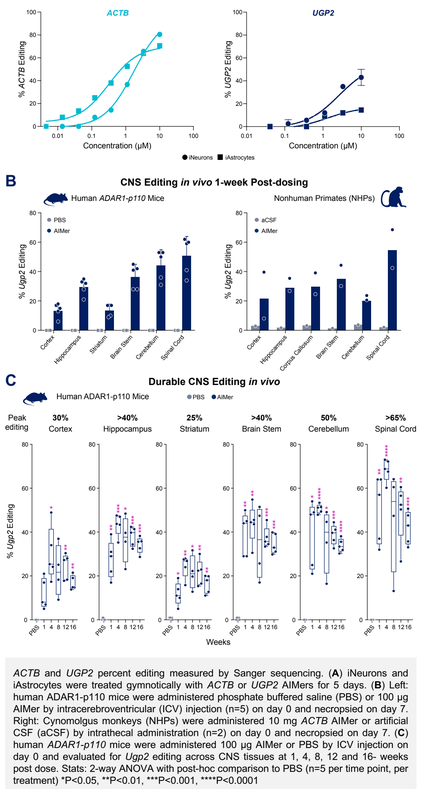
### Figure 3. Impact of AIMer optimization on editing efficiency *in vitro*




(**A**, **B**) Left: Cell-free editing assays: Lysates from 293T cells transfected with human *ADAR-p110* (48h) were incubated with either *UGP2*-targeting AIMer (A) or *ACTB*-targeting AIMer (B) at the concentration indicated for 1h, then RNA was extracted from lysates and RNA editing was quantified by Sanger sequencing. Stats: n=3 per dose, per condition; mean ± SEM shown. Right: Primary murine hepatocytes were treated gymnotically with 3 μM *Ugp2*-targeting AIMers for 6 hours. Cells were refreshed with maintenance media and collected at the indicated time point. RNA editing was quantified by Sanger sequencing. AIMer concentration was quantified by hybridization ELISA 6 hr or 96 hr after the start of the pulse. Stats: A two-way ANOVA was used to calculate statistical significance; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns significant.

- Incorporating stereopure PN linkages in AIMers enhances maximum editing compared to either stereopure PS or stereorandom PN in both cell-free and hepatocyte RNA editing assays (**Figure 3A**).
- The AIMer-D pattern further enhances the editing efficiency benefits of incorporating stereopure PN linkages in AIMers (**Figure 3B**).
- The AIMer-D pattern does not appear to enhance editing in cell-free systems but does lead to an increased cellular concentration of AIMers immediately after treatment (**Figure 3B**).
- Collectively, incorporation of stereopure PN linkages and the AIMer-D pattern improve AIMer-mediated RNA editing efficiency. This impact may occur through multiple mechanisms, including enhancing enzyme activity and AIMer uptake.

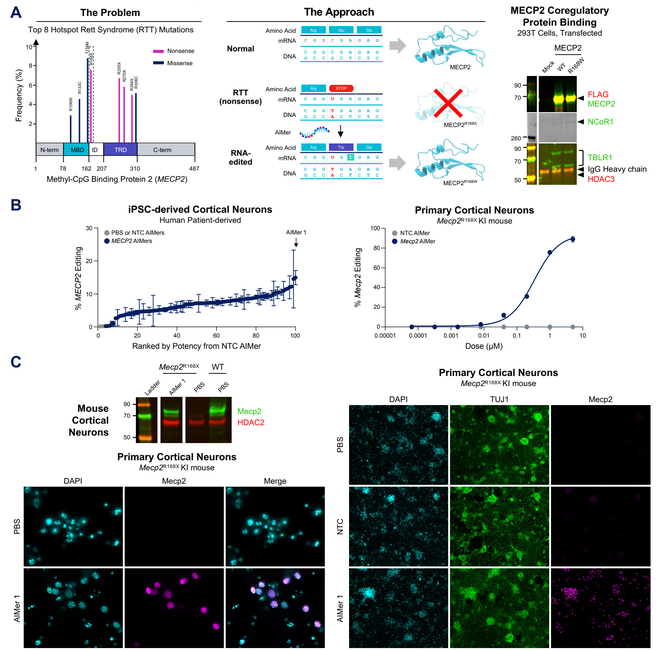
### Figure 4. AIMers facilitate RNA editing in the CNS




*ACTB* and *UGP2* percent editing measured by Sanger sequencing. (**A**) iNeurons and iAstrocytes were treated gymnotically with *ACTB* or *UGP2* AIMers for 5 days. (**B**) Left: human ADAR1-p110 mice were administered phosphate buffered saline (PBS) or 100 μg AIMer by intracerebroventricular (ICV) injection (n=5) on day 0 and necropsied on day 7. Right: Cynomolgus monkeys (NHPs) were administered 10 mg *ACTB* AIMer or artificial CSF (aCSF) by intrathecal administration (n=2) on day 0 and necropsied on day 7. (**C**) human *ADAR1-p110* mice were administered 100 μg AIMer or PBS by ICV injection on day 0 and evaluated for *Ugp2* editing across CNS tissues at 1, 4, 8, 12 and 16- weeks post dose. Stats: 2-way ANOVA with post-hoc comparison to PBS (n=5 per time point, per treatment) *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

- AIMers with the AIMer-S pattern, optimized for the CNS, support dose-dependent editing of ubiquitous housekeeping transcripts in multiple CNS cell lines *in vitro* (**Figure 4A**).
- In a human *ADAR1-p110* transgenic mouse model, and in NHPs, AIMer-directed editing of housekeeping transcripts were observed across the CNS at one week post single dose (**Figure 4B**).
- In human *ADAR1-p110* mice, AIMer-directed editing peaked at 4 weeks and persisted 4 months post-single ICV injection (**AIMer 4C**).

### Figure 5. ADAR editing for MECP2$^{R168X}$ protein restoration




(**A**) Left: Graph adapted from (4) with updated data from RettBASE (data pulled 7 Aug 23). Middle: Proposed MECP2 restoration strategy via AIMer-based A-to-I RNA editing. Right: 293T cells transfected (72 h) with FLAG-MECP2$^{WT}$ or FLAG-MECP2$^{R168W}$, immunoprecipitated with anti-FLAG magnetic beads. Western blot of immunoprecipitated eluates probed for FLAG and NCoR1/SMRT complex members. (**B**) *MECP2* AIMers direct editing in neuronal cells. Percentage editing determined by next-generation sequencing (NGS). Left: Patient iPSC-derived cortical neurons (*MECP2*$^{R168X}$) treated gymnotically with 10 μM AIMer (mean ± SEM; n=2 per AIMer). Right: Primary cortical neurons from *Mecp2*$^{R168X}$ knock-in (KI) mice (E18), treated gymnotically with AIMer for 5 days (mean ± SEM; n=3 per dose/condition). (**C**) AIMer-based editing of *Mecp2*$^{R168X}$ restores protein expression. Western blot of nuclear extracts from mouse primary cortical neurons (E18) treated gymnotically with 10 μM AIMer. Primary cortical neurons from *Mecp2*$^{R168X}$ KI mice (E18) treated with PBS or gymnotic AIMer (30 μM, left; or 1 μM, right) for 5 days. Immunofluorescence staining for nuclei (DAPI, blue) and Mecp2 (magenta) or neuronal marker Tuj1 (Green). Magnification 40X (left) or 10X (right). NTC, nontargeting control. HDAC2, Histone deacetylase 2. WT, wild type.

- We hypothesized that AIMers could be used to correct *MECP2*$^{R168X}$, the most common nonsense mutation found in Rett Syndrome (RTT), by converting the premature stop codon to a Tryptophan (W) codon in *MECP2* mRNA (**Figure 5A**).
- We show that exogenous, plasmid-expressed MECP2$^{R168W}$ protein associates with endogenous co-regulatory proteins NCoR1, TBLR1, and HDAC3, suggesting edited MECP2 may retain wild type MECP2 functionality (**Figure 5A**).
- *MECP2* AIMer incorporating the AIMer-D format directs editing of *MECP2*$^{R168X}$ in human patient-derived cortical neurons and primary cortical neurons isolated from the *Mecp2*$^{R168X}$ KI mouse (**Figure 5B**).
- *MECP2* AIMer incorporating the AIMer-D pattern restores Mecp2 protein expression and localization in primary cortical neurons isolated from the *Mecp2*$^{R168X}$ KI mouse (**Figure 5C**).

**References:** 1. Kandasamy, et al., 2022. *Nuc Acids Res* 50(10):5443-5466; 2. Monian et al., 2022 *Nature Biotech* 40(7):1093-1102. doi: 10.1038/s41587-022-01225-1; 3. Woolf, et al., 1995 *Proc Natl Assoc Sci* 92:8298-8302; 4. Krishnaraj, et al., *Hum Mutat* 2017;00:1-10. **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. Patient fibroblast cells were kindly provided by Rett Syndrome Research Trust and Harvard Stem Cell Institute iPS Core Facility.