# Improving the Potency and Sequence Versatility of RNA Editing Through Oligonucleotide Chemical Modifications

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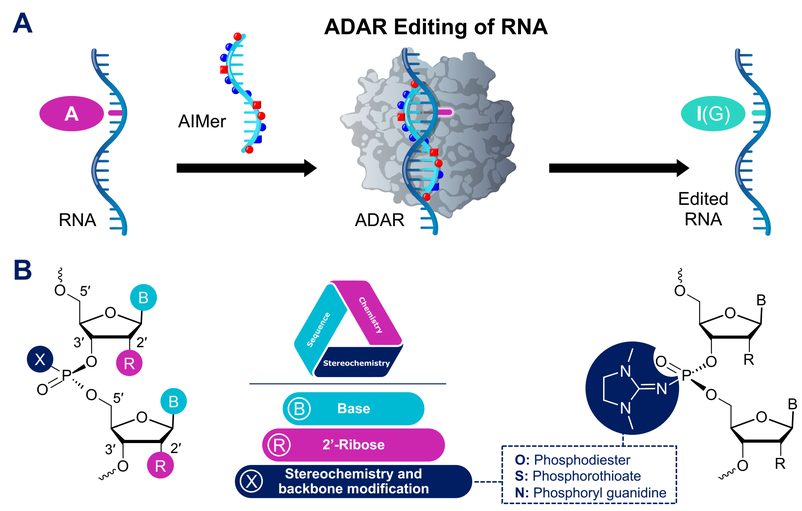
## SUMMARY

- Leveraging our oligonucleotide chemistry platform, we developed relatively short oligonucleotides called AIMers that elicit A-to-I editing with high efficiency using endogenous ADAR (adenosine deaminase acting on RNA) enzymes.
- We have previously demonstrated that AIMers incorporating stereopure design and phosphoryl guanidine (PN) backbone chemistry have overall higher editing efficiencies compared to stereorandom AIMers lacking PN.[1]
- Incorporating stereopure PN in AIMers improves both target engagement and AIMer uptake in cells.
- We identified a sugar and backbone modification pattern that improves editing across many nearest neighbor sequence combinations. This pattern improved editing largely through enhancing AIMer uptake in cells.
- Incorporating an N-3-uridine (N3U) base modification in the AIMer position across from the edited adenosine, known as the orphan site, improved editing efficiency compared to cytosine (C) across all nearest neighbor sequences tested in cells.
- Orphan site N3U increased AIMer-mediated RNA editing in mouse liver compared to orphan site C.
- N3U enhances chemical flexibility of the sugar modification in the AIMer orphan position.

## INTRODUCTION

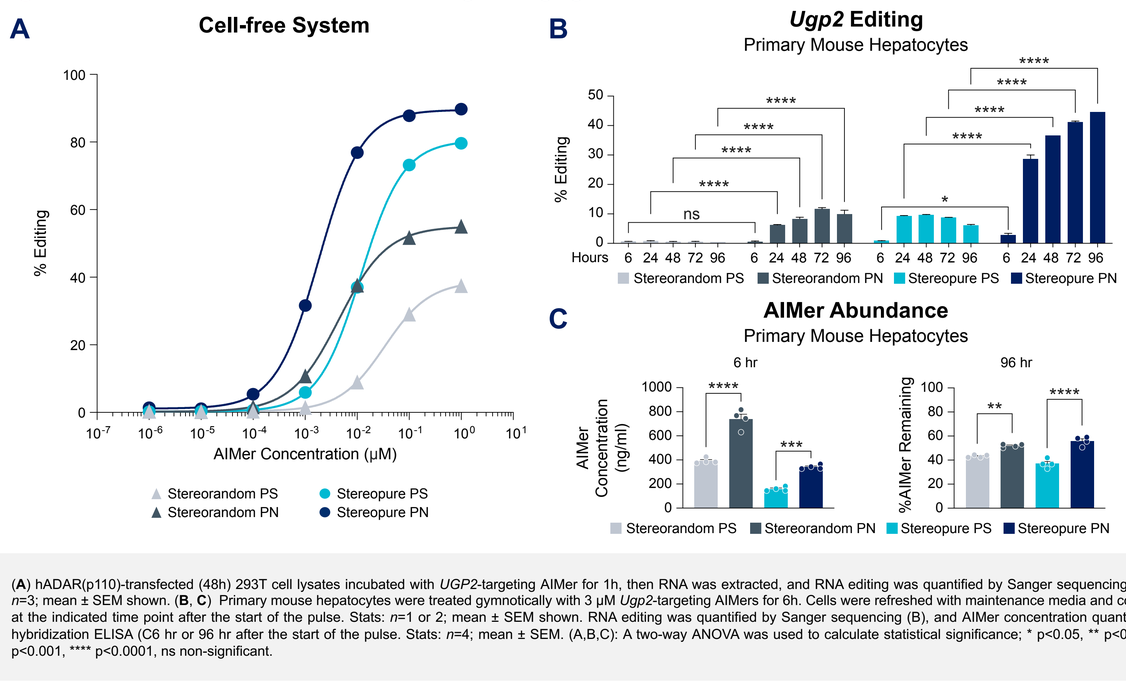
- Wave has developed chemically modified oligonucleotides, called AIMers, which facilitate RNA base editing by recruiting endogenous ADAR enzymes (**Figure 1A**).[1,2]
- We apply PRISM™, our discovery and drug development platform,[3] to generate stereopure AIMers with controlled sequence, chemistry, and stereochemistry (**Figure 1B**).
- A major challenge to advancing RNA editing as a therapeutic modality is that ADAR enzymes exhibit biases toward adenosines positioned within certain 5′- and 3′-nearest neighbor sequence contexts. This results in extremely low editing efficiency for some sequences, limiting the scope of therapeutic applications for RNA editing.
- Here, we apply our PRISM™ platform to advance AIMer chemistry and stereochemistry to support improved RNA editing potency and sequence versatility.

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




## RESULTS

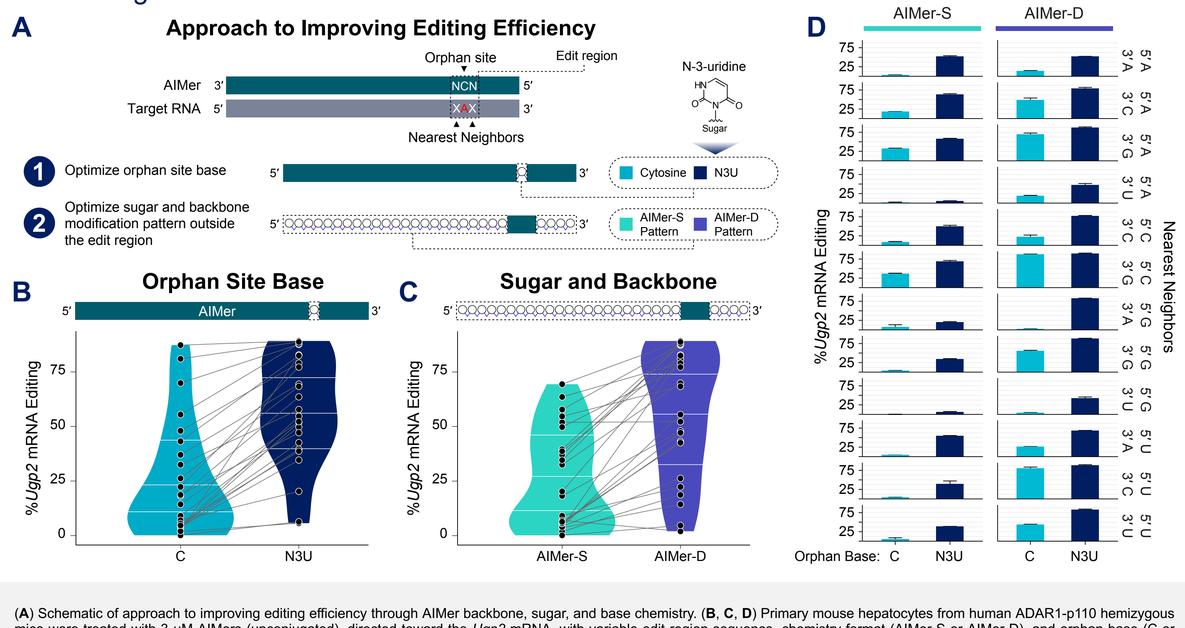
**Figure 2.** Stereopure PN improves target engagement and AIMer uptake *in vitro*




(**A**) hADAR(p110)-transfected (48h) 293T cell lysates incubated with *UGP2*-targeting AIMer for 1h, then RNA was extracted, and RNA editing was quantified by Sanger sequencing. Stats: *n*=3; mean ± SEM shown. (**B**, **C**) Primary mouse hepatocytes were treated gymnotically with 3 μM *Ugp2*-targeting AIMers for 6h. Cells were refreshed with maintenance media and collected at the indicated time point after the start of the pulse. Stats: *n*=1 or 2; mean ± SEM shown. RNA editing was quantified by Sanger sequencing (B), and AIMer concentration quantified by hybridization ELISA (C6 hr or 96 hr after the start of the pulse. Stats: *n*=4; mean ± SEM. (A,B,C): A two-way ANOVA was used to calculate statistical significance; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns non-significant.

- Incorporating stereopure PN in AIMers enhances maximum RNA editing and editing efficiency compared to either stereopure PS or stereorandom PN in cell-free assays (**Figure 2A**).
- Similarly, AIMers with stereopure PN support the greatest mean percent RNA editing in hepatocytes compared to AIMers with stereopure PS, stereorandom PN, or stereorandom PS (**Figure 2B**).
- PN improves cellular uptake of AIMers compared to PS, for both stereopure and stereorandom linkages (**Figure 2C**).
- These results suggest incorporation of stereopure PN improves editing efficiency through both improving target engagement and enhancing cellular uptake.

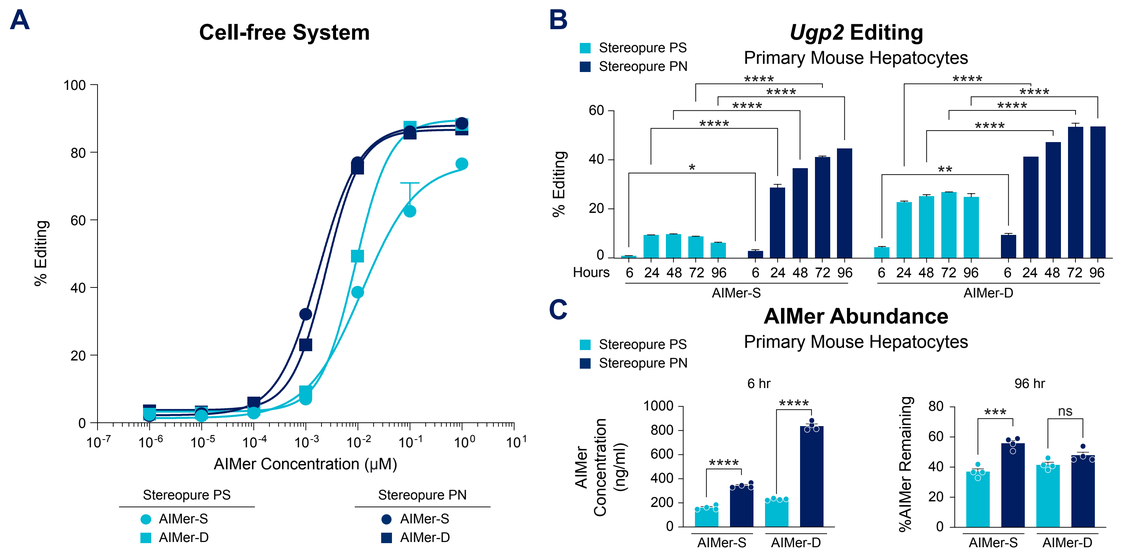
**Figure 3.** AIMer base, sugar and backbone modifications enhance editing efficiency across nearest neighbor combinations in cells




(**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 format (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 3B**).
- The AIMer-D pattern conferred a higher mean percent RNA editing compared to the AIMer-S pattern for most sequences tested (**Figure 3C**).
- The impacts of orphan site N3U base modification and the AIMer-D pattern appear largely additive (**Figure 3D**).
- AIMers with orphan site N3U and the AIMer-D pattern support highly efficient editing for many nearest neighbor combinations in primary mouse hepatocytes.

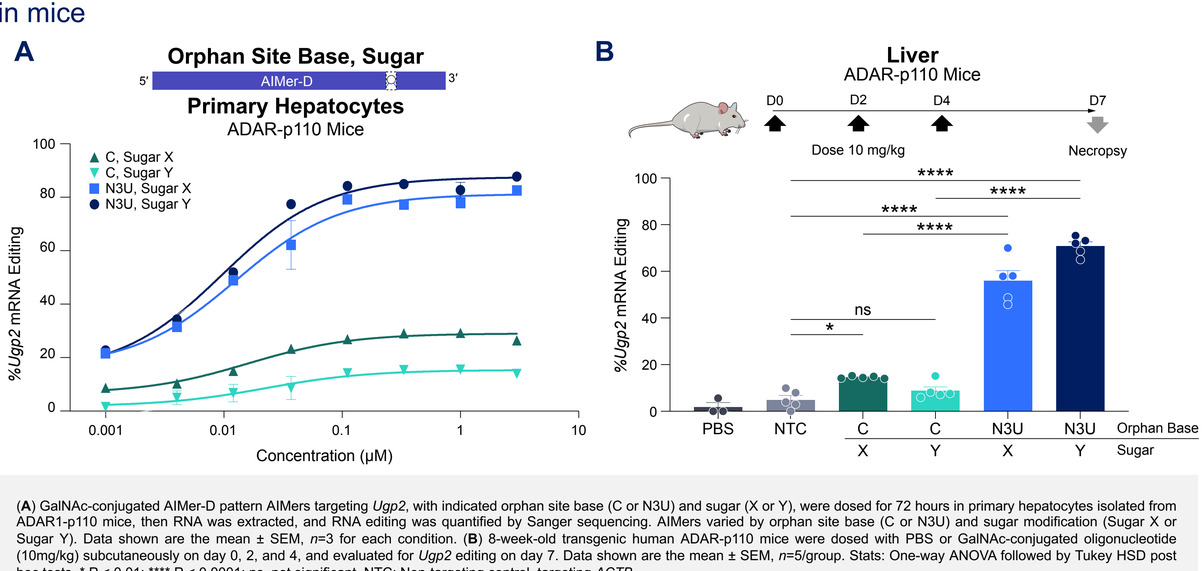
**Figure 4.** AIMer-D pattern enhances editing largely through improved uptake




(**A**) hADAR(p110)-transfected (48h) 293T cell lysates were incubated with *ACTB*-targeting AIMer for 1h, then RNA was extracted and RNA editing was quantified by Sanger sequencing. Stats: *n*=3; mean ± SEM shown. (**B**,**C**) Primary mouse hepatocytes were treated gymnotically with 3 μM *Ugp2*-targeting AIMers for 6h. (B) RNA editing was quantified by Sanger sequencing. (C) AIMer concentration quantified by hybridization ELISA 6-hr or 96-hr after the start of the pulse. Stats: *n*=4, A two-way ANOVA was used to calculate statistical significance, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns non-significant.

- The AIMer-D pattern does not impact maximum editing or editing efficiency in a cell-free editing assay (**Figure 4A**).
- Incorporating stereopure PN linkages enhances editing efficiency compared to stereopure PS linkages alone in cell-free editing assays and in primary hepatocytes (**Figure 4A,B**).
- The AIMer-D pattern with stereopure PN linkages supported enhanced maximum editing in primary hepatocytes compared to the AIMer-S pattern and compared to PS-only linkages (**Figure 4B**).
- At 6 hrs post-dose, AIMers with the AIMer-D pattern and stereopure PN linkages have enhanced concentration in cells compared to AIMers with PS linkages but exhibit similar rates of metabolic clearance by 96-hrs post-dose (**Figure 4C**).
- Together, these data suggest that the AIMer-D pattern improves editing efficiency in cells, compared to the AIMer-S pattern, largely through enhanced AIMer uptake.

**Figure 5.** Incorporation of N3U modification in AIMers supports enhanced editing efficiency in mice




(**A**) GalNAc-conjugated AIMer-D pattern AIMers targeting *Ugp2*, with indicated orphan site base (C or N3U) and sugar (X or Y), were dosed for 72 hours in primary hepatocytes isolated from ADAR1-p110 mice, then RNA was extracted, and RNA editing was quantified by Sanger sequencing. AIMers varied by orphan site base (C or N3U) and sugar modification (Sugar X or Sugar Y). Data shown are the mean ± SEM, *n*=3 for each condition. (**B**) 8-week-old transgenic human ADAR-p110 mice were dosed with PBS or GalNAc-conjugated oligonucleotide (10mg/kg) subcutaneously on day 0, 2, and 4, and evaluated for *Ugp2* editing on day 7. Data shown are the mean ± SEM, *n*=5/group. Stats: One-way ANOVA followed by Tukey HSD post hoc tests. * P < 0.01; **** P < 0.0001; ns, not significant. NTC: Non-targeting control, targeting *ACTB*.

- We next evaluated the impact of N3U on the editing efficiency of *N*-acetylgalactosamine (GalNAc)-conjugated AIMers, and whether N3U is compatible with various sugar modifications in the orphan site position.
- GalNAc-AIMers incorporating orphan site N3U support greater maximum editing compared to AIMers with orphan site C in cells *in vitro* (**Figure 5A**).
- In cells, Sugar Y did not significantly impact editing when N3U was the orphan base, whereas Sugar Y reduced editing when combined with C in AIMers (**Figure 5A**).
- In mice, AIMers with orphan site N3U supported greater *Ugp2* editing in liver than AIMers with C (**Figure 5B**).
- AIMers with N3U and Sugar Y supported similar *Ugp2* RNA editing in liver compared to AIMers with N3U and Sugar X, whereas AIMers with Sugar Y supported reduced *Ugp2* editing compared to AIMers with Sugar X when C was in the orphan position (**Figure 5B**).
- These data suggest that AIMers with orphan site N3U support enhanced editing in mouse liver compared to AIMers with C, and that N3U enhances chemical flexibility for the orphan site sugar.

**References:** 1. Monian et al., 2022. *Nat. Biotechnol.* DOI: 10.1038/s41587-022-01225-1; 2. Woolf et al., 1995 *Proc. Natl. Assoc. Sci.* 92:8298-8302; 3. Kandasamy et al., 2022. *Nuc. Acids. Res.* 50(10):5443-5466. **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.