Effect of Stereochemistry and Backbone Chemistry on AIMer RNA Editing Efficiency

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Summary

- Wave Life Science's RNA base editing platform utilizes single-stranded, chemically modified oligonucleotides (AIMers) to form double-stranded RNA substrates that recruit adenosine deaminase acting on RNA (ADAR) enzymes. We have previously demonstrated that AIMers incorporating stereopure design as well as PN backbone chemistry have overall higher editing efficiencies compared to stereorandom AIMers lacking PN.[1] In this study, we aimed to elucidate the precise mechanisms by which these chemistries improve AIMer activity.
- We evaluated the editing activity of AIMers with various chemistries in cellular and cell-free conditions. In cells, we also assessed editing using gymnotic or transfection conditions to evaluate the impact of cellular uptake on editing. We also quantified AIMers in cells after treatment to understand the impact of AIMer uptake and stability on editing activity.
- We observed that either our stereopure design or the addition of PN chemistry increased cellular and cell-free editing efficiency, with AIMers that are both stereopure and contain PN chemistry exhibiting the greatest activity. Changes to the 2'-ribose modification pattern improved cellular editing but not cell-free editing, suggesting that sugar chemistry may impact stability, uptake, and/or endosomal escape.
- We also observed that PN chemistry improved cellular uptake of AIMers independent of stereopure design.
- PN chemistry improved target engagement in murine hepatocytes, and this may be the major driver for increased editing activity. PN chemistry may also provide a stability benefit to AIMers with low cellular stability.

Introduction

- Wave has developed chemically modified oligonucleotides, called AIMers, which facilitate RNA base editing by recruiting endogenous ADAR enzymes (**Figure 1**).

Figure 1. RNA editing with AIMers: A-to-I editing oligonucleotides

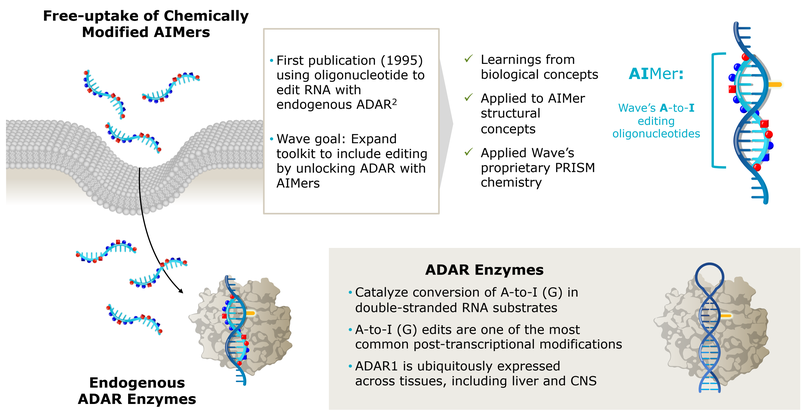
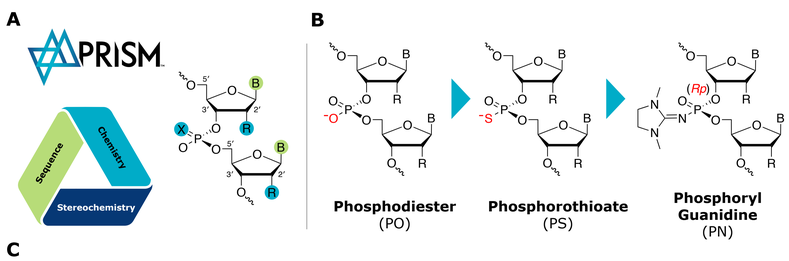


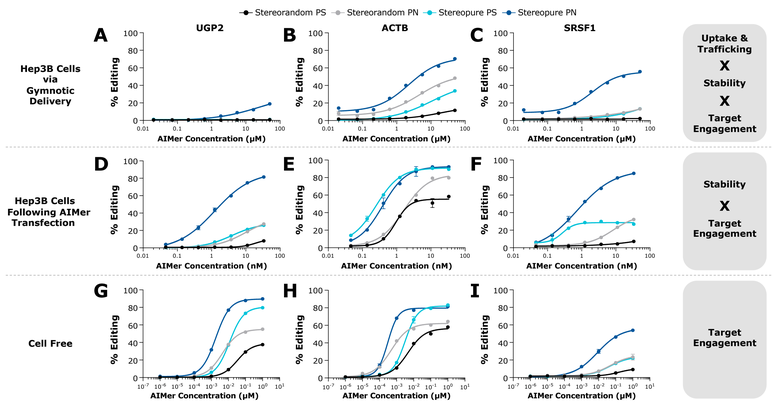
Figure 2. Introduction to PRISM and PN chemistry



- We apply PRISM™, our discovery and drug development platform, to generate stereopure AIMers with controlled sequence, chemistry, and stereochemistry (**Figure 2A**).
- We have developed stereopure AIMers containing PO, PS and PN backbones (**Figure 2B**).
- Here we apply our previous findings that five PN backbone linkages at positions 1, 14, 16, 26 and 29 support robust Actin beta (ACTB) editing[1] onto two other sequences: UDP-glucose pyrophosphorylase 2 (UGP2) and Serine and arginine rich splicing factor 1 (SRSF1).
- We test stereorandom and stereopure versions of each sequence both with and without the inclusion of PN backbone linkages.
- We utilize cellular and *in vitro* assays to investigate the impact of stereopure design and the presence of PN on uptake stability and target engagement (**Figure 2C**).

Results

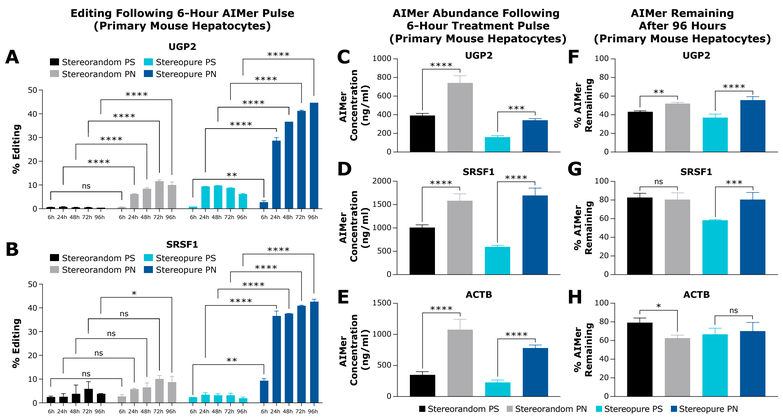
Figure 3. Stereopure design and PN chemistry positively impact editing efficiency independent of delivery method



Hep3B cells were treated gymnotically or by transfection for 48 hr. RNA was collected and Sanger sequencing was used to quantify percentage editing. Cell free editing lysate preparation: Cell free lysate was generated from HEK293 cells which had been transfected with ADARp110. Cells were dissociated and pelleted before being resuspended in cell free editing buffer at an equal volume to the cell pellet. Cells were then sonicated on ice for 5 min before the lysate was cleared by centrifugation. Lysates were stored at -80°C until use. Editing assay: Cell free lysates were thawed on ice before being plated into a 96-well plate. AIMers were added, and the plate was incubated at 30°C for 1 hr. RNA was extracted and editing percentage was calculated by Sanger sequencing.

- The gymnotic datasets (**Figure 3A-C**) show the combined impact of uptake, stability and target engagement. Under gymnotic conditions, there is a clear editing advantage for stereopure AIMers with PN backbone chemistry.
- Under transfection conditions (**Figure 3D-F**), the potencies reflect editing activity when cellular uptake is no longer a challenge. Stereorandom PS AIMers consistently perform poorly compared to either stereopure or PN-containing AIMers.
- The cell free editing assay allows interrogation of RNA editing efficiency without the confounding impacts of cellular uptake and stability. For all targets, the stereopure PN AIMers had the greatest potency (**Figure 3G-I**). For all targets, stereorandom PS AIMers have the lowest potency. The slopes of the stereorandom AIMers are shallow, suggesting an inhibited reaction. This may be due to non-productive stereoisomers present in the stereorandom samples.
- Both PN chemistry and stereopure design have positive impact on AIMer mediated RNA editing, likely reflecting improved target engagement.

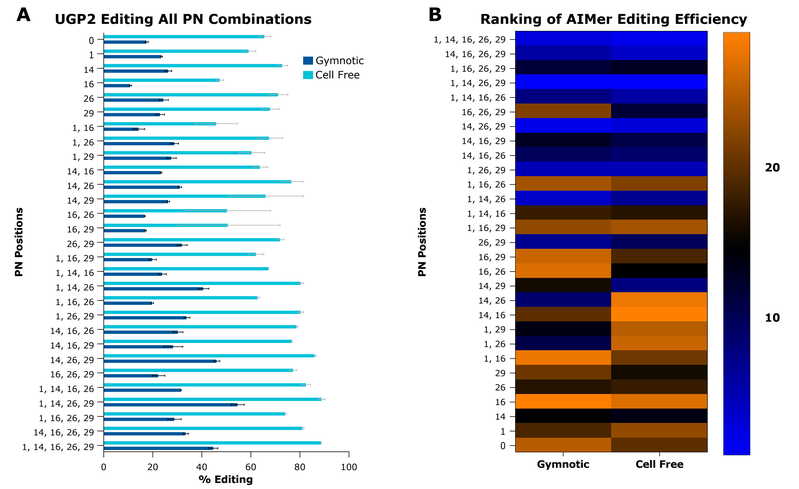
Figure 4. PN chemistry improves AIMer uptake and editing efficiency following a 6-hour dose pulse in primary mouse hepatocytes



Primary murine hepatocytes were treated gymnotically for 6-hr with 3 μM AIMer before being thoroughly washed with PBS. Cells were refreshed with maintenance media and collected at the indicated time point. For editing data, RNA was extracted, reverse transcribed and Sanger sequenced. For measuring AIMer abundance, cells were lysed in RIPA, and AIMer concentration was quantified using a hybrid ELISA. For A-B, a mixed effects models was used to test for statistical significance. For C-H, a One-way ANOVA was used to test for significance, Stats: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns not significant.

- In primary murine hepatocytes, following a gymnotic 6-hr pulse of AIMer, stereopure, PN-containing AIMers yielded more effective editing of SRSF1 and UGP2 than stereorandom AIMers or those lacking PN chemistry (**Figure 4A, B**).
- Independent of AIMer sequence or stereochemistry, the presence of PN chemistry increased crude uptake of AIMers following a 6-hr dose pulse (**Figure 4C-E**).
- In this study, stereorandom AIMers have equivalent or better uptake than stereopure AIMers (**Figure 4C-E**), though they do not effectively edit (**Figure 4A, B**).
- SRSF1- and ACTB-targeting AIMers are largely stable in the cellular environment, with ~80% of AIMer remaining after 96 hr (**Figure 4G-H**).
- UGP2 AIMers are less stable, with ~40% remaining after 96 hr with PN AIMers having slightly more AIMer remaining than PS AIMers (**Figure 4G-H**), suggesting that PN chemistry may confer a stability benefit to AIMers with lower cellular stability.

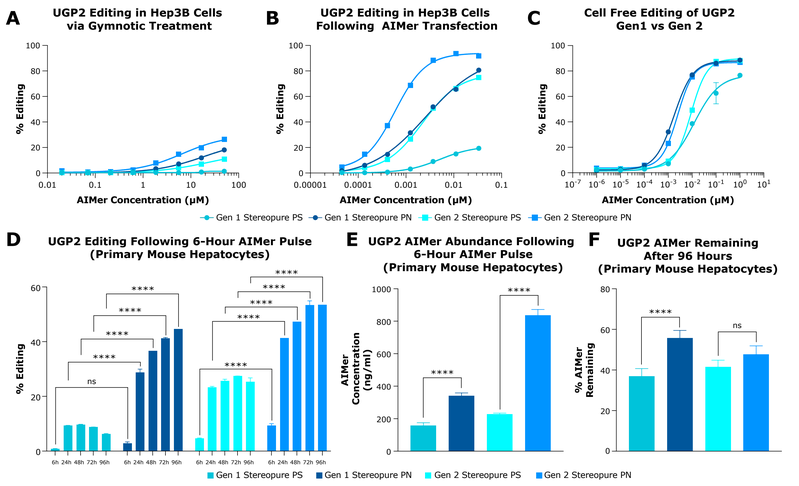
Figure 5. Improved target engagement is a major factor in PN-induced gain in editing activity



32 stereopure AIMers were designed with all combinations of the five PN backbone positions in the UGP2-targeting AIMer. Of these, 29 were selected for testing in primary hepatocytes under gymnotic conditions at 6 μM, as well as in the cell free system at 1 μM. Sanger sequencing was used to quantify RNA editing. The ranked ratio of percentage editing for each AIMer to the AIMer containing all five PN linkages is depicted in the heat map.

- In general, we observed that increasing the number of PN linkages in a UGP2-targeting AIMer increased editing (**Figure 5A**).
- There is good correlation between cell-free editing and editing in primary hepatocytes under gymnotic conditions (**Figure 5B**), suggesting that target engagement may be the driving factor for how PN chemistry improves editing efficacy.

Figure 6. Changes to the 2'-chemistry and backbone enhance cellular editing partially through improved uptake



Hep3B cells were treated either gymnotically or by transfection with UGP2-targeting AIMers. Cell-free editing was measured as described in Figure 3. Primary murine hepatocytes were treated gymnotically for 6 hr with 3 μM of AIMer before being thoroughly washed with PBS, refreshed with maintenance media and collected at the reported time point. RNA was collected, reverse transcribed, and editing was quantified by Sanger sequencing. Cells were lysed in RIPA for use in a hybrid ELISA to quantify AIMer concentration immediately after or 96-hr after dosing. For E-F, a One-way ANOVA was used to test for significance. For D a mixed effects models was used to test for statistical significance. Stats: **** p<0.0001, ns significant.

- We performed additional chemical optimization to improve the activity of our AIMers. On a stereopure background, significant changes to the 2'-chemistry, addition of PO linkages, and changes to the positions of the PN linkages yielded our second-generation of AIMers with improved cellular editing.
- Editing efficacy of PS/PO AIMers with our second-generation design approximates that of first-generation PN-containing AIMer (**Figure 6A, B**). Addition of PN to the second-generation AIMer improved cellular editing under gymnotic and transfection conditions (**Figure 6A, B**).
- There is no difference in editing efficiency between the two different generations of AIMers in the cell-free system, suggesting that improved target engagement is not responsible for the increase in editing (**Figure 6C**).
- In primary hepatocytes following a 6-hr dose pulse, second-generation AIMers edit more efficiently than the first-generation AIMers (**Figure 6D**). The uptake of second-generation AIMers is improved over the first generation (**Figure 6E**).
- Stability of first-generation AIMers is similar to that of second-generation AIMers (**Figure 6F**), so it is unlikely to explain the gain in efficiency observed for second-generation AIMers.
- Changes to the 2'-chemistry, addition of PO linkages, and moving the PN linkages in our second-generation AIMers improved editing partially by increasing cellular uptake.

References: 1. Monian et al., 2022, *Nat. Biotechnol.* 40:1093-1102; 2. Woolf et al., 1995, *Proc. Natl. Acad. Sci.* 92:8298-8302. **Acknowledgments:** The authors are grateful to Amy Donner (Wave Life Sciences) and Eric Smith for editorial and graphical support, respectively. This work was funded by Wave Life Sciences.