Application of ADAR-mediated RNA Editing to Modulate Gene Expression

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Summary

- AIMers can be applied to modulate gene expression by altering protein-protein interactions or transcript stability.
- AIMers were designed to modulate Nrf2-Keap1 protein-protein interactions and activate Nrf2-dependent gene expression. The AIMers directed up to 80% editing of Nrf2 or Keap1 transcripts in multiple cell types *in vitro* and led to dose-dependent activation of Nrf2-dependent gene expression.
- In mice expressing human *ADAR1*, AIMers led to ≥40% transcript editing and activation of Nrf2-dependent gene expression in the liver.
- AIMers were designed to modify regulatory elements in RNA that impact transcript stability. The AIMers increased mRNA expression 2-15-fold *in vitro* in cultured cells.
- GalNAc-AIMers that supported 40-60% editing in the mouse liver resulted in a corresponding ~5-fold increase in mRNA and protein expression 1-week after treatment.

Introduction

- AIMers are chemically modified oligonucleotides that direct sequence-specific adenosine (A) to inosine (I)/guanosine (G) RNA editing using endogenous ADAR enzymes (**Figure 1A**).
- In addition to correcting disease-causing mutations, application of AIMers to modulate protein-protein, protein-RNA, and RNA-RNA interactions can impact numerous biological outcomes (**Figure 1B**).

Figure 1. AIMers direct RNA editing with endogenous ADAR enzymes

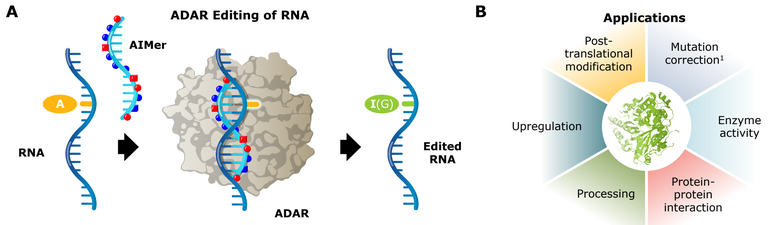
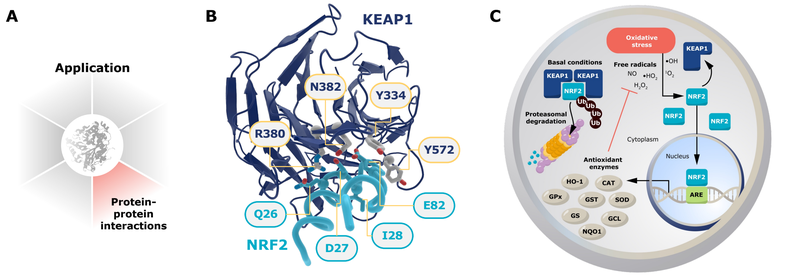


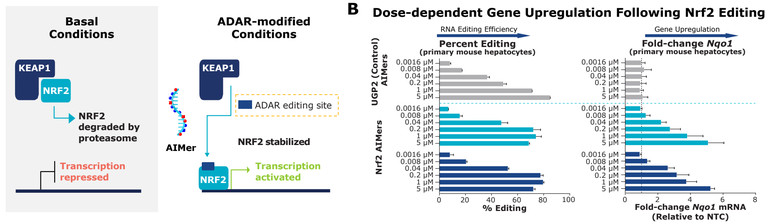
Figure 2. Applying AIMers to modulate protein-protein interactions



- We developed AIMers to modify the Nrf2-Keap1 interface (**Figure 2A**).
- Nrf2 is an antioxidant transcription factor negatively regulated by Keap1 through Nrf2-Keap1 binding (**Figure 2B, C**).

(B) adapted from Ref 2. (C) adapted from Ref 3. Note: Q26, D27, and I28 appear on the Nrf2 DLG motif; E82 appears on the Nrf2 ETGE motif.

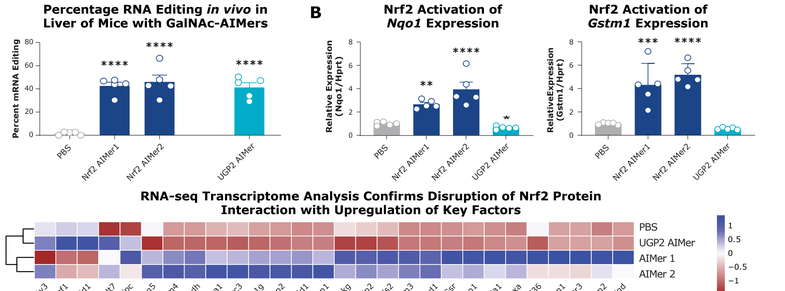
Figure 3. Dose-dependent modulation of Nrf2-Keap1 interaction *in vitro*



Primary mouse hepatocytes treated gymnotically with the indicated concentration of AIMer. RNA was collected 48 h later. Editing was quantified by PCR and Sanger sequencing. Gene expression was quantified by qPCR (n=2).

- Keap1 interacts directly with Nrf2, leading to its degradation. When this protein-protein interaction is disrupted, Nrf2 translocates to the nucleus and activates gene expression (**Figure 3A**).
- We generated GalNAc-AIMers to target mouse *Nrf2* mRNA, altering amino acids at the Nrf2-Keap1 interface.
- In cultured primary mouse hepatocytes, AIMers supported dose-dependent editing of either mouse *Ugp2* (control RNA) or *Nrf2* mRNA (**Figure 3B**). In the same cells, treatment with AIMers targeting *Ugp2* had no effect on expression of *Nqo1*, a gene downstream of Nrf2 (**Figure 3B**).
- The AIMers targeting *Nrf2* led to dose-dependent increases in *Nqo1* expression (**Figure 3B**) consistent with disruption of the protein-protein interaction in mouse cells.

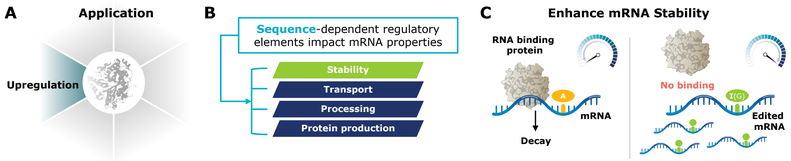
Figure 4. AIMers enable gene expression changes *in vivo* with a single edit



C57BL/6 mice expressing human *ADAR1* were dosed with 10 mg/kg of the indicated GalNAc-AIMer subcutaneously on days 0, 2, and 4. Livers were harvested (day 7) and analyzed for (A) RNA editing and (B) *Nqo1* and *Gstm1* expression via Sanger sequencing and qPCR, respectively. Data were analyzed via One-way ANOVA with Tukey's multiple comparisons test. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 (C) RNA-seq was performed on livers from mice treated as indicated. Expression of each gene was assigned a z-score based on mean expression and the number of sequencing reads. A higher z-score indicates more expression relative to other groups.

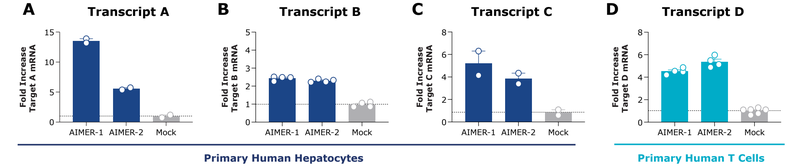
- In mouse liver 1-week after first dose, GalNAc-AIMers led to ~50% editing of the targeted mRNA (*Nrf2* or *Ugp2*) (**Figure 4A**).
- At the same time, expression of Nrf2-dependent RNAs *Nqo1* and *Gstm1* increased significantly after GalNAc-Nrf2 AIMer treatment compared with PBS, whereas expression of these same RNAs did not increase after treatment with GalNAc-Ugp2 AIMer (**Figure 4B**).
- RNA-seq evaluation of liver gene expression 1-week after first dose shows that Nrf2-dependent genes generally increased after GalNAc-Nrf2 AIMer treatment, whereas the expression of these same genes did not increase in response to PBS or GalNAc-Ugp2 AIMer treatment (**Figure 4C**).
- These data provide preclinical proof-of-concept that AIMer-mediated RNA editing can be applied to modulate PPIs in mice.

Figure 5. Applying AIMers to edit RNA motifs to restore or upregulate gene expression



- We developed AIMers to upregulate mRNA expression (**Figure 5A**).
- Multiple sequence-dependent regulatory elements in RNA can be edited to impact gene expression (**Figure 5B**).
- AIMers were designed to edit RNA sequence elements that impact mRNA stability, with the aim of upregulating gene expression (**Figure 5C**).

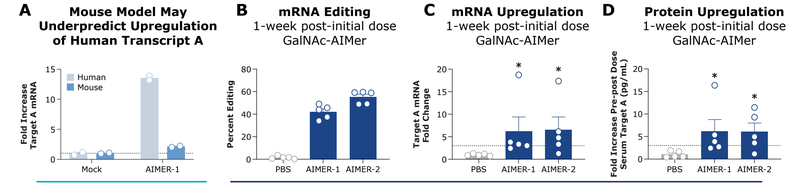
Figure 6. AIMers can edit RNA motifs to upregulate gene expression in hepatocytes and T cells *in vitro*



(A-C) Primary human hepatocytes were treated with 10 μM AIMer or water (mock treatment) for 48 hours. (D) Primary human T cells were treated with 10 μM AIMer for 48 hours. In all panels, mRNA expression was quantified by qPCR.

- In cultured primary human hepatocytes, AIMers designed to edit regulatory elements in different transcripts led to increased expression of the edited transcripts (2->10-fold upregulation) compared with mock-treated cells (**Figure 6A-C**).
- In cultured primary human T cells, multiple AIMers that edit RNA regulatory elements led to increased expression of the edited transcript (4-6-fold) compared with mock treatment (**Figure 6D**).

Figure 7. AIMers upregulate mRNA and downstream serum protein *in vivo*



(A) Human or mouse primary hepatocytes were treated with 10 μM GalNAc-AIMers for 48 hours. (B-D) Mice expressing human ADAR (n=5) were treated subcutaneously with 10 mg/kg GalNAc-AIMer or PBS on days 0, 2, and 4. Editing (Sanger sequencing), mRNA expression (qPCR), and protein expression (ELISA), were evaluated on day 7. Data were analyzed via the Kruskal-Wallis test followed by a Dunn post hoc test comparing to PBS-treated samples with Bonferroni adjustment for multiple comparisons. * p<0.05

- We compared Transcript A expression in response to RNA editing in cultured mouse and human cells. After GalNAc-AIMer treatment, Transcript A expression is increased more in human hepatocytes (~14-fold more than mock) compared to mouse hepatocytes (~2-fold more than mock), indicating that the mouse model may underpredict the impact of AIMers on this transcript (**Figure 7A**).
- We evaluated mouse AIMers *in vivo* in mice expressing a human *ADAR* transgene. One-week after the first dose, we detected 40-60% RNA editing in the liver (**Figure 7B**), which corresponded to a ~5-fold upregulation of Transcript A expression (**Figure 7C**) and a ~5-fold upregulation of protein expression (**Figure 7D**).

References: 1. Monian et al., 2022. *Nat Biotechnol* DOI: 10.1038/s41587-022-01225-1; 2. Francisqueti-Ferron et al., 2019. *Int. J. Mol. Sci.* DOI: 10.3390/ijms20133208; 3. Canning et al., 2015. *Free Radic Biol. Med* DOI: 10.1016/j.freeradbiomed.2015.05.034. **Acknowledgments:** Editorial and graphical support provided by Amy Donner (Wave Life Sciences) and Eric Smith, respectively.