An oligonucleotide design platform for ADAR-guided mRNA editing

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Introduction

Site-directed mRNA editing using endogenous Adenosine Deaminase acting on RNA (ADAR) offers a programmable approach to modify transcripts without permanent genomic alteration. ADAR enzymes catalyze the deamination of adenosine-to-inosine in double stranded RNA structures, which is interpreted as guanosine during translation. By recruiting endogenous ADAR to selected adenosine through a complementary guide editing oligonucleotide (EON), RNA editing enables precise and transient modulation of RNA. However, the development of oligonucleotide therapeutics has been limited by challenges in intracellular delivery[1], productive target engagement[2,3], and control of off-target effects[3,4]. The cellular and molecular determinants that drive on-target editing remain to be fully explored. To address these, we investigated the intracellular fate of Axiomer™ EONs in primary human hepatocytes (PHH) and evaluated how these insights could inform EON optimization.

Known biological barriers for ASO activity

Following cellular uptake, oligonucleotides can be retained within endosomal compartments, trafficked toward lysosomes, or degraded before reaching sites of productive RNA engagement. Efficient activity requires sufficient intracellular accumulation, endosomal release, access to the relevant subcellular compartment, and productive interaction with the target mRNA. For EON-guided RNA editing, these same barriers may influence the amount of EON available to recruit endogenous ADAR, thereby enabling site-directed RNA editing. (**Fig. 1**)

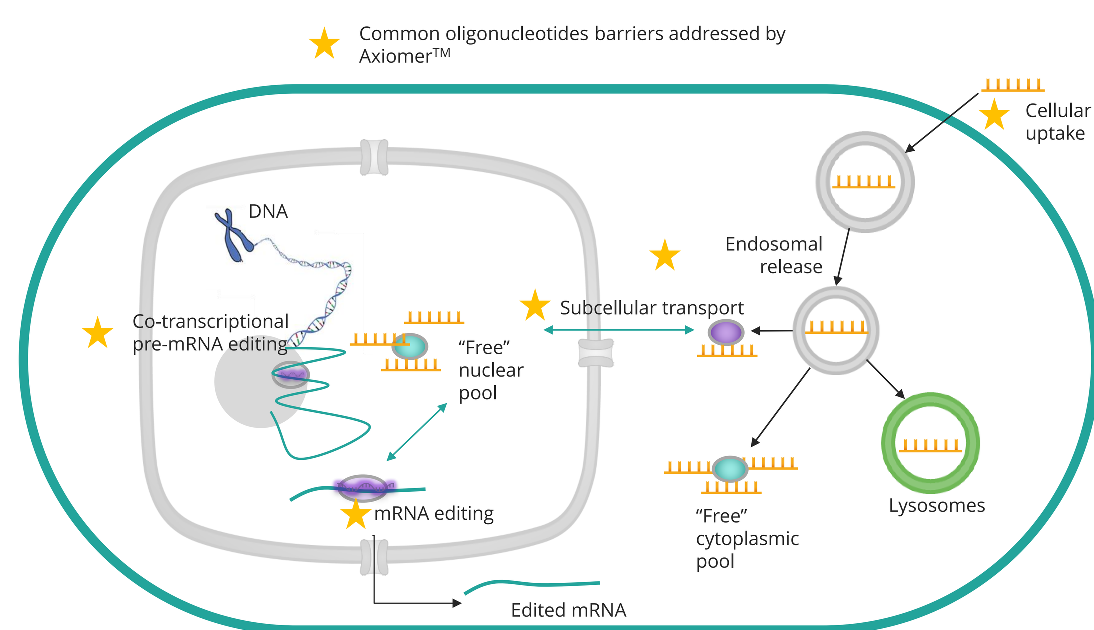


Figure 1. Intracellular fate and common barriers of oligonucleotides therapeutics

Objectives

- **Determine whether EONs are efficiently taken up by cells and support sustained editing**
- **Determine whether EONs gain cytosolic access and are subsequently transported to the nucleus to support RNA editing**
- **Determine whether endosomal escape is a limiting step for achieving efficient EON-mediated mRNA editing**
- **Use mechanistic insights and historical editing datasets to support AI-supported EON design and improve editing performance**
- **Evaluate whether improved EON performance is achieved with minimal transcriptomic perturbation and limited off-target editing**

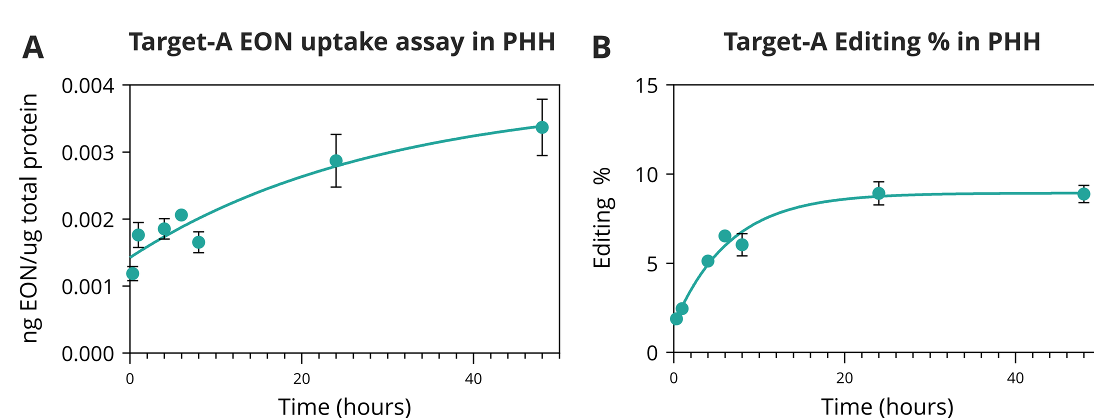
Results

Rapid EON uptake supports durable and co-transcriptional mRNA editing in PHH

EON for model Target-A was rapidly taken up by PHH gymnotically, with intracellular EON levels increasing over time. (**Fig. 2A and B**) This uptake was sufficient to drive Target-A mRNA editing, which increased within hours and reached a near-plateau by 24h.

A 4h pulse exposure was sufficient to trigger editing and maintain measurable activity through 72h, indicating that brief EON exposure can support durable ADAR-mediated editing in PHH. (**Fig. 2C**)

Target-A mRNA editing was detected across subcellular RNA fractions following gymnotic EON treatment in PHH. Using a nuclear run-on assay, edited Target-A transcripts were detected in nascent RNA fractions, demonstrating that EON-guided ADAR editing can occur co-transcriptionally. (**Fig. 2D**) This indicates that Target-A EON can support editing of newly synthesized RNA in PHH, consistent with productive nuclear editing activity.



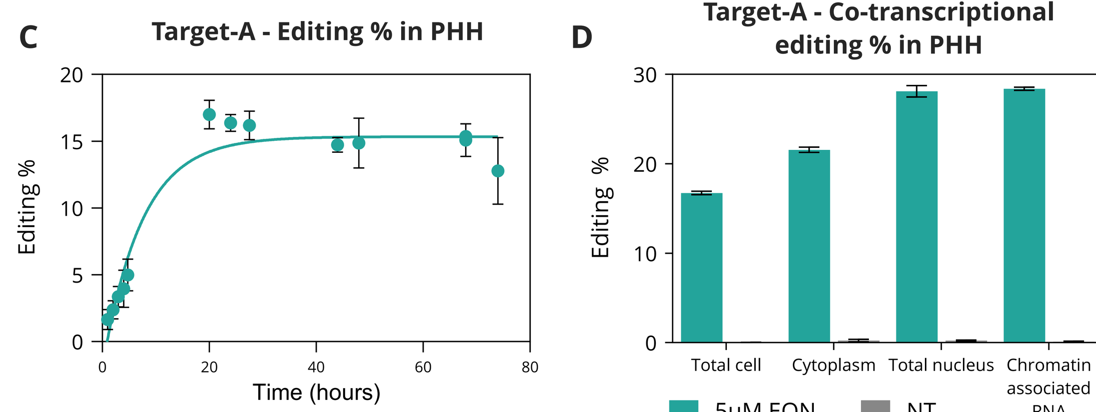


Figure 2. (**A and B**) Temporal intracellular EON levels were quantified by bioanalysis and mRNA editing was measured by dPCR over time following 1 µM EON treatment. N=3. (**C**) Target-A mRNA editing was measured by dPCR following a 4 h pulse treatment with 4 µM EON. N=3, data are shown as mean ± SEM. (**D**) Co-transcriptional editing was assessed by NRO assay following EON treatment in PHH. N=2.

EONs with distinct editing activities achieve similar nuclear accumulation in PHH

Three distinct EONs with different editing efficiencies were selected to assess whether subcellular biodistribution explains differences in activity. (**Fig. 3A and B**) As expected, EON-3 showed markedly lower Target-A editing than the other two EONs. Subcellular fractionation and bioanalysis showed that all three EONs were detected in the nucleus following treatment, irrespective of their editing efficiency. Notably, the low-editing EON-3 also accumulated in the nuclear fraction. These data indicate that nuclear exposure alone does not account for differences in editing activity, suggesting that factors such as EON design or productive target interaction, contribute to editing performance.

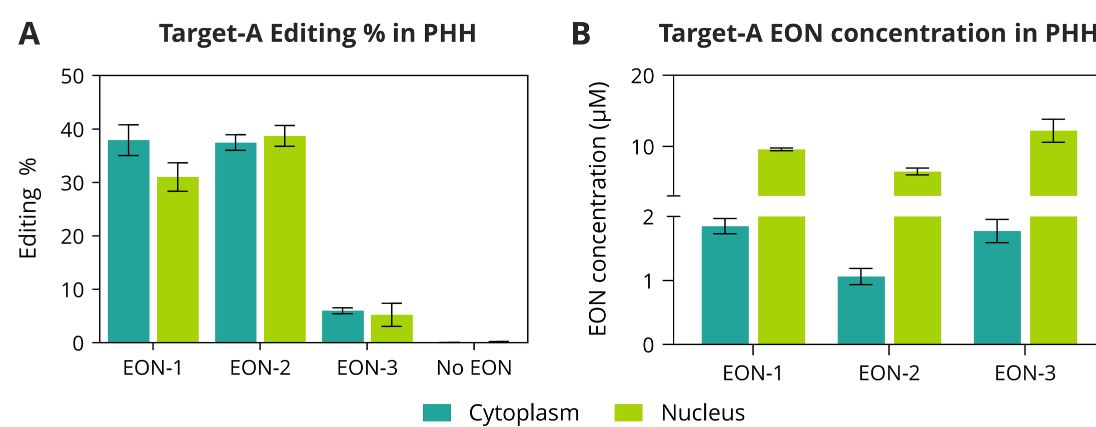


Figure 3. (**A**) Target-A editing was quantified in PHH treated with 5µM EONs of three distinct designs for 72h with different editing efficiencies, followed by estimation of editing % in subcellular fractions using dPCR, N=3. (**B**) Subcellular biodistribution of the same EONs was assessed by fractionation and bioanalysis using similar conditions. N=3, data are shown as mean ± SEM.

CAPA assay shows that cytosol accessible EONs efficiently access the nucleus in HeLa cells

Chloroalkane penetration assay (CAPA) detects compartment-accessible EONs using chloroalkane-tagged EONs and localized HaloTag reporters. OMM-HaloTag and H2B-HaloTag reporters measure cytosolic and nuclear access, respectively; reduced fluorescent dye labeling indicates greater EON accessibility. (**Fig. 4A**) CAPA showed that CA-tagged EONs generated detectable signal with the OMM-anchored HaloTag reporter, indicating that a fraction of internalized EONs is accessible to the cytosol after endosomal escape. (**Fig. 4B**)

Importantly, EONs also generated signal with the H2B-fused nuclear HaloTag reporter. Comparable cytosolic and nuclear CAPA signals suggest that cytosol-accessible EONs efficiently reach the nucleus. (**Fig. 4C**) Together, these results support that nuclear access is not a major barrier once EONs become cytosol-accessible. This supports nuclear access of productive EONs, where ADAR-mediated editing can occur.

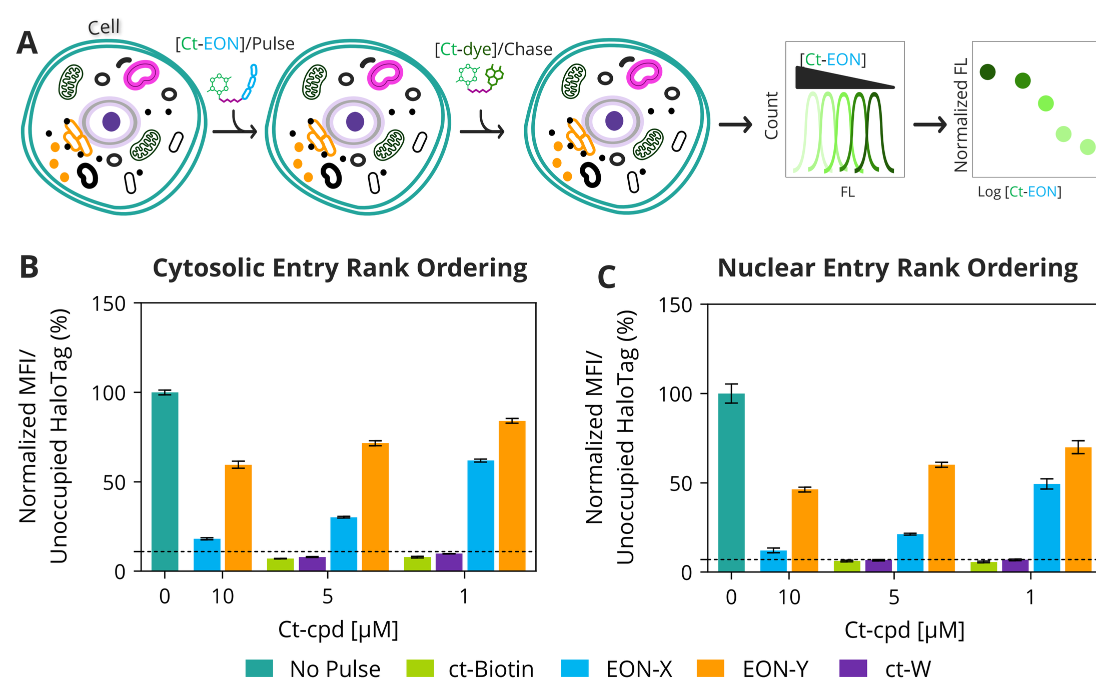


Figure 4. (**A**) Schematic of CAPA for detecting compartment accessible chloroalkane-tagged EONs using cytosolic-facing OMM-HaloTag and nuclear H2B-HaloTag reporters. (**B**) CAPA signal quantification showing chloroalkane-tagged EON detection in cytosolic and nuclear compartments, compared with tryptophan-chloroalkane and biotin-chloroalkane small-molecule controls. 4h pulse followed by 1h chase was performed. N=3, data are shown as mean ± SEM.

Chloroquine does not substantially enhance nuclear EON accumulation or editing in PHH

Chloroquine, a known endosome disruptor[5], was used to test whether additional release of endosomally trapped EONs enhances productive nuclear delivery and Target-A editing in PHH. Primary human hepatocytes were treated with 5 µM EON with or without (50 µM) chloroquine, followed by assessment of nuclear EON accumulation and editing activity. (**Fig. 5A and B**)

Chloroquine caused little to no increase in nuclear EON levels and produced only minimal changes in Target-A editing. A modest increase in editing was observed at 50 µM chloroquine after 24 h, but this condition may induce cytotoxicity. Together, these data suggest that chloroquine provided limited editing enhancement, and that additional mechanisms beyond endosomal release influence EON activity.

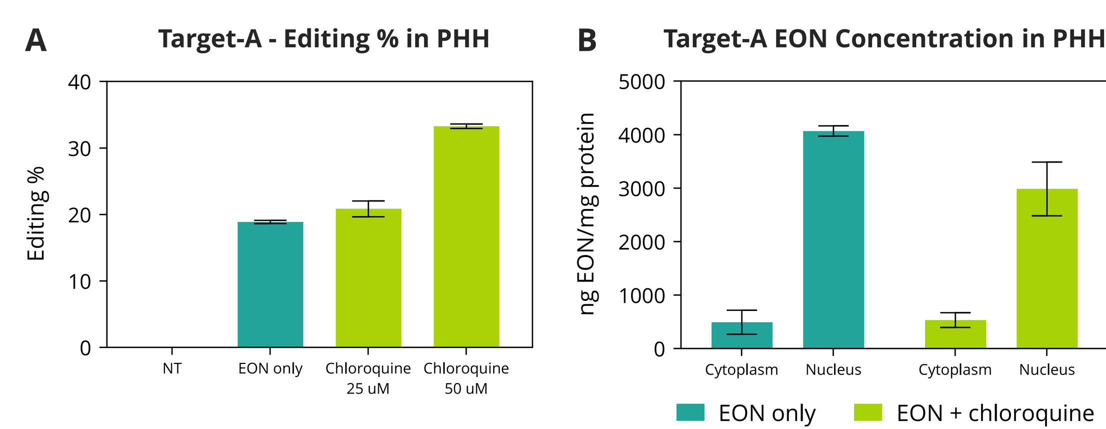
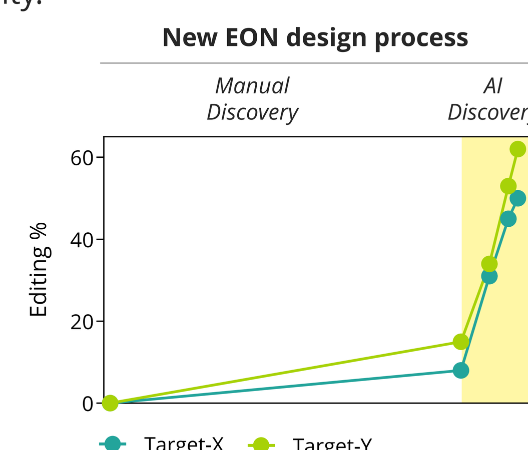


Figure 5. (**A**) Target-A editing was quantified in PHH after 24h treatment with 5 µM EON alone or in combination with chloroquine at the indicated concentrations and mRNA editing was measured by dPCR, N=2. (**B**) Target-A EON levels were quantified by bioanalysis in cytoplasmic and nuclear fractions from PHH treated with 5 µM EON for 24h, with or without a 4h chloroquine pulse treatment, N=3, data are shown as mean ± SEM.

AI-supported EON design accelerates discovery: ~90% faster discovery and up to 6× improvement in EON performance

Since uptake, nuclear access, and endosomal escape did not appear to fully explain differences in EON activity, we used AI-guided design to improve EON performance. Models trained on historical, experimentally validated Axiomer™ editing datasets were used to generate new EON designs with improved predicted activity.

AI-guided discovery reduced design-test timelines compared with manual discovery and identified EONs with substantially improved editing performance across targets. These results support AI-guided EON optimization as an effective strategy to accelerate discovery and enhance editing activity.



Trained on 12+ years of **PROPRIETARY AXIOMER DATA**	AI enables discovery of **BETTER-PERFORMING EONs**	Robotics-enabled HTS **ACCELERATES DESIGN-TEST CYCLES**
Trained on experimentally-validated editing outcomes of numerous EONS and targets	*Models trained on our in-house data generate EONs with higher editing efficiency and greater sequence diversity*	*Enabling rapid iteration per target and amplifying AI-driven learning through continuous model improvement*

Figure 6. Editing performance of EON designs generated during manual discovery and AI-guided discovery phases for representative targets.

Axiomer™ EON designs show minimal off-target editing and limited transcriptomic perturbation

RNA-seq analysis in PHH showed minimal differential gene expression following EON treatment, indicating limited transcriptomic perturbation.

Off-target editing analysis identified only low-frequency, single-digit off-target events, primarily at non-consequential sites. Together, these data support that improved EON activity can be achieved while maintaining transcriptomic specificity and a low off-target profile.

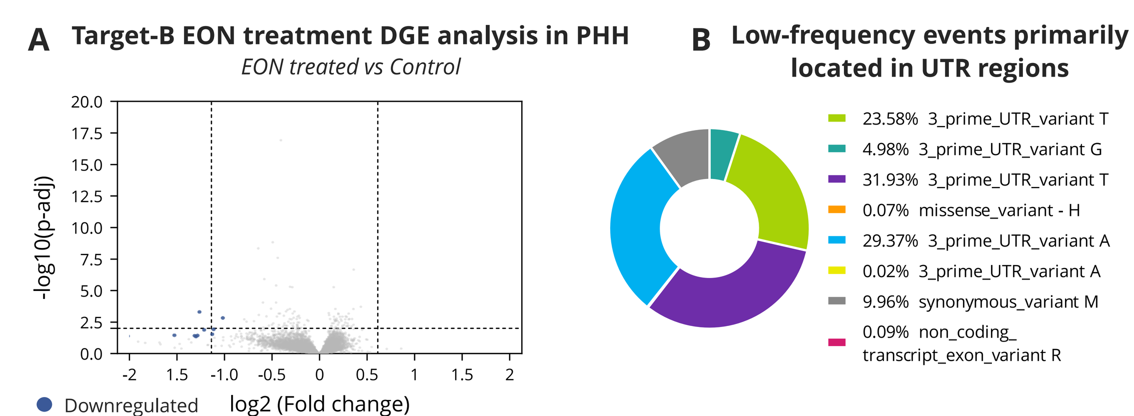


Figure 7. (**A**) Volcano plot showing differential gene expression in PHH following EON treatment compared with control. (**B**) Distribution of detected off-target editing sites, showing a limited number of low-frequency events primarily located in UTR regions.

Conclusions

- **Axiomer™ EONs rapidly enter PHH and enable sustained, co-transcriptional mRNA editing *in vitro***
- **Nuclear access is not the primary determinant of editing efficiency across tested EONs**
- **Chloroquine provided limited editing enhancement, suggesting additional mechanisms beyond endosomal release influence EON activity.**
- **AI-supported EON design accelerates discovery and improves editing performance**
- **Optimized EONs maintain minimal off-target activity and limited transcriptomic perturbation.**

Literature

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2. G Lu et al., *Nucleic Acids Research*, 2024
3. H M Khosravi et al., *RNA Biol*, 2021
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5. C Del Toro Runzer et al., *Mol Ther Nucleic Acids*, 2023