Preclinical Efficacy of ENTR-801 for the Treatment of *USH2A*-Associated Retinitis Pigmentosa Amenable to Exon 13 Skipping

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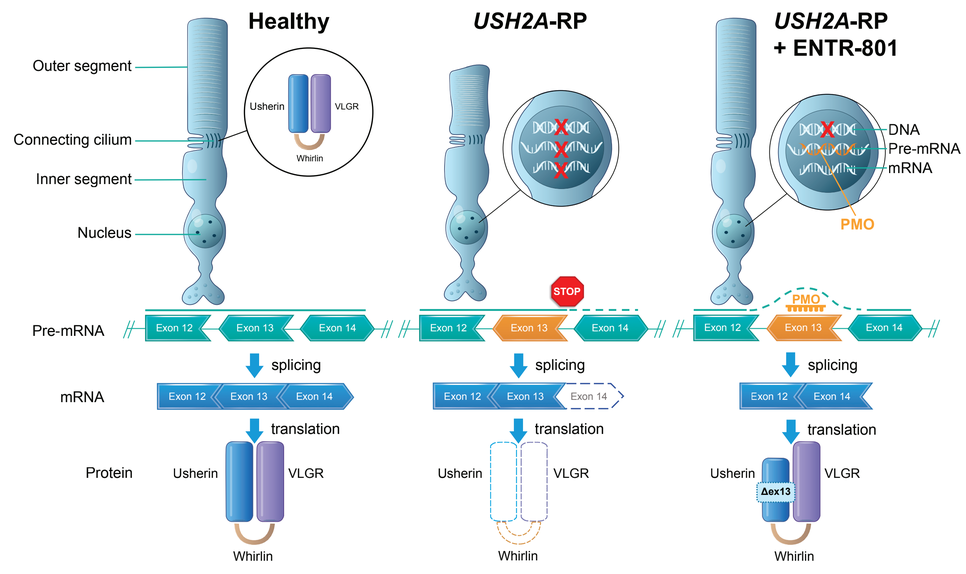
INTRODUCTION

- *USH2A* variants are a leading cause of non-syndromic autosomal recessive retinitis pigmentosa and of Usher syndrome type 2A, in which retinitis pigmentosa occurs with congenital hearing loss.[1]
- Exon 13 is one of the most frequently mutated regions of the USH2A gene.[2]
- The most common mutations in exon 13 introduce a premature stop codon in messenger RNA (mRNA), which in turn triggers nonsense-mediated decay of the transcript, resulting in no usherin protein production (**Figure 1**).
  - Upon exon 13 skipping, a truncated protein is generated, which has been reported to retain functionality.[3]
- Results of preclinical studies have shown that antisense oligonucleotide-mediated *USH2A* exon 13 skipping can produce a shorter but functional usherin protein.[3]
- A neutrally charged phosphorodiamidate morpholino oligomer (PMO) approach was developed to induce exon 13 skipping and address safety considerations associated with negatively charged ASOs.

MATERIALS AND METHODS

- Neutrally charged PMOs, including ENTR-801, were synthesized and evaluated for *USH2A* exon 13 skipping potency and efficacy.
- *USH2A* exon 13 skipping was assessed by digital droplet reverse transcription-polymerase chain reaction in a human retinoblastoma cell line (WERI-Rb-1), human induced pluripotent stem cell–derived retinal organoids (iPSC-ROs), human retinal explants, and partially humanized mice expressing wild-type human *USH2A* (ph*USH2A* mice).
- Usherin protein production in ph*USH2A* mice following a single intravitreal (IVT) injection of ENTR-801 was evaluated via Western immunoblot and quantified via densitometric analysis in ImageJ.
- PMO biodistribution in the retina of ph*USH2A* mice after a single IVT injection of ENTR-801 was evaluated via immunofluorescence with a custom-made anti-PMO antibody.
- Human explants were obtained from VisionGift (Portland, OR) or Vision Share (Chicago, IL). Wild-type iPSCs were licensed from StemCell Technologies (Cambridge, MA) and engineered by EditCo Bio (Redwood City, CA) using CRISPR-Cas9 to introduce the mutation.
- All animal procedures were conducted in accordance with guidelines for the care and use of laboratory animals.

Figure 1. *USH2A*-PMO Construct Mechanism of Action



mRNA, messenger RNA; PMO, phosphorodiamidate morpholino oligomer; RP, retinitis pigmentosa; VLGR, very large G protein-coupled receptor.

OBJECTIVE

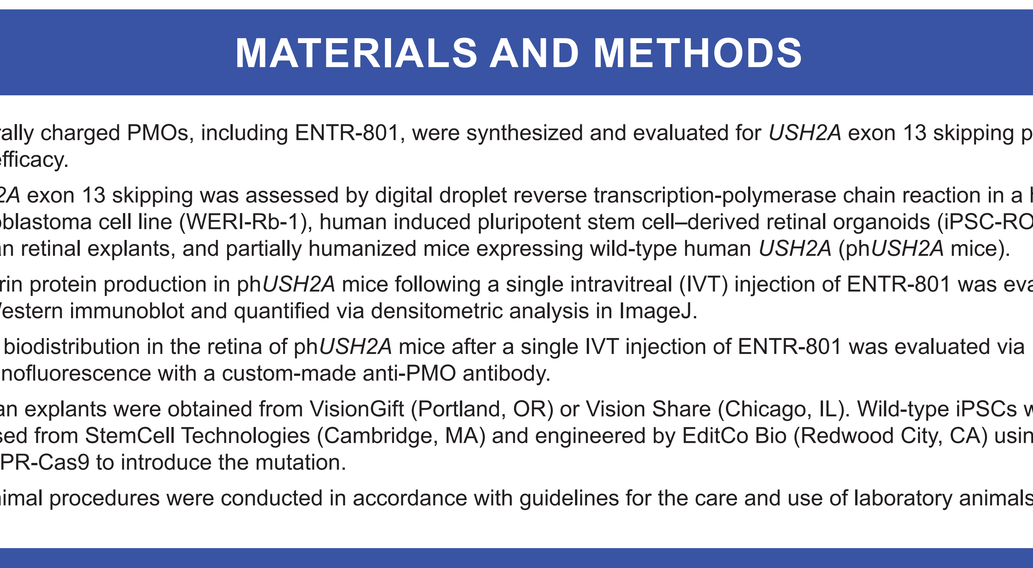
- To evaluate the preclinical efficacy and therapeutic potential of ENTR-801 to induce exon 13 skipping in human *USH2A* mRNA.

RESULTS

Evaluation of PMOs for Exon 13 Skipping Potency in a Human Retinoblastoma Cell Line

- When comparing EC-50 (ie, half-maximal effective concentration), ENTR-801 was among the top performing PMOs tested for *USH2A* exon 13 skipping in WERI-Rb1 cells (**Figure 2**).

Figure 2. Exon 13 Skipping Potency of ENTR-801 and Other PMO Candidates in Human Retinoblastoma Cells

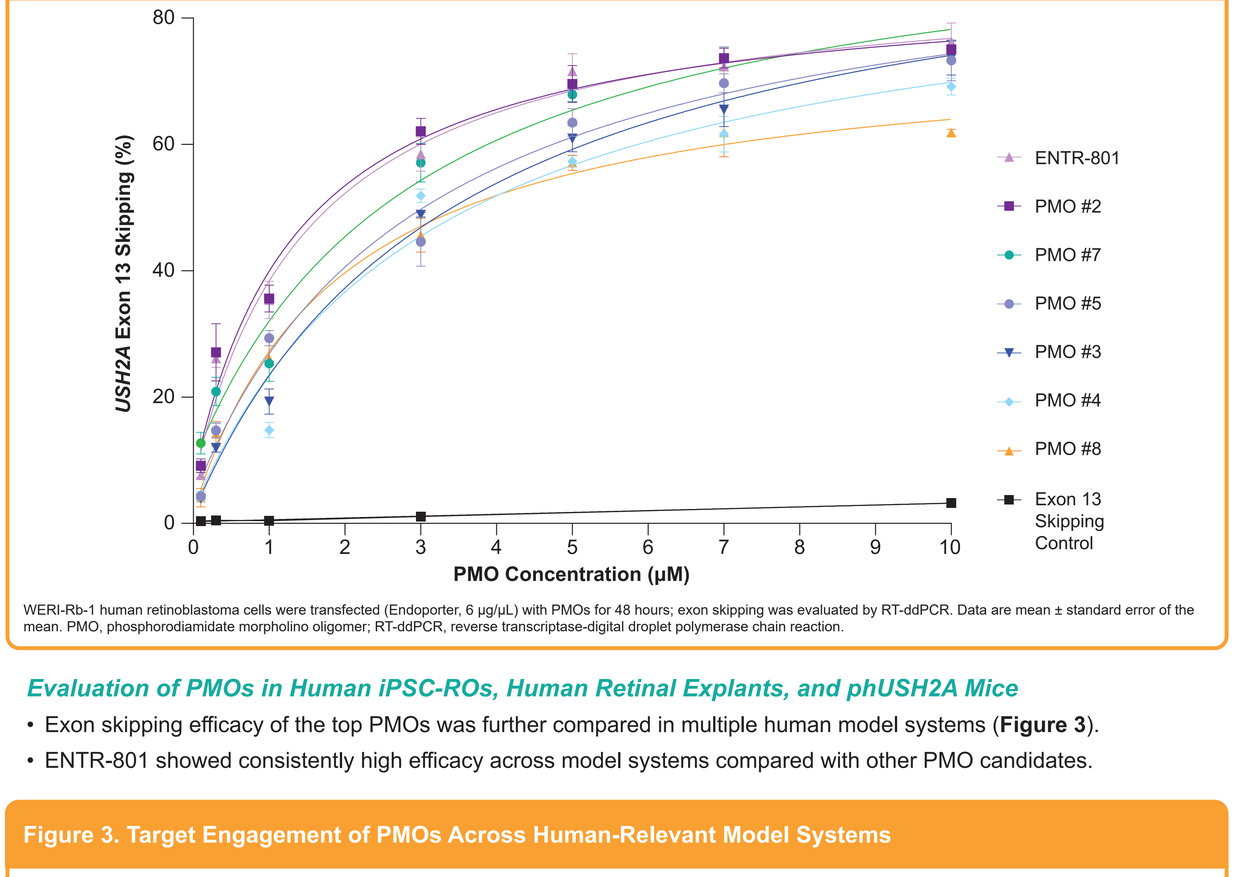


WERI-Rb-1 human retinoblastoma cells were transfected (Endoporter, 6 μg/μL) with PMOs for 48 hours; exon skipping was evaluated by RT-ddPCR. Data are mean ± standard error of the mean. PMO, phosphorodiamidate morpholino oligomer; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction.

Evaluation of PMOs in Human iPSC-ROs, Human Retinal Explants, and phUSH2A Mice

- Exon skipping efficacy of the top PMOs was further compared in multiple human model systems (**Figure 3**).
- ENTR-801 showed consistently high efficacy across model systems compared with other PMO candidates.

Figure 3. Target Engagement of PMOs Across Human-Relevant Model Systems



Exon skipping was evaluated with RT-ddPCR on (**left**) day 7 after treatment of wild-type human iPSC-ROs (n=3) with low- and high-dose PMO, with no statistically significant difference between PMOs at the same doses; (**middle**) on day 3 after treatment of human retinal explants (n=3) with med-dose PMO, (**right**) and on day 7 after a single bilateral IVT injection at a low dose/eye in ph*USH2A* mice (n=10). Data are mean ± standard error of the mean. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. iPSC-RO, induced pluripotent stem cell-derived retinal organoid; IVT, intravitreal; Med, medium; ns, not significant; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; PMO, phosphorodiamidate morpholino oligomer; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction; WT, wild-type.

Dose Dependency of ENTR-801 Efficacy in Human-Relevant Model Systems

- ENTR-801 demonstrated dose-dependent efficacy across both human retinal explants and humanized mice (**Figure 4**).

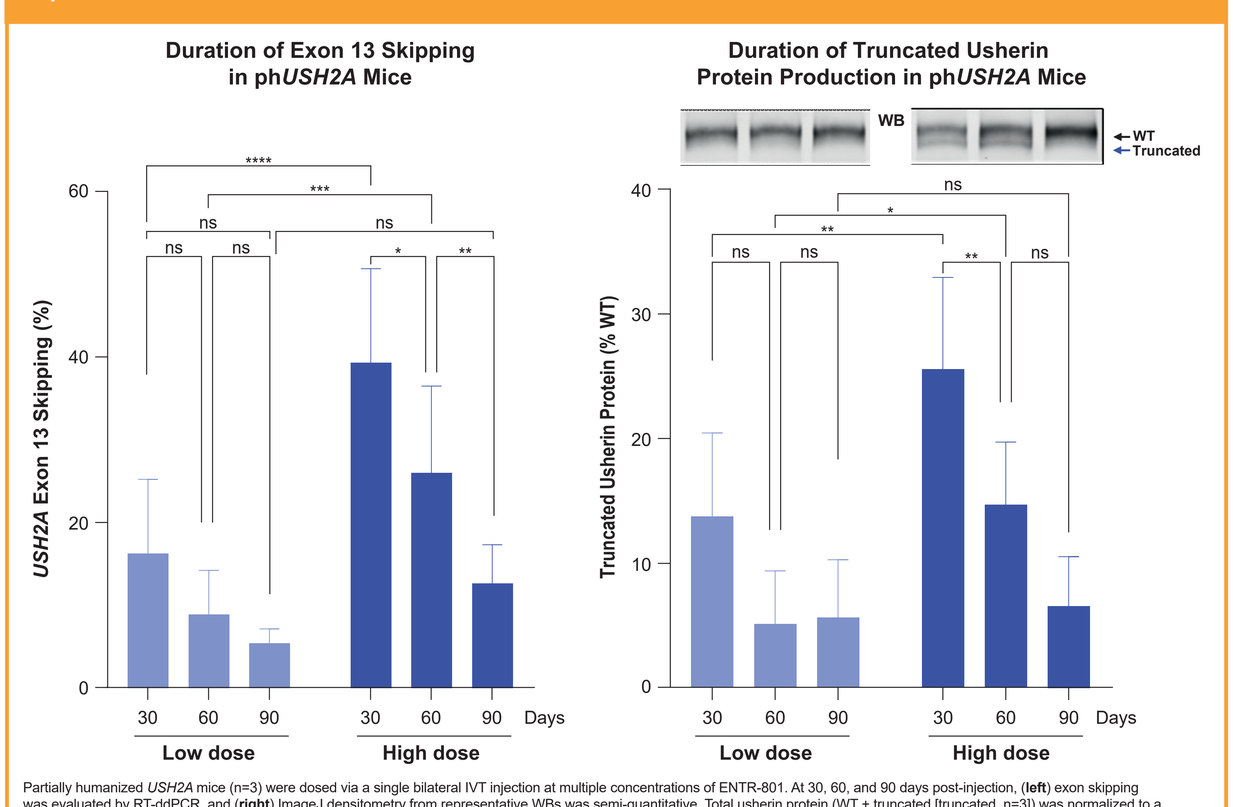
Figure 4. Efficacy of ENTR-801 in Human Retinal Explants and in ph*USH2A* Mice

Exon skipping was evaluated with RT-ddPCR on (**left**) day 3 after treatment of human retinal explants (n=3) with low, med or high doses of ENTR-801, (**middle**) and on day 30 after a single bilateral IVT injection of ENTR-801 at low to high doses per eye in ph*USH2A* mice (n=8). (**right**) Truncated usherin protein from ph*USH2A* mice (n=2) was assessed at day 30 via WB and quantified via densitometric analysis in ImageJ. One-way ANOVA was used for statistical comparison. Data are mean ± standard error of the mean. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ANOVA, analysis of variance; IVT, intravitreal; Med, medium; ns, not significant; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction; WB, Western immunoblot; WT, wild-type.

Duration of Effect of a Single Dose of ENTR-801 in phUSH2A Mice

- Treatment of partially humanized mice with ENTR-801 resulted in durable exon skipping and truncated usherin protein production (**Figure 5**).

Figure 5. Duration of Target Engagement and Truncated Usherin Protein Production with ENTR-801 in ph*USH2A* Mice

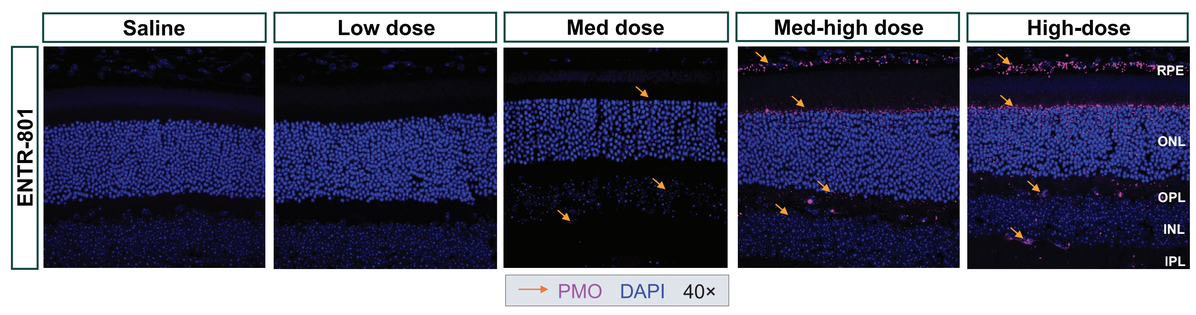


Partially humanized *USH2A* mice (n=3) were dosed via a single bilateral IVT injection at multiple concentrations of ENTR-801. At 30, 60, and 90 days post-injection, (**left**) exon skipping was evaluated by RT-ddPCR, and (**right**) ImageJ densitometry from representative WBs was semi-quantitative. Total usherin protein (WT + truncated [truncated, n=3]) was normalized to a housekeeping protein, and the percentage of truncated protein was calculated as the intensity of the truncated band divided by the total protein intensity. Data are mean ± standard error of the mean. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. IVT, intravitreal; ns, not significant; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction; WB, Western immunoblot; WT, wild-type.

Retinal Localization 30 Days After Intravitreal Injection of ENTR-801 in phUSH2A Mice

- Dose-dependent increases in ENTR-801 were detected in mouse retinal target cells 30 days after IVT injection (**Figure 6**).

Figure 6. Dose-Dependent Biodistribution of ENTR-801 in ph*USH2A* Mouse Retina



Partially humanized *USH2A* mice were dosed via a single bilateral IVT injection at multiple concentrations. On day 30 post-injection, eyes were fixed overnight with Hartman's fixative, embedded in OCT, and cryosectioned. PMO biodistribution was assessed via immunostaining using custom-made anti-PMO antibody. Immunostained sections were imaged at 40× (LSM-880 Zeiss confocal). DAPI, 4′,6-diamidino-2-phenylindole; INL, inner nuclear layer; IPL, inner plexiform layer; IVT, intravitreal; Med, medium; OCT, optimal cutting temperature compound; ONL, outer nuclear layer; OPL, outer plexiform layer; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; PMO, phosphorodiamidate morpholino oligomer; RPE, retinal pigment epithelium.

CONCLUSIONS

- ENTR-801 demonstrated substantial exon 13 skipping across human iPSC-ROs, retinal explants, and ph*USH2A* mice, confirming consistent target engagement in vitro, ex vivo, and in vivo.
- At a medium dose per eye of ENTR-801, exon skipping and truncated usherin protein were detectable through 90 days after a single IVT injection.
- ENTR-801 exhibited dose-dependent retinal biodistribution across all layers after a single IVT injection.
- These results support further development of ENTR-801 for *USH2A*-associated retinitis pigmentosa.

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1. McGee TL, et al. *J Med Genet*. 2010. 2. Su B-N, et al. *Front Aging Neurosci*. 2022. 3. Dulla K, et al. *Mol Ther*. 2021.