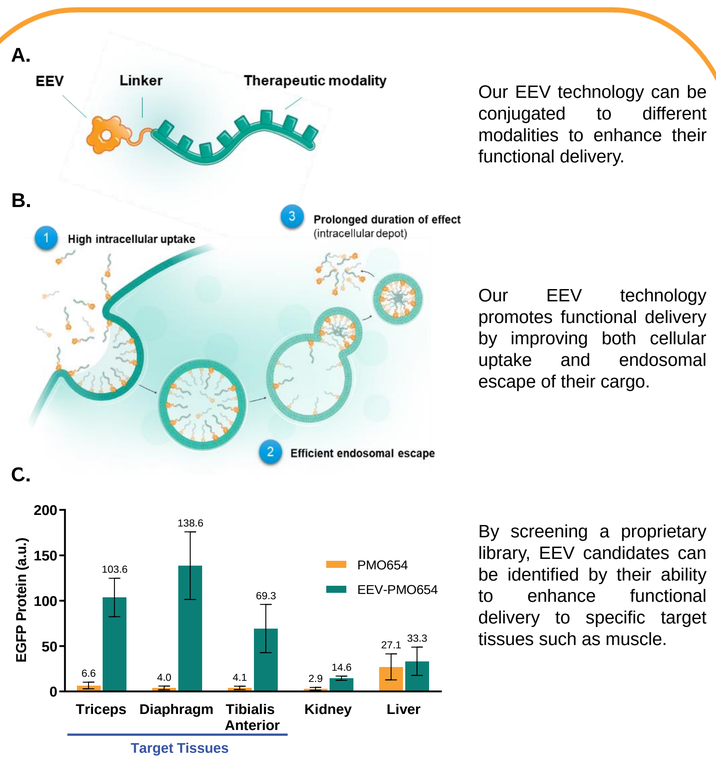
Development of Endosomal Escape Vehicles to Enhance the Intracellular Delivery of Oligonucleotides

Xiang Li, Patrick G. Dougherty, Mahboubeh Kheirabadi, Ajay Kumar, Amy Hicks, Mark Wysk, Kimberli J. Kamer, Nelsa L. Estrella, Xiulong Shen, Wenlong Lian, Nanjun Liu, Matthew Streeter, Suresh Peddigari, Chance Brandt, Roshni Mukundan, Vyoma Patel, Phallika Mon, Arianna Bonilla, Andy Stadheim, Mohanraj Dhanabal, Mahasweta Girgenrath, Natarajan Sethuraman, Leo Ziqing Qian

Entrada Therapeutics, Inc, Boston, MA

INTRODUCTION

- Intracellular delivery of oligonucleotides is challenging due to poor cell entry and limited escape from the endosome in the target cell
- To improve cellular uptake and enhance endosomal escape of oligonucleotides and other biologics, we developed we developed our Endosomal Escape Vehicle (EEV) delivery platform based on cyclic cell penetrating peptides (cCPPs).[1-2] (**Figure 1**)
- To determine the therapeutic applicability of our EEV-PMO (phosphorodiamidate morpholino oligomer) platform, we utilized two pre-clinical models of neuromuscular disorders
  - Duchenne muscular dystrophy (DMD) is an X-linked, progressive disease caused by mutations in the *DMD* gene, resulting in the production of nonfunctional dystrophin protein[3-4]. Currently approved unconjugated PMO therapies were designed to restore the reading frame and produce dystrophin by exon skipping but have shown modest improvements[5-6].
  - Myotonic dystrophy type 1 (DM1) is an autosomal dominant, multisystemic disease caused by a mutation in the *DMPK* (DM1 protein kinase) gene causing trinucleotide CUG repeats in DMPK mRNA.[7] The mutant *DMPK* mRNA forms hairpin loops that sequester MBNL (Muscleblind like splicing regulator) proteins[8], which prevent MBNL from performing its downstream splicing functions[9-10]. There are currently no approved therapies for DM1.



Our EEV technology can be conjugated to different modalities to enhance their functional delivery.

Our EEV technology promotes functional delivery by improving both cellular uptake and endosomal escape of their cargo.

By screening a proprietary library, EEV candidates can be identified by their ability to enhance functional delivery to specific target tissues such as muscle.

Figure 1. A) Structure of EEV-conjugated therapeutic consisting of cyclic cell-penetrating peptide (cCPP)-based EEV, linker, and therapeutic modality. **B**) Mechanism of EEV-mediated delivery of therapeutics. **C**) Functional delivery in EGFP-transgenic mice. EGFP-654 mice contain an EGFP gene interrupted by human beta-globin intron 2 with mutated nt654[16]; Data shown as mean ± standard deviation.

OBJECTIVES

To evaluate the effectiveness of disease-specific EEV-PMO conjugates to correct splicing deficits in preclinical models of Duchenne muscular dystrophy (DMD) and myotonic dystrophy type 1 (DM1).

METHODS

DMD Studies

- EEV-PMO-23, a *DMD* exon 23 skipping PMO conjugated to our EEV platform, was administered intravenously (IV) to assess exon skipping and dystrophin production in D2-*mdx*[11] mice. These mice contain a nonsense mutation in exon 23.
- ENTR-601-44 is a *DMD* exon 44 skipping PMO conjugated to our EEV platform and was administered IV and evaluated for exon 44 skipping efficacy in the hDMD[12] mouse model and non-human primates (NHPs, Macaca fascicularis).
- Exon skipping efficiency was analyzed by RT-PCR. Dystrophin restoration was evaluated by western blotting and immunofluorescence. Serum creatine kinase (CK) activity was measured to assess muscle membrane integrity.

DM1 Studies

- ENTR-701 is a CUG-repeat blocking PMO conjugated to our EEV platform, was evaluated for number of nuclear foci, CUG-repeat expansion transcript level and correction of aberrant splicing in HSA-LR[13] mouse model. The HSA-LR mice carry a transgene resulting in CUG repeat expansion and recapitulates DM1 phenotype and molecular pathology.

ACKNOWLEDGEMENTS

Corresponding author: Leo Ziqing Qian (qian@entradatx.com). This research was funded by Entrada Therapeutics, Inc. (Boston, MA). The authors would like to thank Aji Nair, Fatima Kazmi, Emmanuelle Sizensky, Sara Blake, Ge Zhang, and Cecilia Sun of Entrada Therapeutics and Eric T. Wang's lab at University of Florida. References: 1. Qian Z. *Biochemistry* 2016. 2. Sahni A. *ACS Chem Biol* 2020. 3. Hoffman EP. *Cell* 1987. 4. Birnkrant DJ. *Lancet Neurol* 2018. 5. EXONDYS 51® Prescribing Information. 6. VILTEPSO® Prescribing Information. 7. Philips AV. *Science* 1998. 8. Pettersson OJ. *NAR* 2015. 9. Wagner SD. *PLoS Genet* 2016. 10. Tanner M. *Nucleic Acids Res* 2021. 11. Sazani P. *Nature Biotech* 2002. 12. Bulfield G. *Proc Natl Acad Sci* 1984. 13. 't Hoen P. *J Biol Chem* 2008.

RESULTS

DMD Preclinical Results

Exon Skipping and Dystrophin Restoration in D2-*mdx* mice

- Administration of EEV-PMO-23 produced robust exon 23 skipping in cardiac and skeletal muscles of D2-*mdx* mice after four monthly doses (**Figure 2**)

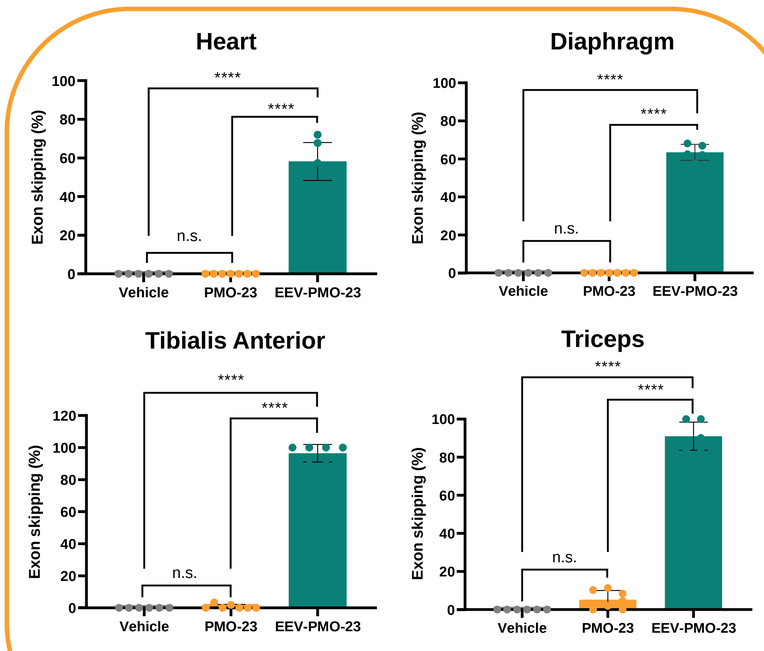


Figure 2. Exon Skipping with EEV-PMO-23 in D2-*mdx* mice. D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO or 20 mg/kg PMO equivalent of EEV-PMO, and the data were collected ~4 weeks after the last dose. Data shown as shown as mean ± standard deviation. ****p<0.0001; n.s., not significant.

- Broad dystrophin expression and restoration of skeletal and cardiac muscle integrity were observed with EEV-PMO-23 compared to PMO-23 alone (**Figure 3**)

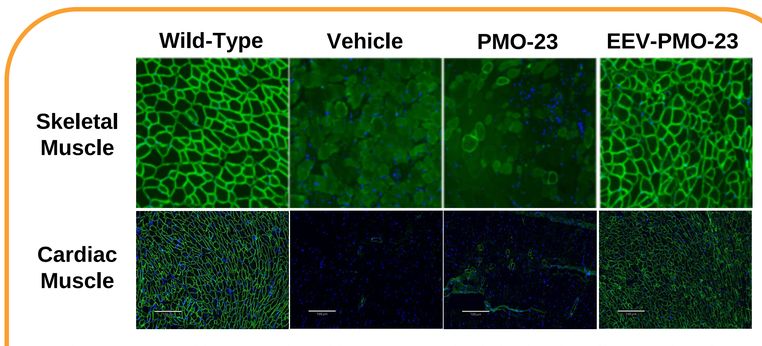


Figure 3. Dystrophin Restoration with EEV-PMO-23 in skeletal and cardiac muscles of *D2-mdx* mice. D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO or 20 mg/kg PMO equivalent of EEV-PMO, and the data were collected ~4 weeks after the last dose. Dystrophin in green and DAPI in blue.

CK Correction and Functional Improvement in D2-*mdx* Mice

- EEV-PMO-23 administration normalized serum CK levels and showed significant improvements in muscle function when compared to PMO alone after four monthly IV doses in D2-*mdx* mice (**Figure 4**)

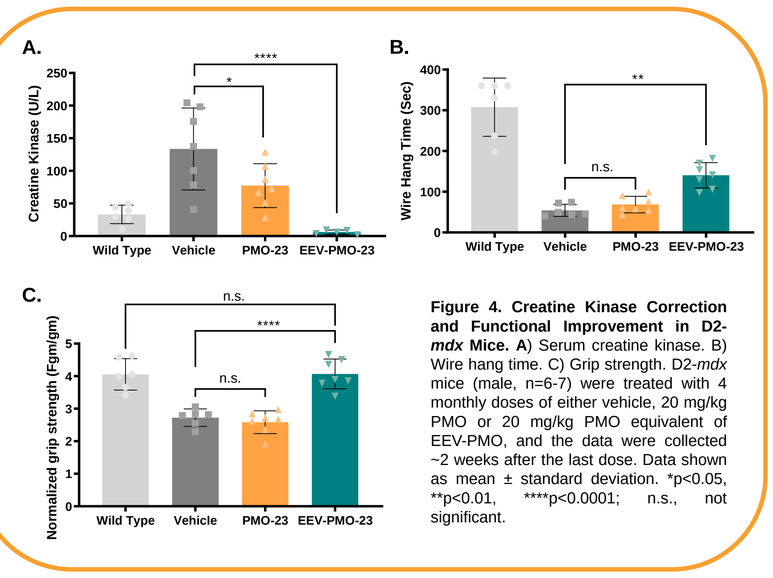


Figure 4. Creatine Kinase Correction and Functional Improvement in D2-*mdx* Mice. A) Serum creatine kinase. B) Wire hang time. C) Grip strength. D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO or 20 mg/kg PMO equivalent of EEV-PMO, and the data were collected ~2 weeks after the last dose. Data shown as mean ± standard deviation. *p<0.05, **p<0.01, ****p<0.0001; n.s., not significant.

Durable Exon Skipping with ENTR-601-44 DMD Clinical Candidate

- A single dose of ENTR-601-44 resulted in robust exon skipping in both skeletal and cardiac muscles in NHP (**Figure 5A**), as well as prolonged duration of effect for at least 12 weeks (**Figure 5B**)

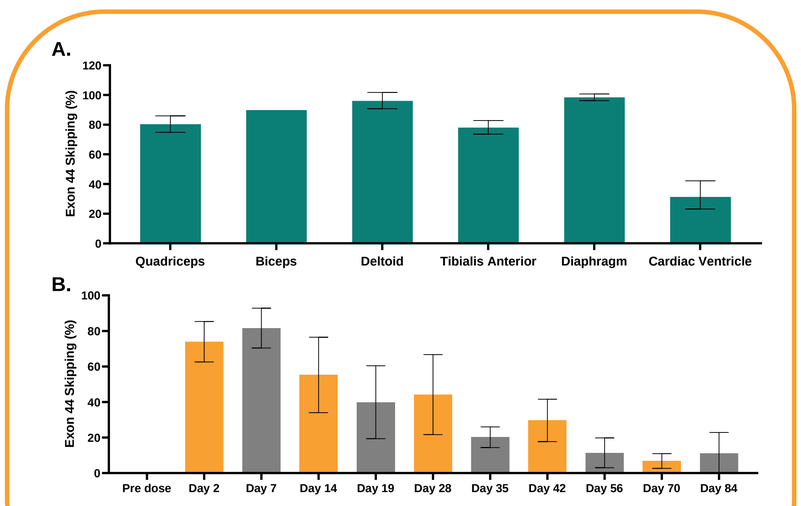


Figure 5. Durable Exon Skipping with ENTR-601-44 in NHP. A) Exon skipping in NHP skeletal and cardiac muscle 7 days post IV infusion of 30 mg/kg PMO equivalent. **B**) Duration of effect in biceps of NHP for up to 12 weeks post IV infusion of 35 mg/kg PMO equivalent. Data shown as mean ± standard deviation.

DM1 Preclinical Results

Efficacy of ENTR-701 DM1 Clinical Candidate in HSA-LR Mice

- ENTR-701 treatment reduced the number of nuclear foci (**Figure 6A, 6B**), corrected aberrant splicing of *Atp2a1* (**Figure 6C**) and reduced HSA-r(CUG)220 mRNA in HSA-LR mice (**Figure 6D**)

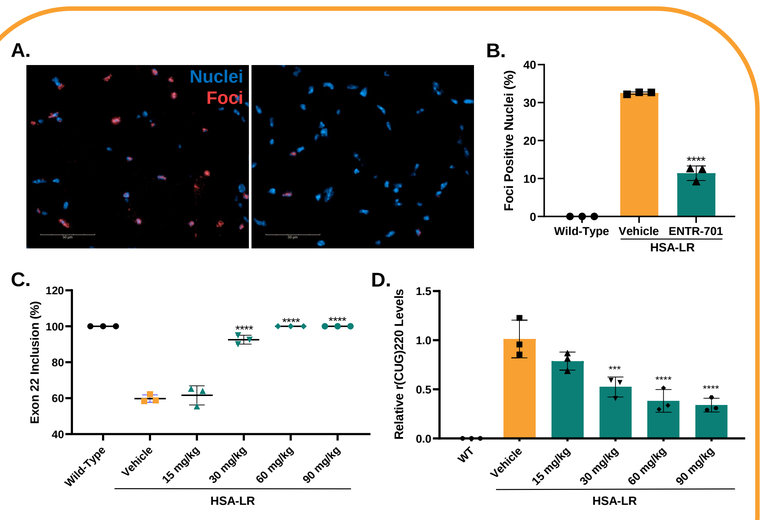


Figure 6. Efficacy of ENTR-701 in HSA-LR Mice. Reduction of nuclear foci in tibialis anterior by immunofluorescence (**A**, **B**). **C**) Correction of *Atp2a1* (sarcoplasmic/endoplasmic reticulum calcium ATPase) splicing. D) Reduction of HSA-r(CUG)220 with repeat expansion. Data shown as mean ± standard deviation. **p<0.01, ***p<0.001, ****p<0.0001 relative to HSA-LR vehicle.

Durable Efficacy of ENTR-701 in HSA-LR Mice

- A single dose of ENTR-701 corrected splicing deficits for *Mbnl1* (**Figure 7A**), *Atp2a1* (**Figure 7B**), and *Nfix* (**Figure 7C**) for at least 8 weeks post-dose. Pinch-induced myotonia was also ameliorated for at least 8 weeks post-dose.

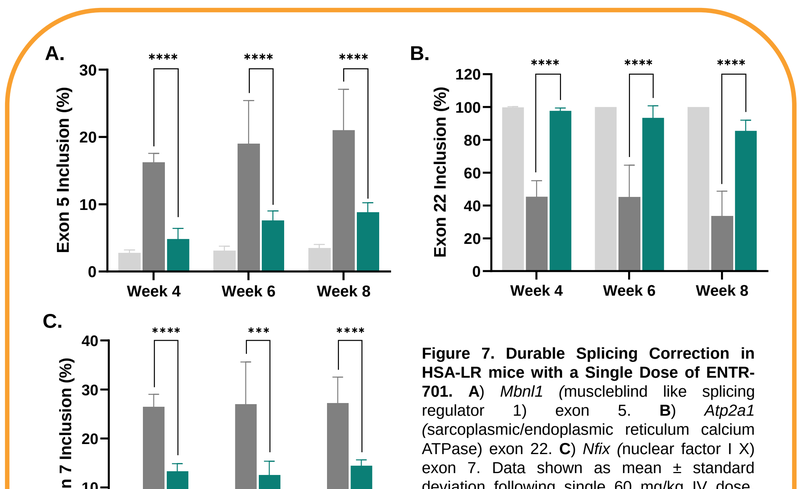


Figure 7. Durable Splicing Correction in HSA-LR mice with a Single Dose of ENTR-701. A) *Mbnl1 (*muscleblind like splicing regulator 1) exon 5. **B**) *Atp2a1 (*sarcoplasmic/endoplasmic reticulum calcium ATPase) exon 22. **C**) *Nfix (*nuclear factor I X) exon 7. Data shown as mean ± standard deviation following single 60 mg/kg IV dose. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

CONCLUSIONS

- ENTR-601-44, our clinical candidate for DMD exon 44 amenable patients, produces durable and robust exon skipping in both cardiac and skeletal muscle of mice and NHP
- ENTR-701, our clinical candidate for DM1, corrects splicing deficits and improves myotonia in mice. A durable effect was observed 8 weeks following a single dose.
- These findings validate our proprietary EEV-PMO approach in two models of neuromuscular disorders and support further study in clinical trials