Endosomal Escape Vehicle–Oligonucleotide Conjugates for the Targeted Upregulation and Downregulation of Gene Expression

Poster # P081

Ziqing Leo Qian, Xiang Li, Xiulong Shen, Mahboubeh Kheirabadi, Amy N. Hicks, Mark Wysk, Haoming Liu, Ajay Kumar, Nelsa L. Estrella, Patrick G. Dougherty, Suresh Peddigari, Anushree Pathak, Kimberli J. Kamer, Wenlong Lian, Nanjun Liu, Matthew Streeter, Sara L. Blake, Christopher M. Brennan, Ning Li, Vlad Batagui, Keyede Oye, Ningguo Gao, Daniel Wang, Arianna Bonilla, Mohanraj Dhanabal, Mahasweta Girgenrath, and Natarajan Sethuraman

Entrada Therapeutics, Boston, MA

INTRODUCTION

- Intracellular delivery of oligonucleotides is challenging because of poor cell entry and limited escape from the endosome in the target cell.[1,2]
- To improve cellular uptake and enhance endosomal escape of oligonucleotides and other biologics, we developed our endosomal escape vehicle (EEV)™ delivery platform based on cyclic cell-penetrating peptides (**Figure 1**).
- Here, we assessed the broad applicability of our EEV platform to deliver splice-modulating oligonucleotides (phosphorodiamidate morpholino oligomers [PMOs]) to different target cell types.

OBJECTIVES

- To evaluate the ability of our EEV platform to deliver PMOs in preclinical models of Duchenne muscular dystrophy (DMD), myotonic dystrophy type 1 (DM1), and murine macrophages.
- To evaluate functional ability of EEV-PMO conjugates to upregulate or downregulate target genes.

MATERIALS AND METHODS

EEV Library Screening and Optimization

- EEVs with robust target cell uptake and efficacy were screened in vitro, and functional validation was assessed. Well-tolerated EEVs with desired tissue functional delivery were then assessed in wild-type and disease animal models. Finally, an EEV candidate with desired therapeutic effect was selected.

DMD Studies

- DMD is 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]
- EEV-PMO-23, a DMD exon 23 skipping PMO conjugated to our EEV platform, was administered intravenously to assess exon skipping and dystrophin production in D2-*mdx*[7] mice (**Figure 2A**). These mice contain a nonsense mutation in exon 23. Additional information will be presented in Poster #P157.

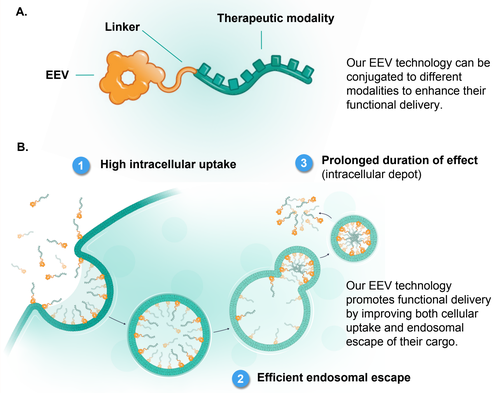
DM1 Studies

- DM1 is a multisystemic disease caused by a mutation in the *DMPK* (DM1 protein kinase) gene causing trinucleotide CUG repeats in DMPK mRNA[8] (**Figure 3A**). The mutant DMPK mRNA forms hairpin loops that sequester MBNL (Muscleblind-like splicing regulator) proteins,[9] which prevent MBNL from performing its downstream splicing functions.[10,11]
- ENTR-701, 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 an HSA-LR[11,12] mouse model. HSA-LR mice carry a transgene resulting in CUG-repeat expansion and recapitulates DM1 phenotype and molecular pathology. Additional information will be presented in Poster #P159.

IRF5 Studies

- Interferon regulatory factor 5 (IRF5) is a transcription factor that promotes production of several proinflammatory cytokines in macrophages, and overexpression of IRF5 has been implicated in several autoimmune and inflammatory diseases.[13-16]
- IRF5 experiments used several EEVs conjugated to an exon skipping PMO.[17] The PMO in this conjugate facilitates exon 4 skipping of IRF5 mRNA that produces a premature stop codon, thereby reducing translation of IRF5 mRNA (**Figure 4A**).[17]

Figure 1. EEV-PMO Structure and Mechanism of Action.



(**A**) Structure of EEV-conjugated therapeutic consisting of cCPP-based EEV, linker, and therapeutic modality. (**B**) Mechanism of EEV-mediated delivery of therapeutics. EEV, endosomal escape vehicle; cCPP, cyclic cell-penetrating peptide.

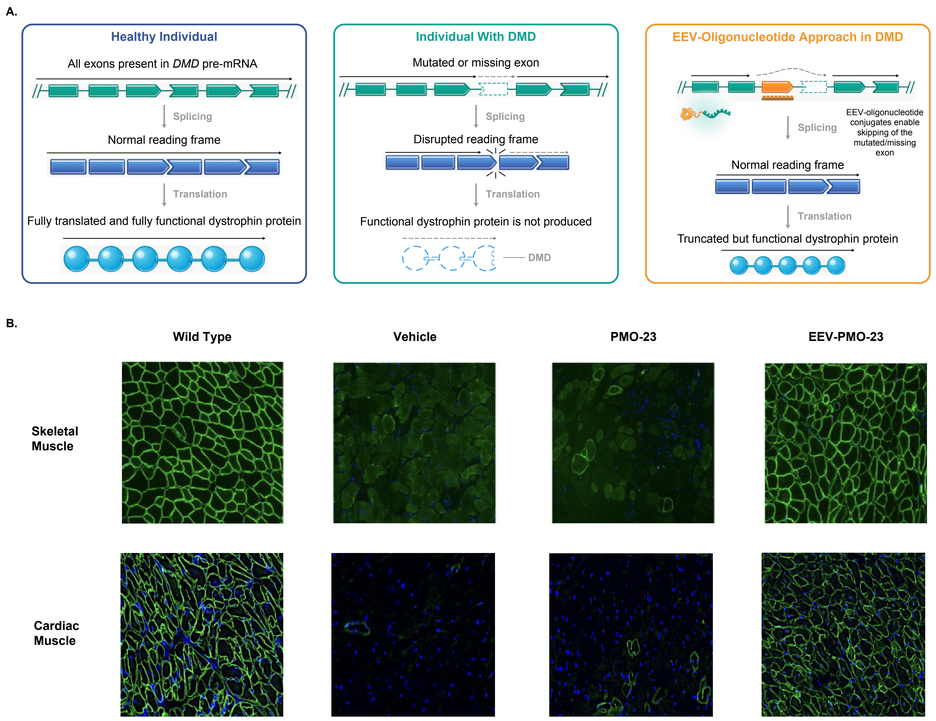
RESULTS

Upregulation of Target Genes

Exon Skipping and Dystrophin Restoration in D2-*mdx* Model of DMD

Administration of EEV-PMO-23 produced robust exon 23 skipping in cardiac and skeletal muscles of D2-*mdx* mice after 4 monthly doses. Broad dystrophin expression and restoration of skeletal and cardiac muscle integrity were observed with EEV-PMO-23 compared with PMO-23 alone (**Figure 2B**).

Figure 2. Exon Skipping and Dystrophin Restoration With EEV-PMO-23 in D2-*mdx* Mice.



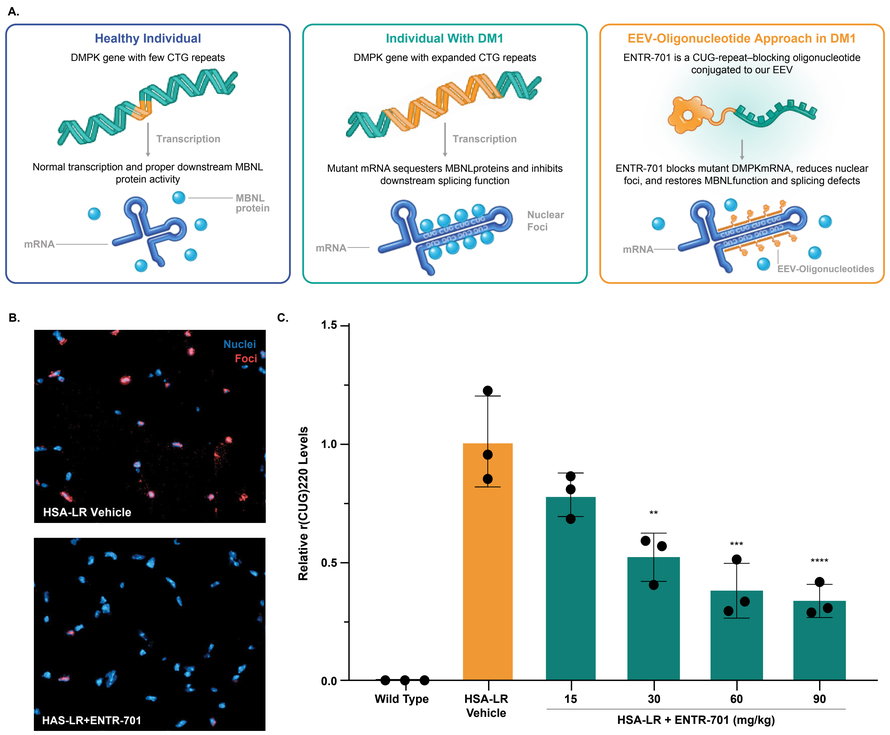
(**A**) Mechanism of EEV-PMO conjugates in DMD models. (**B**) D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO or 20 mg/kg PMO equivalent of EEV-PMO, and the data were collected ~4 weeks after the last dose. Dystrophin in green and DAPI in blue. DAPI, 4′,6-diamidino-2-phenylindole; DMD, Duchenne muscular dystrophy; EEV, endosomal escape vehicle; PMO, phosphorodiamidate morpholino oligomer.

Downregulation of Target Genes

Durable Efficacy of ENTR-701 in HSA-LR Model of DM1

ENTR-701 treatment reduced the number of nuclear foci (**Figure 3B**) and HSA-r(CUG)220 mutant mRNA (**Figure 3C**) in HSA-LR mice. Furthermore, a single dose of ENTR-701 corrected splicing deficits for up to 8 weeks.

Figure 3. Efficacy of ENTR-701 in HSA-LR Mice.

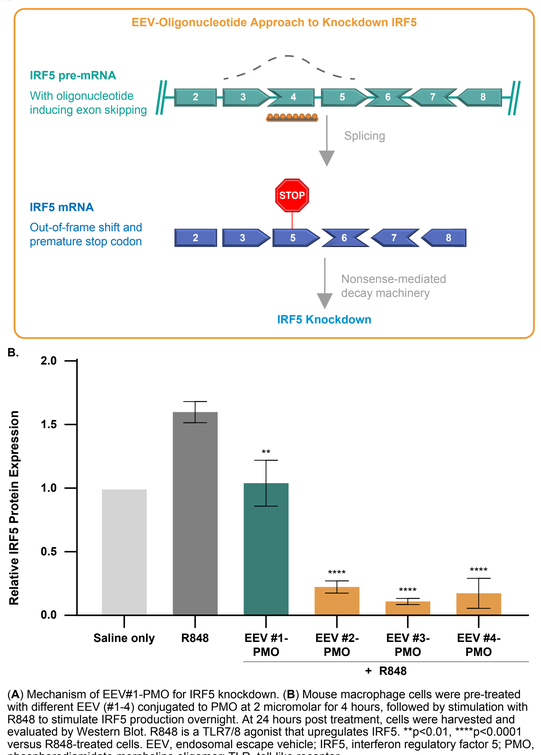


(**A**) Mechanism of EEV-PMO conjugates in DM1 models. (**B**) Reduction of nuclear foci in tibialis anterior by immunofluorescence. (**C**) Reduction of HSA-r(CUG)220 with repeat expansion. **p<0.01, ***p<0.001, ****p<0.0001 relative to HSA-LR vehicle. DM1, myotonic dystrophy type 1; *DMPK*, DM1 protein kinase gene; EEV, endosomal escape vehicle; MBNL, Muscleblind-like splicing regulator; PMO, phosphorodiamidate morpholino oligomer.

EEV-PMO Treatment Reduced IRF5 Expression In Vitro and In Vivo

Four different EEV-PMO conjugates reduced IRF5 expression in mouse macrophages stimulated to produce IRF5 (**Figure 4B**). EEV#1-PMO also induced downregulation of IRF5 in liver, small intestine, and skeletal muscle of wild-type mice.

Figure 4. EEV-PMO Knockdown of IRF Expression.



(**A**) Mechanism of EEV#1-PMO for IRF5 knockdown. (**B**) Mouse macrophage cells were pre-treated with different EEV (#1-4) conjugated to PMO at 2 micromolar for 4 hours, followed by stimulation with R848 to stimulate IRF5 production overnight. At 24 hours post treatment, cells were harvested and evaluated by Western Blot. R848 is a TLR7/8 agonist that upregulates IRF5. **p<0.01, ****p<0.0001 versus R848-treated cells. EEV, endosomal escape vehicle; IRF5, interferon regulatory factor 5; PMO, phosphorodiamidate morpholino oligomer; TLR, toll-like receptor.

ACKNOWLEDGMENTS

This research was funded by Entrada Therapeutics, Inc. (Boston, MA). Editorial and graphics support for the development of this poster was provided by Ashfield MedComms, an Inizio Company, and funded by Entrada Therapeutics, Inc. 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. Sazani P. *Nature Biotech.* 2002. 8. Philips AV. *Science.* 1998. 9. Pettersson OJ. *NAR.* 2015. 10. Wagner SD. *PLoS Genet.* 2016. 11. Tanner M. *Nucleic Acids Res.* 2021. 12. 't Hoen P. *J Biol Chem.* 2008. 13. Takaoka A. *Nature*. 2005. 14. Graham RR. *Nat Genet*. 2006. 15. Krausruber T. *Nat Immunol.* 2011. 16. Yang Y. *Inflamm Bowel Dis.* 2021. 17. Data on File, Entrada Therapeutics, Inc.

CONCLUSIONS

- Our EEV™ platform consists of a library of proprietary cyclic peptides with unique chemistry that enable improved cellular uptake, endosomal escape, and consistent translation across species.
- The results presented here demonstrate that our EEV platform successfully and efficiently delivers oligonucleotides to specific cell and tissue types.
- This approach has broad applicability to upregulate or downregulate target genes through distinct mechanisms of action.
- In future clinical trials, the therapeutic potential of our EEV-PMO approach will be examined in patients with DMD (ENTR-601-44 program) and DM1 (ENTR-701 program).