Optimization and Application of the Endosomal Escape Vehicle (EEV™) Platform for Enhanced Delivery of Oligonucleotides to Skeletal and Cardiac Muscle

Xiang Li, Patrick G. Dougherty, Mahboubeh Kheirabadi, Nelsa L. Estrella, Ajay Kumar, Amy N. Hicks, Ashweta Sahni, Mark Wysk, Kimberli J. Kamer, Wenlong Lian, Nanjun Liu, Ning Li, Matthew Streeter, Kyle Totaro, Ming Zhou, Christopher M. Brennan, Anushree Pathak, Catherine Kugler, Alison Burman, Sara L. Blake, Mohanraj Dhanabal, Andy Stadheim, Mahasweta Girgenrath, Ziqing Leo Qian

Entrada Therapeutics, Boston, MA

INTRODUCTION

- Currently approved phosphorodiamidate morpholino oligomer (PMO) therapies for Duchenne muscular dystrophy (DMD) were designed to restore the messenger RNA (mRNA) reading frame and produce dystrophin by exon skipping, but have shown modest improvements.[1,2]
  - Intracellular delivery of oligonucleotides is challenging because of poor cell entry and limited escape from the endosome in the target cell.[3,4]
- To enhance PMO delivery to target tissues, we designed a family of proprietary cyclic cell–penetrating peptides that form the core of the Endosomal Escape Vehicle (EEV™) platform[5] (**Figure 1**).
  - The medicinal chemistry of cell-penetrating peptides is integral to their ability to efficiently deliver therapeutic cargo. As such, EEV peptides have been optimized for the efficient delivery of antisense oligonucleotides to target cells and tissue.[6]
- Here, we examined the EEV-PMO approach in multiple preclinical models of DMD.

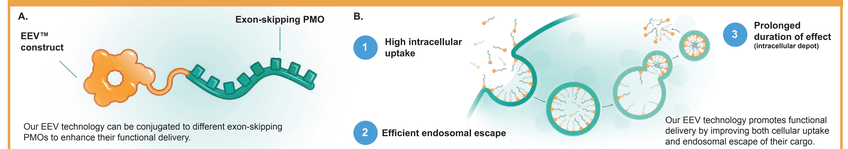
OBJECTIVE

- To assess the therapeutic potential of exon-skipping EEV-PMO constructs in preclinical models of Duchenne muscular dystrophy.

MATERIALS & METHODS

- EEV constructs and PMOs were prepared by solid phase synthesis following established procedure and conjugated by amide bond formation or click chemistry followed by ion exchange chromatography and/or reverse phase liquid chromatography.[6]
- *mdx* mice carry a nonsense mutation in *DMD* exon 23 and were evaluated for exon skipping 7 days following a single 20-mg/kg intravenous (IV) injection of PMO-23, EEV1-PMO-23, or EEV2-PMO-23 (**Figure 2B**).
- EGFP-654 mice[7] were administered three once-weekly IV doses of 10 mg/kg PMO654 or EEV3-PMO654 and were evaluated for EGFP mRNA splice correction 1 week after the last dose (**Figure 2D**).
- EEV-PMO-23, a *DMD* exon 23–skipping PMO conjugated to the EEV platform, was administered IV every 6 weeks (Q6W) in D2-*mdx* mice (**Figure 3**). These mice carry a nonsense mutation in *DMD* exon 23.
- del44hDMD.*mdx* are human dystrophin (hDMD)–expressing mice engineered with a deletion in the hDMD exon 44 transgene on the *mdx* background, resulting in an exon 45 skip–amenable mouse line. These mice were treated with a human exon 45–skipping EEV-PMO construct (EEV-PMO-45) (**Figure 4**).
- Exon-skipping efficiency was analyzed by reverse-transcriptase polymerase chain reaction (PCR) and LabChip (Perkin Elmer, Santa Clara, CA) (**Figures 2** and **3**) or reverse-transcriptase digital droplet PCR (**Figure 4**). Dystrophin restoration was evaluated by Simple Western Jess (Bio-Techne, Minneapolis, MN).

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



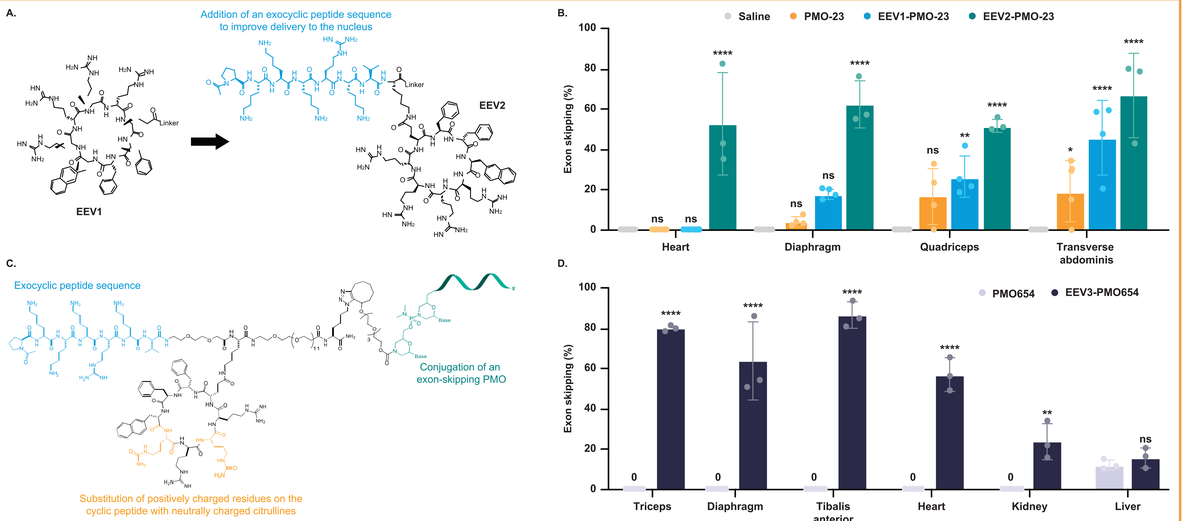
(A) Structure of EEV-PMO construct consisting of a cyclic cell-penetrating peptide-based EEV-construct and exon-skipping PMO; **(B)** Mechanism of EEV construct-mediated delivery of PMOs.
EEV, endosomal escape vehicle construct; PMO, phosphorodiamidate morpholino oligomer.

RESULTS

EEV Peptides to Efficiently Deliver PMOs to Skeletal and Cardiac Muscle

- The addition of an exocyclic peptide sequence to a first-generation EEV (EEV1) (**Figure 2A**) increased exon skipping in cardiac and skeletal muscle of *mdx* mice (**Figure 2B**).
- The EEV platform was further optimized (EEV3) by replacing positively charged residues with neutrally charged residues (**Figure 2C**). These modifications resulted in robust functional delivery to skeletal and cardiac muscle (**Figure 2D**).

Figure 2. EEV Peptides to Efficiently Deliver PMOs to Skeletal and Cardiac Muscle.

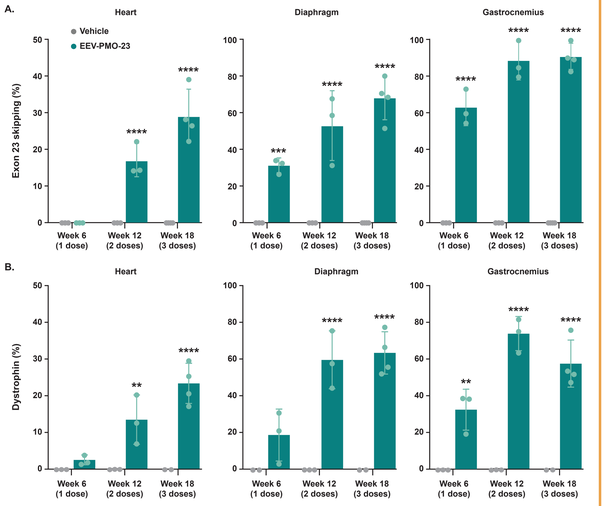


(A) Development of a second-generation EEV (EEV2); **(B)** EEV2 enhances PMO-23 exon skipping in *mdx* mice (**p<0.01, ****p<0.0001 vs. vehicle); **(C)** Development of a third-generation EEV (EEV3); **(D)** EEV3 efficiently delivers PMO654 to target tissues in EGFP-654 mice (**p<0.01, ****p<0.0001 vs. PMO654).
EEV, Endosomal Escape Vehicle; PMO, phosphorodiamidate morpholino oligomer; ns, not significant.

Exon Skipping and Dystrophin Production With EEV-PMO-23 in the D2-mdx Mouse Model of DMD

- Significant exon 23 skipping (**Figure 3A**) and dystrophin production (**Figure 3B**) was observed in cardiac and skeletal muscle following three Q6W doses of EEV-PMO-23.
  - Improvements over vehicle-treated mice were observed after the first dose in skeletal muscle and after the second dose in cardiac muscle.

Figure 3. Exon 23 Skipping and Dystrophin Production With EEV-PMO-23 in D2-*mdx* Mice.

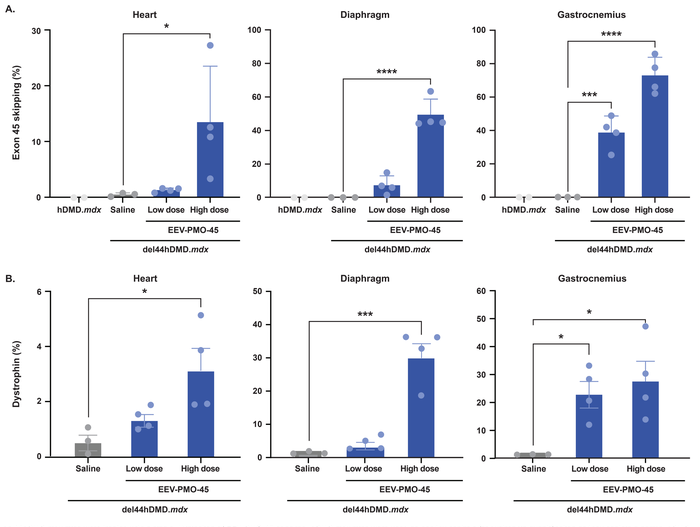


D2-*mdx* mice were treated with 80-mg/kg EEV-PMO-23 or vehicle Q6W. Exon skipping **(A)** and dystrophin production **(B)** were analyzed 6 weeks after each dose. Data shown as mean ± SD. One-way ANOVA was used for statistical comparison; **p<0.01, ***p<0.001,****p<0.0001 vs. vehicle. ANOVA, analysis of variance; EEV, Endosomal Escape Vehicle; PMO, phosphorodiamidate morpholino oligomer; Q6W, every 6 weeks; SD, standard deviation.

Exon Skipping and Dystrophin Production in a Mutation-Specific Murine Model

- EEV-PMO-45 produced robust exon 45 skipping and dystrophin production in del44hDMD.*mdx* mice harboring a *DMD* exon 45 skip–amenable mutation (**Figure 4**).

Figure 4. Efficacy of EEV-PMO-45 in del44hDMD.*mdx* Mice With an Exon 45 Skip–Amenable Mutation.



del44hDMD.*mdx* mice were treated with a single IV injection of EEV-PMO-45 or saline. hDMD.*mdx* mice were used as healthy controls for dystrophin quantification as they contain a normal hDMD transgene on the *mdx* background. Exon 45 skipping **(A)** and dystrophin protein expression **(B)** were analyzed in the gastrocnemius, diaphragm, and heart 2 weeks after dosing. Data shown as mean ± SD. One-way ANOVA was used for statistical comparison; *p<0.05, ***p<0.001,****p<0.0001 vs. vehicle. ANOVA, analysis of variance; EEV, Endosomal Escape Vehicle; hDMD, human dystrophin; IV, intravenous; PMO, phosphorodiamidate morpholino oligomer; SD, standard deviation.

ACKNOWLEDGMENTS

This research was funded by Entrada Therapeutics, Inc (Boston, MA). The del44hDMD.*mdx* and the hDMD.*mdx* mouse models were all acquired and licensed from Leiden University (Leiden, The Netherlands). The authors would like to thank Aji Nair for assistance with poster development (Entrada Therapeutics, Inc). Editorial and studio support for this poster was provided by Ashfield MedComms (US), an Inizio company, and was funded by Entrada Therapeutics, Inc. References: 1. EXONDYS 51® Prescribing Information. 2. VILTEPSO® Prescribing Information. 3. Qian Z. *Biochemistry*. 2016. 4. Sahni A. *ACS Chem Biol*. 2020. 5. Qian Z, et al. *ACS Chem*. 2013. 6. Li X, et al. *Mol Ther Nucleic Acids*. 2023. 7. Sazani P, et al. *Nat Biotechnol*. 2002.

CONCLUSIONS

- The EEV platform efficiently delivered exon-skipping PMOs to skeletal and cardiac muscle in preclinical models of DMD.
  - These results underscore the importance of the medicinal chemistry of cell-penetrating peptides for successful delivery of PMOs to target tissues.
- These findings support earlier studies demonstrating the preclinical efficacy of ENTR-601-44 and ENTR-601-45, and support further study of these EEV-PMO constructs in patients with exon 44 and 45 skip–amenable DMD, respectively.
- A phase 1 clinical trial of ENTR-601-44 in healthy subjects is ongoing with expected completion in the second half of 2024.