240

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

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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.

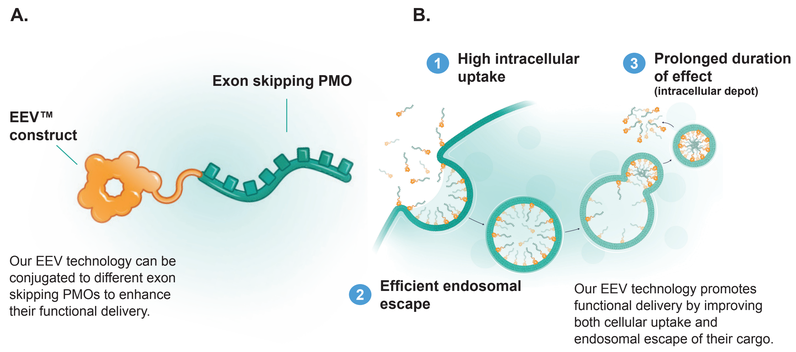
MATERIALS AND METHODS

- EEV construct and PMO 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 23 skipping 7 days following a single 20-mg/kg intravenous (IV) injection of saline, PMO-23, EEV1-PMO-23, or EEV2-PMO-23 (**Figure 2B**).
- Enhanced green fluorescent protein (EGFP)-654 mice[7] were administered three once-weekly IV doses of 10-mg/kg PMO-654 or EEV3-PMO-654 and were evaluated for EGFP mRNA splice correction 1 week after the last dose (**Figure 2D**).
- ENTR-601-44, a *DMD* exon 44–skipping PMO conjugated to the EEV platform, was administered to human dystrophin (hDMD)–producing mice[8] and nonhuman primates (NHPs) to assess exon 44 skipping in cardiac and skeletal muscles (**Figure 3**).
- ENTR-601-45, a *DMD* exon 45–skipping PMO conjugated to the EEV platform, was evaluated for exon skipping in hDMD mice to assess exon 45 skipping in cardiac and skeletal muscle (**Figure 4**).
- Exon-skipping efficiency was analyzed by reverse-transcriptase polymerase chain reaction and LabChip (Perkin Elmer, Santa Clara, CA).

OBJECTIVE

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

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



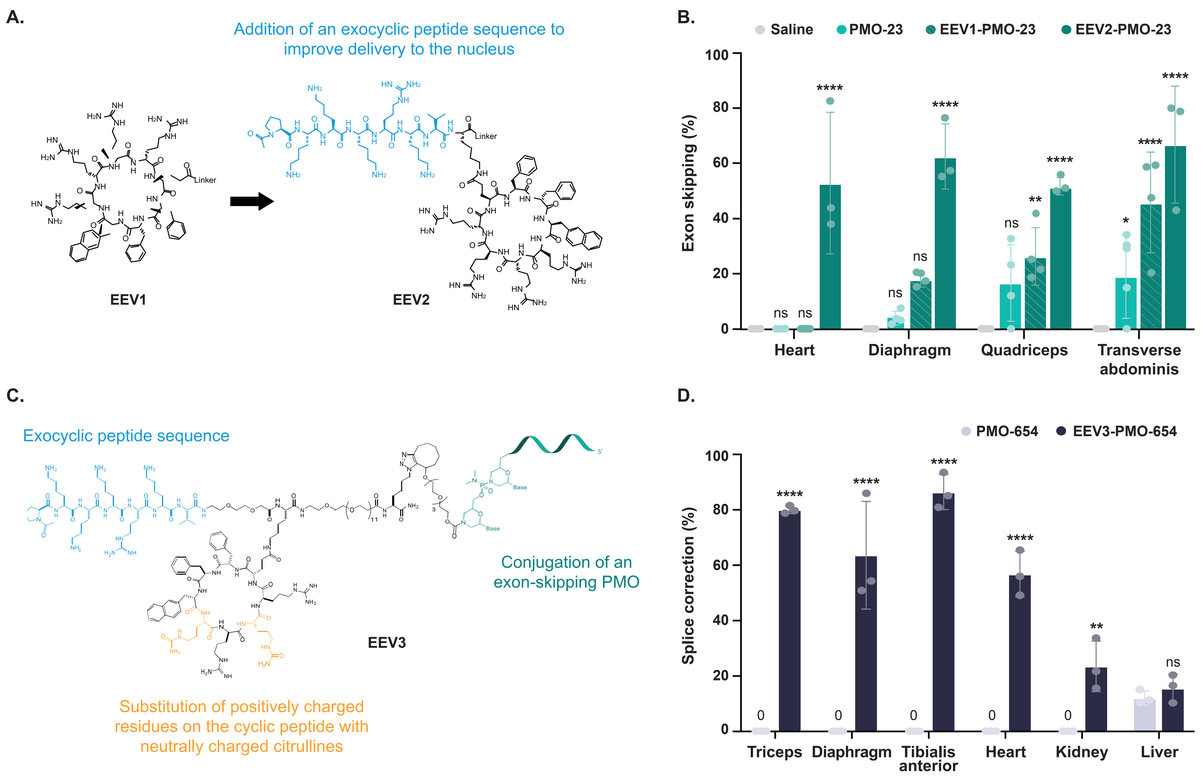
(A) Structure of EEV-PMO construct consisting of a cyclic cell-penetrating peptide-based EEV-construct and exon-skipping PMO; **(B)** Mechanism of EEV construct-mediated delivery of PMOs. EEV, Endosomal Escape Vehicle; PMO, phosphorodiamidate morpholino oligomer.

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 enhanced splice correction in both skeletal and cardiac muscle (**Figure 2D**).

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

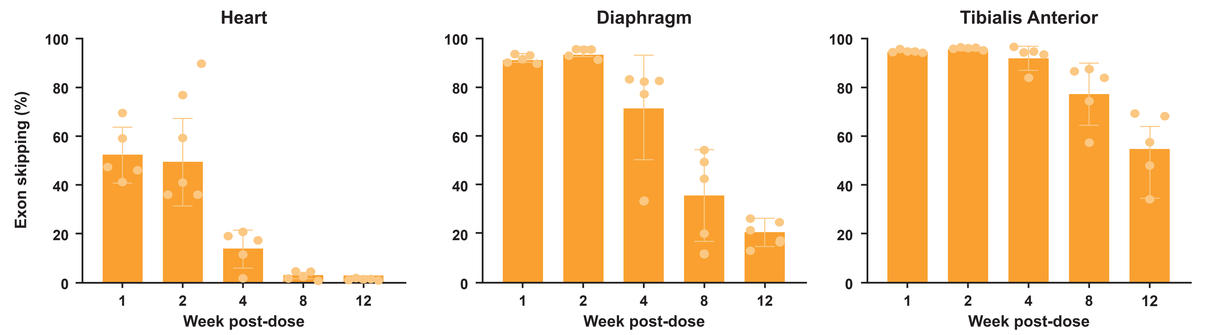


(A) Development of a second-generation EEV (EEV2); **(B)** EEV2 enhances PMO-23 exon skipping in *mdx* mice (*p<0.05, **p<0.01, ****p<0.0001 vs. vehicle); **(C)** Development of a third-generation EEV (EEV3); **(D)** EEV3 efficiently delivers PMO-654 to target tissues in EGFP-654 mice (**p<0.01, ****p<0.0001 vs. PMO-654). EEV, Endosomal Escape Vehicle; EGFP, enhanced green fluorescent protein; ns, not significant; PMO, phosphorodiamidate morpholino oligomer.

Exon Skipping and Durable Efficacy of ENTR-601-44 and ENTR-601-45 in hDMD Mice

- A single IV dose of ENTR-601-44 resulted in high and durable levels of exon 44 skipping across major muscle groups in hDMD mice (**Figure 3**) for at least 12 weeks.

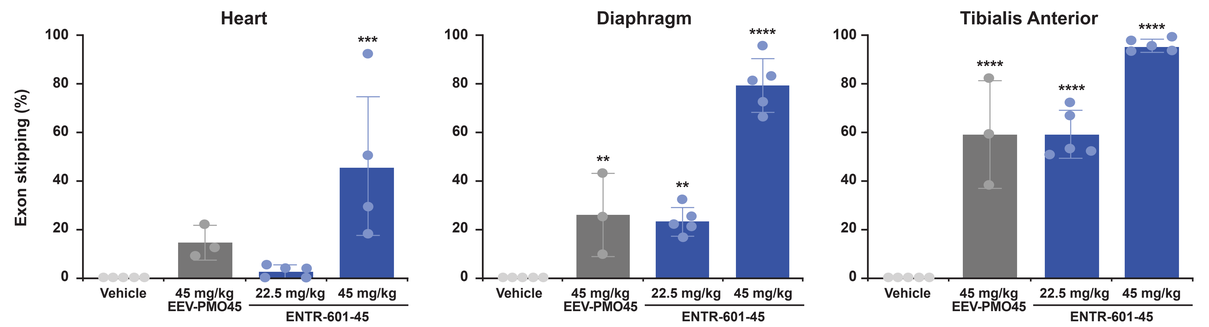
Figure 3. Exon 44 Skipping With ENTR-601-44 in hDMD Mice.



hDMD mice were treated with a single 60-mg/kg IV dose of ENTR-601-44. hDMD, human dystrophin; IV, intravenous.

- A single dose of ENTR-601-45 delivered up to a two-fold improvement in exon skipping when compared with an equivalent dose of the same EEV conjugated to a casimersen sequence (EEV-PMO45) (**Figure 4**)

Figure 4. ENTR-601-45 Target Engagement in hDMD Mice.



hDMD mice were treated with a single IV dose of EEV-PMO45 or ENTR-601-45 and assessed for exon skipping 7 days later. Values are shown as mean ± standard deviation (n=3–5). ENTR-601-45 is a *DMD* exon 45–skipping EEV-oligonucleotide construct. hDMD transgenic mice express full-length human dystrophin gene.[8] Casimersen is an exon 45–skipping PMO approved in the United States.[9] **p<0.01, ***p<0.001, ****p<0.0001 vs. vehicle. *DMD*, dystrophin gene; EEV, Endosomal Escape Vehicle; hDMD, human dystrophin; IV, intravenous; PMO, phosphorodiamidate morpholino oligomer.

ACKNOWLEDGMENTS

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CONCLUSIONS

- Development of the EEV platform led to efficient delivery of 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.
- ENTR-601-44 and ENTR-601-45 showed robust exon-skipping efficacy in animal models of DMD.
- Together, these findings support the potential for further study in patients with exon 44 and exon 45 skip–amenable DMD.
  - A phase 1 clinical trial of ENTR-601-44 in healthy volunteers demonstrated that ENTR-601-44 showed dose-dependent exon 44 skipping with no adverse events related to study drug.[10]