Endosomal Escape Vehicle (EEV™)-Oligonucleotide Conjugates Produce Exon Skipping and Dystrophin Production in Preclinical Models of Duchenne Muscular Dystrophy

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INTRODUCTION

- Currently approved phosphorodiamidate morpholino oligomer (PMO) therapies for Duchenne muscular dystrophy (DMD) were designed to restore the 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)**.
  - Results of preliminary studies in *mdx* mice demonstrated that EEV-PMO constructs produce dystrophin in skeletal and cardiac muscle by exon skipping.[6]
- Here, we further examined the EEV-PMO approach in multiple preclinical models of DMD.

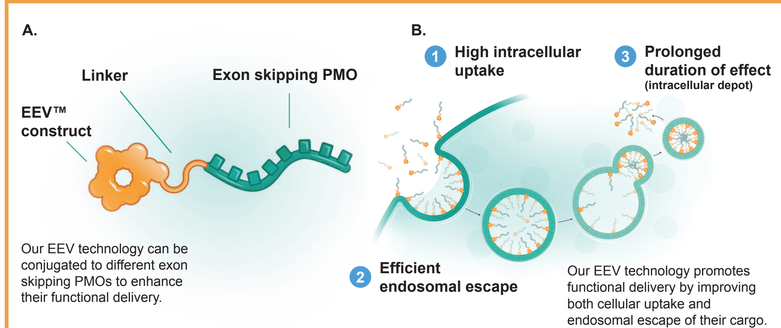
MATERIALS AND METHODS

- The efficacy of 3 EEV-PMO constructs was assessed in the following animal and cell models:
  - EEV-PMO-23, a DMD exon 23 skipping PMO conjugated to the EEV platform, was administered intravenously to assess exon skipping and dystrophin production in D2-*mdx*[7] mice (**Figure 2**). These mice contain a nonsense mutation in exon 23.
  - ENTR-601-44, a DMD exon 44 skipping PMO conjugated to the EEV platform, was administered to human dystrophin (hDMD) producing mice and nonhuman primates (NHPs) to assess exon 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 and dystrophin production in DMD patient-derived skeletal and cardiac muscle cells (**Figure 4**).
- Exon-skipping efficiency was analyzed by reverse-transcriptase polymerase chain reaction and LabChip (Perkin Elmer, Santa Clara, CA). Dystrophin restoration was evaluated by Simple Western Jess (Bio-Techne, Minneapolis, MN) and immunofluorescence.

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.



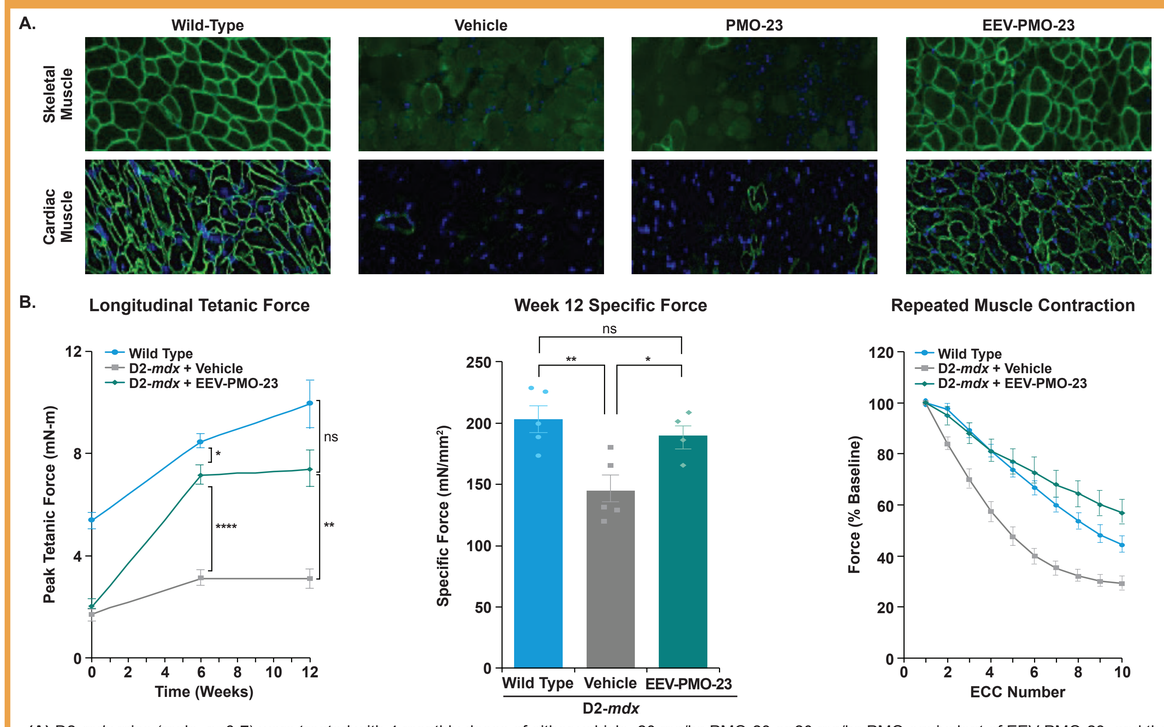
(A) Structure of EEV-PMO construct consisting of a cyclic cell-penetrating peptide-based EEV-construct, linker, and exon skipping PMO; **(B)** Mechanism of EEV construct-mediated delivery of PMOs. EEV, endosomal escape vehicle construct; PMO, phosphorodiamidate morpholino oligomer.

RESULTS

Exon Skipping and Dystrophin Restoration With EEV-PMO-23 in D2-mdx Model of DMD

- Broad dystrophin expression and restoration of skeletal and cardiac muscle integrity were observed with monthly EEV-PMO-23 administration compared with PMO-23 alone (**Figure 2A**). Biweekly treatment with EEV-PMO-23 improved skeletal muscle contractile force in D2-*mdx* mice that was not significantly different than that in wild-type mice at week 12 (**Figure 2B**).

Figure 2. Dystrophin Restoration and Improved Muscle Contractility With EEV-PMO-23.

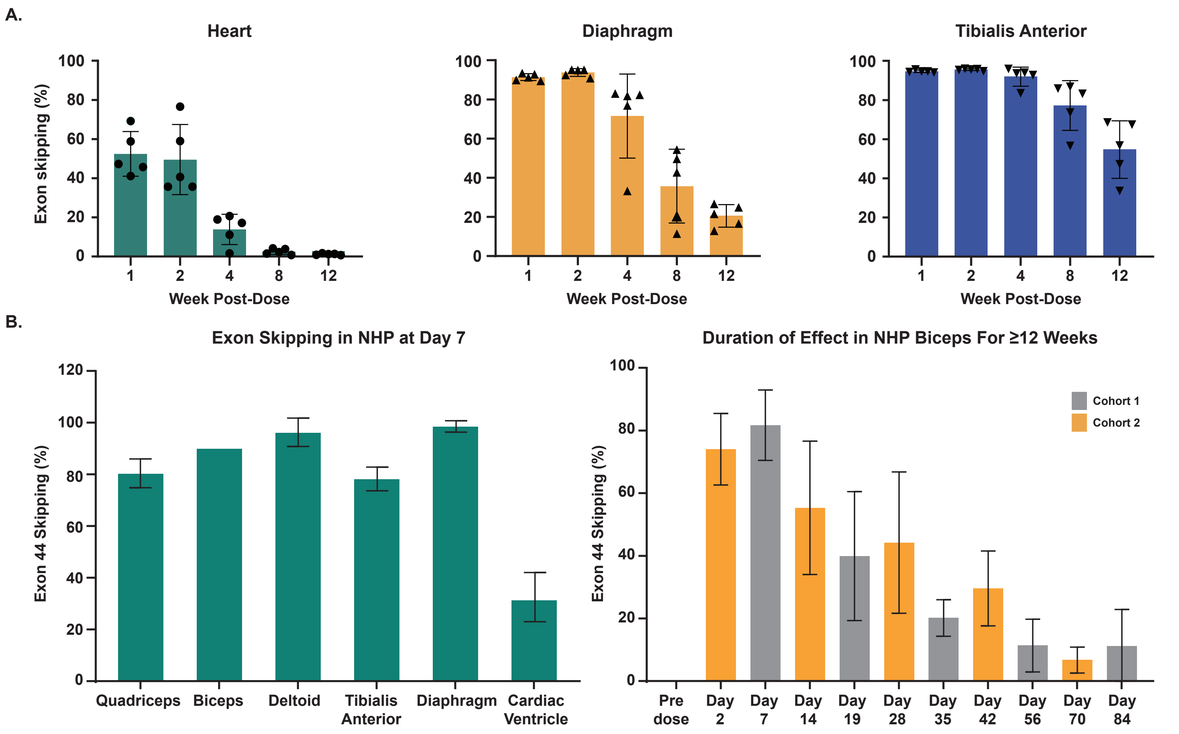


(A) D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO-23 or 20 mg/kg PMO equivalent of EEV-PMO-23, and the data were collected ~4 weeks after the last dose. Dystrophin in green and DAPI in blue. **(B)** D2-*mdx* mice were treated with vehicle or 80 mg/kg (once every 2 weeks) EEV-PMO-23. Muscle contractility was assessed at weeks 6 and 12 via isometric force generated by tetanic contraction of plantar flexor muscle group and ECC generated by repeated tetanic contraction of the tibialis anterior muscle. *p<0.05, **p<0.01, ****p<0.0001; ns, not significant; shown as mean ± SEM. DAPI, 4′,6-diamidino-2-phenylindole; EEC, eccentric force; EEV, endosomal escape vehicle; PMO, phosphorodiamidate morpholino oligomer.

Exon Skipping and Durable Efficacy of ENTR-601-44 in Murine and NHP Models of DMD

- 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 3A**) and NHPs (**Figure 3B**) for at least 12 weeks.

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

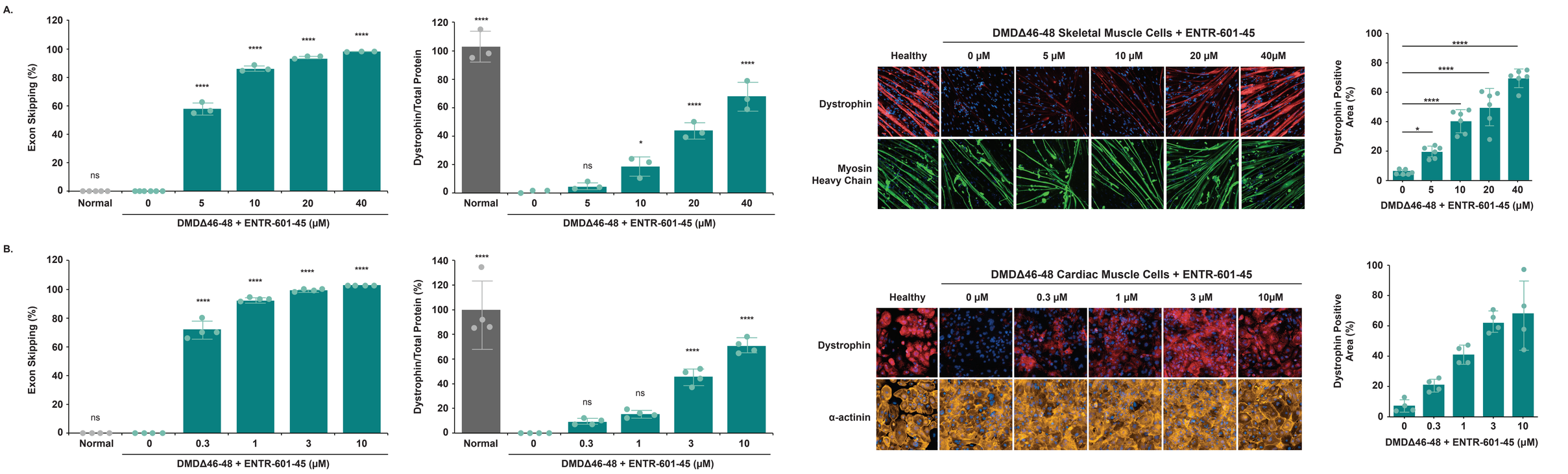


A (hDMD mice). A single 60 mg/kg (PMO equivalent) IV dose of ENTR-601-44 was administered to hDMD mice. **B (NHPs).** At 7 days post-IV infusion of 30 mg/kg ENTR-601-44 (PMO equivalent), robust exon 44 skipping observed across different muscle groups isolated from the ENTR-601- 44–treated NHPs (left graph). Post-intravenous infusion of 35 mg/kg ENTR-601-44 (PMO equivalent), robust exon 44 skipping observed in biceps in the ENTR-601-44–treated NHPs (n=3 per cohort) for ≥12 weeks (right graph). hDMD, human dystrophin; IV, intravenous; NHP, nonhuman primate; PMO, phosphorodiamidate morpholino oligomer.

Exon Skipping and Dystrophin Restoration With ENTR-601-45 in Patient-Derived Cells

- ENTR-601-45 showed robust exon skipping and dystrophin production in patient-derived skeletal (**Figure 4A**) and cardiac (**Figure 4B**) muscle cells.

Figure 4. Efficacy of ENTR-601-45 in Skeletal and Cardiac Muscle Cells.



DMDΔ46-48 patient-derived skeletal **(A)** and cardiac **(B)** muscle cells were incubated with ENTR-601-45. Myotubes were treated for 24 hours and analyzed after 5 days of differentiation. Cardiomyocytes were treated for 24 hours and analyzed 48 hours later. Data are shown as mean (SD); 1-way ANOVA used for exon skipping and dystrophin/total protein data (n=3 per cohort for skeletal and n=4 per cohort for cardiac); 1-way ANOVA and Dunnett's multiple comparison test used for dystrophin-positive area analyses (n=6 per cohort for skeletal and n=4 per cohort for cardiac); *p<0.05, ****p<0.0001; ns, not significant; relative to untreated DMDΔ46-48 cells.

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CONCLUSIONS

- The results presented here demonstrate that the EEV platform efficiently delivers exon skipping oligonucleotides to skeletal and cardiac muscle in preclinical models of DMD.
  - Studies in D2-*mdx* mice demonstrated that the EEV-PMO approach has the potential to improve muscle contractility.
- ENTR-601-44 and ENTR-601-45 in cell and animal models showed robust exon skipping and increased dystrophin production.
- Together, these findings support the potential for further study in patients with DMD amenable to exon 44 and 45 skipping.