Preclinical Efficacy of ENTR-601-50, a Novel EEV™-Oligonucleotide Construct for the Treatment of Exon 50 Skip–Amenable Duchenne Muscular Dystrophy

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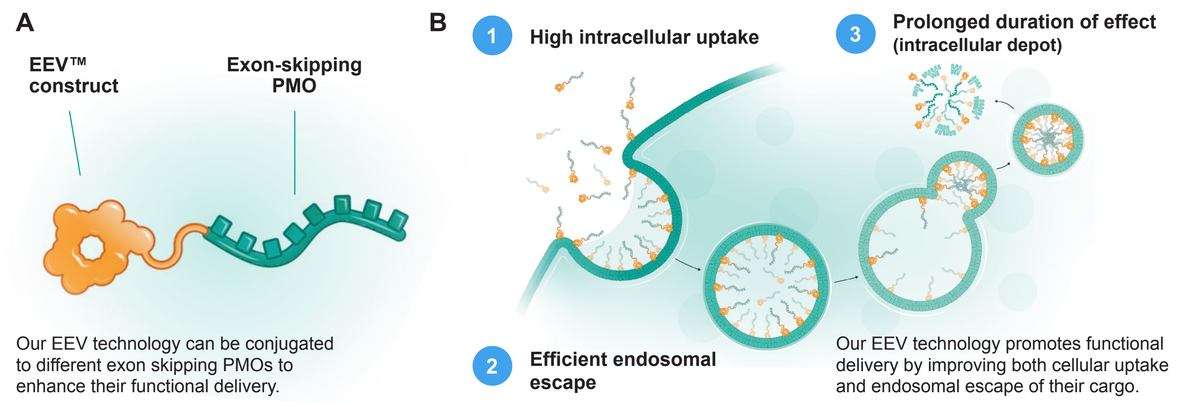
BACKGROUND

- The Endosomal Escape Vehicle (EEV™) platform consists of a family of cyclic cell-penetrating peptides developed to enhance the delivery of therapeutics to target cells by overcoming barriers to cell entry and endosomal escape[1] (**Figure 1**).
  - The EEV platform has been shown to improve antisense phosphorodiamidate morpholino oligomer (PMO) delivery to skeletal and cardiac muscle in preclinical models of Duchenne muscular dystrophy (DMD).[2]
- EEV-PMO therapeutics designed for exon 44 (ENTR-601-44) and exon 45 (ENTR-601-45) skip–amenable DMD, respectively, demonstrated robust exon skipping and dystrophin production in both cell and animal models, suggesting their therapeutic potential in patients with DMD.[3,4]
  - Furthermore, ENTR-601-44 was well tolerated and demonstrated significant exon 44 skipping compared with placebo in a phase 1 clinical trial of healthy human volunteers.[5]
- Here, we assessed the preclinical efficacy of ENTR-601-50, an exon 50 skipping EEV-PMO construct in development for the treatment of DMD amenable to exon 50 skipping.

MATERIALS AND METHODS

- ENTR-601-50 is a *DMD* exon 50 skipping PMO conjugated to the EEV platform, and is in development for the treatment of exon 50 skip–amenable DMD. EEV-PMO-50, an exon 50 skipping EEV-PMO tool compound, was used for the satellite cell study in Figure 6.
- Skeletal muscle cells were derived from a patient with exon 50 skip–amenable DMD harboring a deletion of *DMD* exons 51, 52, 53, 54, and 55 (DMDΔ51-55).
- Cardiomyocytes (CM) were derived from a patient with exon 50 skip-amenable DMD harboring a deletion of *DMD* exon 51 (DMDΔ51).
- del51hDMD.*mdx* mice (Taconic Biosciences, Germantown, NY) express human dystrophin (hDMD) and are engineered with a deletion in the *hDMD* exon 51 transgene on the *mdx* background, resulting in an exon 50 skip–amenable mouse line. hDMD.*mdx* mice were used as healthy controls for dystrophin quantification, as they contain a normal *hDMD* transgene on the *mdx* background.
- Exon-skipping efficiency was analyzed by digital droplet RT-PCR. 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; PMO, phosphorodiamidate morpholino oligomer.

OBJECTIVE

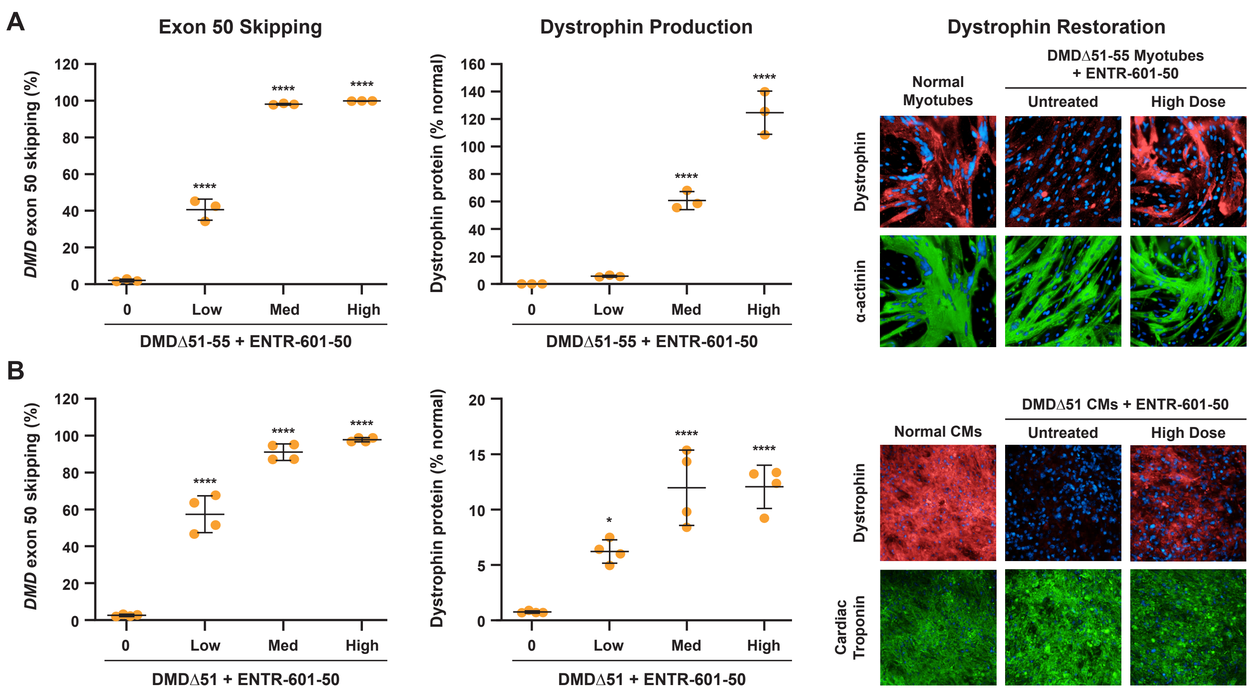
- To assess the efficacy and therapeutic potential of ENTR-601-50 in cell and animal models of DMD amenable to exon 50 skipping.

RESULTS

Exon Skipping and Dystrophin Restoration With ENTR-601-50 in DMD Patient-Derived Cells

- Treatment of patient-derived skeletal (**Figure 2A**) and cardiac (**Figure 2B**) muscle cells with ENTR-601-50 resulted in dose-dependent *DMD* exon 50 skipping and dystrophin protein restoration.

Figure 2. Efficacy of ENTR-601-50 in DMD Patient-Derived Skeletal and Cardiac Muscle Cells

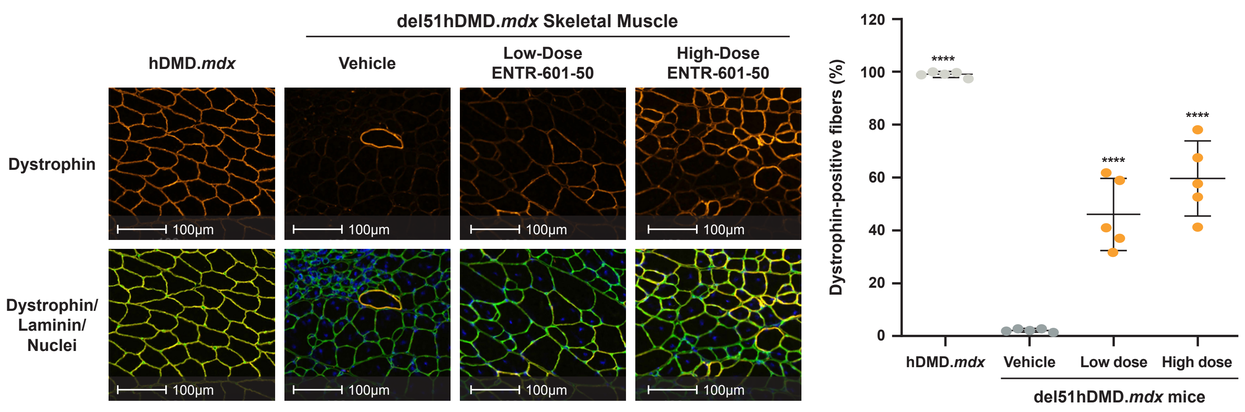


DMDΔ51-55 patient-derived skeletal (A) and DMDΔ51 patient-derived cardiac (B) muscle cells were incubated with ENTR-601-50. Skeletal myotubes (A) were treated for 24 hours and analyzed after 5 days of differentiation. Cardiomyocytes (B) were treated for 24 hours and analyzed 48 hours later. Dystrophin protein is shown in red, and nuclei appear blue. Data are shown as mean ± standard deviation; one-way ANOVA was used for exon skipping and dystrophin/total protein data (n=4 per cohort); *p<0.05, ****p<0.0001 compared with untreated DMDΔ51-55 cells in Panel A and untreated DMDΔ51 cells in Panel B. ANOVA, analysis of variance; CM, cardiomyocyte.

ENTR-601-50 Increased Dystrophin-Positive Fibers in Skeletal Muscle of Mice With an Exon 50 Skip–Amenable Mutation

- A single IV dose of ENTR-601-50 produced dose-dependent increases in dystrophin-positive muscle fibers, localizing in the gastrocnemius sarcolemma of del51hDMD.*mdx* mice 2 weeks post-dose (**Figure 4**). Significant increases in dystrophin-positive muscle fibers, evenly distributed across the tissue, were observed with both doses of ENTR-601-50 compared with vehicle.

Figure 4. Dystrophin Distribution and Localization in Skeletal Muscle of del51hDMD.*mdx* Mice With ENTR-601-50



del51hDMD.*mdx* mice were treated with a single IV injection of ENTR-601-50 or vehicle. Dystrophin protein distribution and cellular localization (red) were analyzed by immunofluorescence in the gastrocnemius 2 weeks after dosing. Quantification via Halo Image Analysis Software is shown as the percentage of dystrophin-positive muscle fibers relative to the total number of muscle fibers, as determined by laminin staining (green) and dystrophin staining (red); co-localization to the sarcolemma appears yellow, and nuclei appear blue. Data shown as mean ± standard deviation. One-way ANOVA was used for statistical comparison; ****p<0.0001 vs. vehicle. ANOVA, analysis of variance; hDMD, human dystrophin transgene; IV, intravenous.

CONCLUSIONS

- ENTR-601-50 demonstrated significant dose-dependent exon skipping and dystrophin production in both cell and animal models of DMD amenable to exon 50 skipping.
- In animals treated with ENTR-601-50, dystrophin was correctly localized at the sarcolemma of muscle fibers.
- Treatment with ENTR-601-50 resulted in the expression of a functional protein, as evidenced by enhanced skeletal muscle function in a mouse model amenable to exon 50 skipping.
- Additionally, EEV-PMO-50 effectively penetrated the satellite cell compartment in mouse skeletal muscle, potentially enhancing dystrophin expression in functionally impaired satellite cells and promoting regeneration.
- These findings demonstrate the therapeutic potential of ENTR-601-50 and support further studies in patients with DMD amenable to exon 50 skipping.

ACKNOWLEDGMENTS

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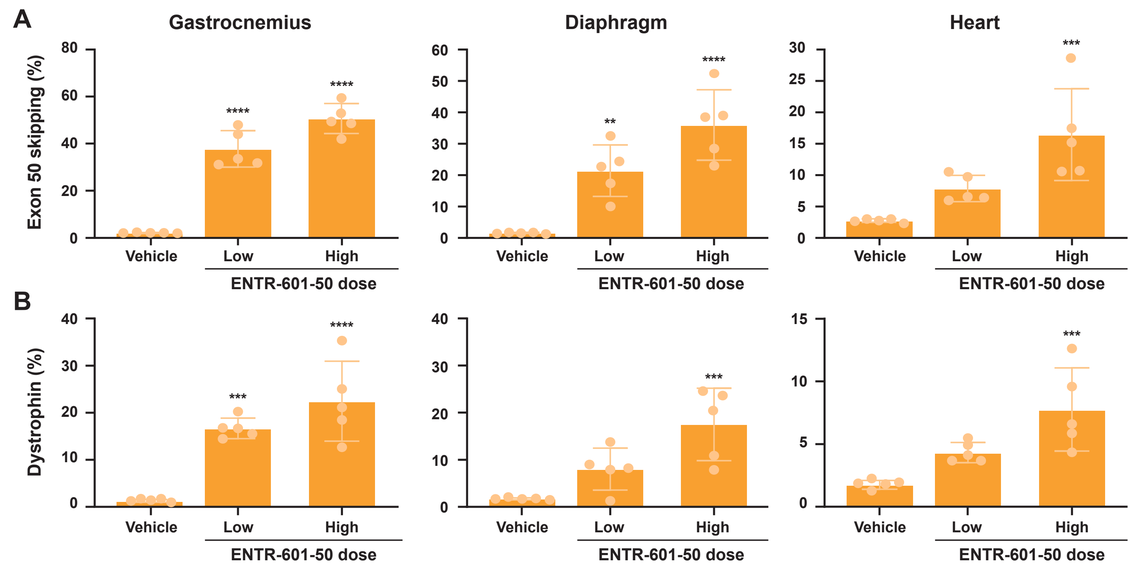
Exon Skipping and Dystrophin Production With ENTR-601-50 in Mice With an Exon 50 Skip–Amenable Mutation

- A single intravenous (IV) dose of ENTR-601-50 led to robust human *DMD* exon 50 skipping (**Figure 3A**) and dystrophin production (**Figure 3B**) in del51hDMD.*mdx* mice harboring an exon 50 skip–amenable mutation 2 weeks after dosing.

Figure 3. Efficacy of ENTR-601-50 in del51hDMD.*mdx* Mice

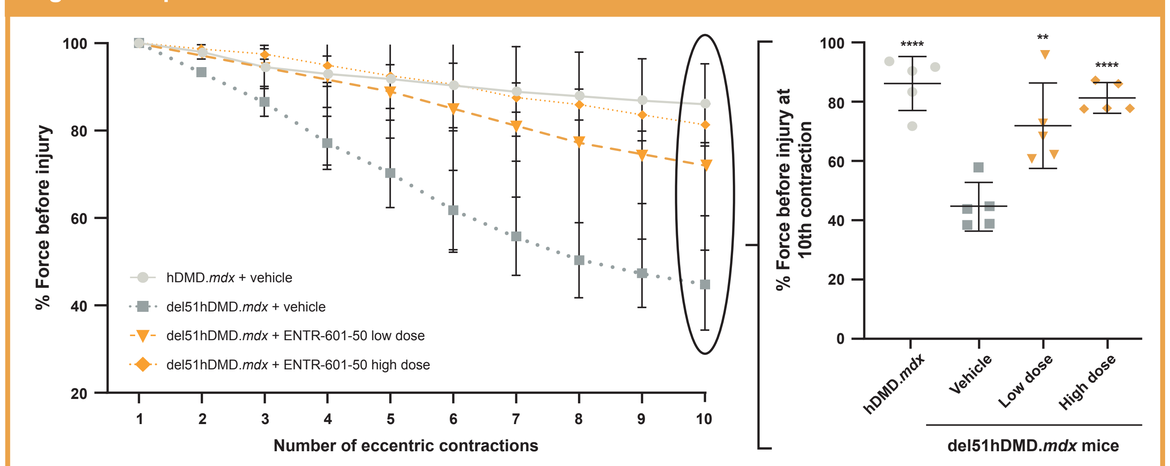


del51hDMD.*mdx* mice were treated with a single IV injection of ENTR-601-50 or vehicle. Human *DMD* exon 50 skipping (A) and dystrophin protein expression (B) were analyzed in the gastrocnemius, diaphragm, and heart 2 weeks post-dose. Percent dystrophin protein restoration is normalized to total protein and normalized to hDMD.*mdx* controls. Data shown as mean ± standard deviation. One-way ANOVA was used for statistical comparison; **p≤0.01, ***p≤0.001, ****p≤0.0001 vs. vehicle. ANOVA, analysis of variance; hDMD, human dystrophin transgene; IV, intravenous.

ENTR-601-50 Improved Skeletal Muscle Function in Mice With an Exon 50 Skip–Amenable Mutation

- A significant and dose-dependent increase in resistance to membrane damage was observed following the tenth contraction with both doses of ENTR-601-50 two weeks after dosing (**Figure 5**).

Figure 5. Improved Skeletal Muscle Function With ENTR-601-50 in del51hDMD.*mdx* Mice

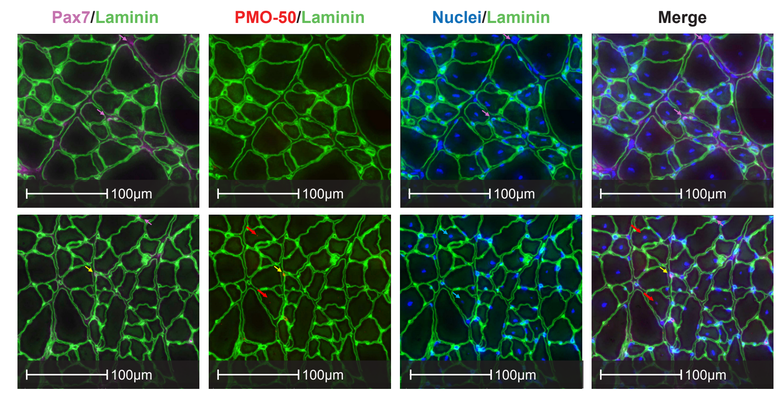


del51hDMD.*mdx* mice were treated with a single IV injection of ENTR-601-50 at low or high dose or with vehicle. ECC-induced muscle force loss generated by repeated ECC contraction of the gastrocnemius muscle was assessed 2 weeks after dosing. Data (mean ± standard deviation) are shown across 10 ECC contractions normalized as a percentage of the initial force before any ECC contractions (left) and as the percentage of force retained after the 10th contraction (right). Vehicle-treated hDMD.*mdx* mice were used as a control group for normal muscle function. One-way ANOVA was used for statistical comparison to vehicle-treated del51hDMD.*mdx* mice; **p≤0.01, ****p≤0.0001. ANOVA, analysis of variance; ECC, eccentric force; hDMD, human dystrophin transgene; IV, intravenous.

*Localization of PMO-50 to Satellite Cells in Skeletal Muscle of del51hDMD.*mdx *Mice*

- PMO-50 co-localized with satellite cells and newly generated centrally nucleated fibers in skeletal muscle 2 weeks post-dose.

Figure 6. PMO-50 Co-Localizes With Satellite Cells and Centrally Nucleated Fibers in Skeletal Muscle



del51hDMD.*mdx* mice were treated with a single IV dose of EEV-PMO-50 or vehicle. Gastrocnemius muscle was analyzed with immunohistochemistry for Pax7 (purple arrows, satellite cell marker), PMO-50 (red), and laminin (green) 2 weeks post-dose. Nuclei appear blue. Red arrows show PMO-50-positive centrally nucleated fibers. Yellow arrows show co-localization of PMO-50 with satellite cells. EEV, Endosomal Escape Vehicle; IV, intravenous; Pax7, paired box protein 7; PMO, phosphorodiamidate morpholino oligomer.