Exon 45 Skipping, Dystrophin Production, and Functional Improvement With ENTR-601-45 in Preclinical Models of Duchenne Muscular Dystrophy

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INTRODUCTION

- Intracellular delivery of oligonucleotide therapeutics for the treatment of Duchenne muscular dystrophy (DMD) is challenging because of poor cell entry and limited escape from the endosome in the target cell, necessitating high therapeutic doses.[1,2]
- To address these limitations, we designed a family of cyclic cell-penetrating peptides that form the core of our Endosomal Escape Vehicle (EEV™) platform, which has been shown to efficiently deliver exon skipping phosphorodiamidate morpholino oligomers (PMOs) to skeletal and cardiac muscle.[3,4] (**Figure 1**)
- Preclinical proof of concept studies in D2-*mdx* mice showed robust exon skipping and dystrophin production in skeletal and cardiac muscle following monthly or every 6 weeks (Q6W) administration of an EEV-exon 23 skipping PMO construct.[5]
- To further assess the therapeutic potential of EEV-PMO constructs, we examined the preclinical efficacy of ENTR-601-45, a *DMD* exon 45 skipping PMO conjugated to the EEV platform, developed for the treatment of exon 45 skip–amenable DMD.

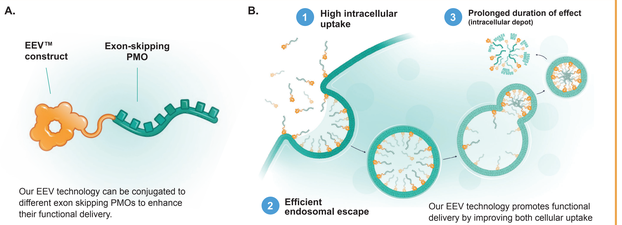
MATERIALS AND METHODS

- ENTR-601-45 is a *DMD* exon 45 skipping PMO conjugated to the EEV platform and is in development for the treatment of exon 45 skip–amenable DMD.
- Skeletal muscle cells and cardiomyocytes were derived from a patient with exon 45 skip–amenable DMD harboring a deletion of DMD exons 46, 47, and 48 (DMDΔ46-48).
- 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. 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 either reverse-transcriptase polymerase chain reaction (RT-PCR) and LabChip (Perkin Elmer, Santa Clara, CA) (**Figure 2**) or digital droplet RT-PCR (**Figure 3**). Dystrophin restoration was evaluated by Simple Western Jess (Bio-Techne, Minneapolis, MN).
- Resistance towards eccentric-induced muscle damage via repeated eccentric (ECC) contractions was measured in the gastrocnemius muscle using a 3-in-1 Whole Animal Muscle Physiology system from Aurora Scientific (Aurora, ON, Canada).

OBJECTIVE

- To assess the efficacy and therapeutic potential of ENTR-601-45 in preclinical models of DMD amenable to exon 45 skipping.

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.

CONCLUSIONS

- ENTR-601-45 produced robust dose-dependent exon skipping and dystrophin restoration in both in vitro and in vivo models of exon 45 skip–amenable DMD.
- Improved skeletal muscle function in an exon 45 skip–amenable DMD mouse model suggests that ENTR-601-45 is capable of producing functional dystrophin protein in vivo.
  - At the highest dose of ENTR-601-45 examined, dystrophin production and muscle function were similar to healthy control mice.
- Together, these results demonstrate the therapeutic potential of ENTR-601-45 and support further study in patients with DMD amenable to exon 45 skipping.

ACKNOWLEDGMENTS

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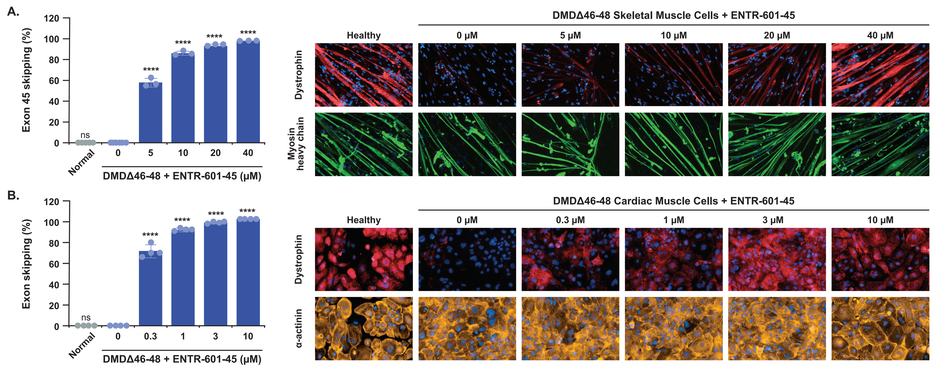
References: 1. Qian Z. *Biochemistry*. 2016. 2. Sahni A. *ACS Chem Biol*. 2020. 3. Qian Z, et al. *ACS Chem*. 2013. 4. Li X, et al. *Mol Ther Nucleic Acids*. 2023. 5. Kumar A. MDA 2022. Poster 126.

RESULTS

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

- ENTR-601-45 showed robust exon skipping and dystrophin production in patient-derived skeletal (**Figure 2A**) and cardiac (**Figure 2B**) muscle cells.

Figure 2. Efficacy of ENTR-601-45 in DMD Patient-Derived Skeletal and Cardiac Muscle Cells.

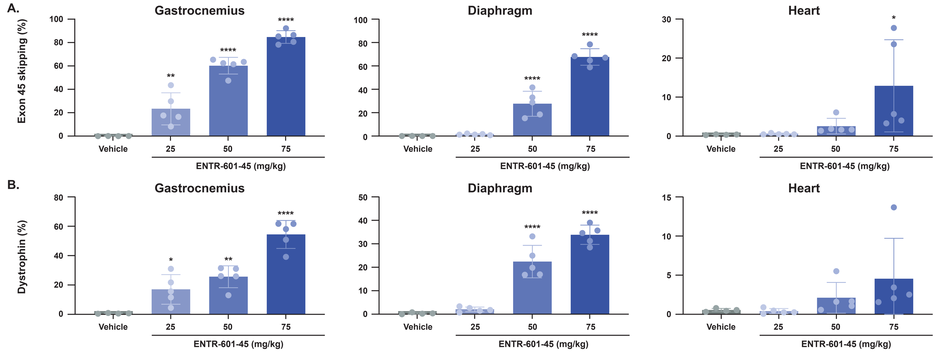


DMDΔ46-48 patient-derived skeletal **(A)** and cardiac **(B)** muscle cells were incubated with ENTR-601-45. Skeletal myotubes (A) were treated for 24 hours and analyzed after 5 days of differentiation. Cardiomyocytes **(B)** were treated for 24 hours and analyzed 48 hours later. Data are shown as mean ± standard deviation; 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<0.0001 compared to untreated DMDΔ46-48 cells. ANOVA, analysis of variance; DMD, Duchenne muscular dystrophy; ns, not significant.

Exon Skipping and Dystrophin Restoration of ENTR-601-45 in Mice With an Exon 45 Skip–Amenable Mutation

- Three Q6W doses of ENTR-601-45 led to robust human *DMD* exon 45 skipping and dystrophin production in del44hDMD.*mdx* mice harboring an exon 45 skip–amenable mutation 6 weeks after the final dose (**Figure 3**).

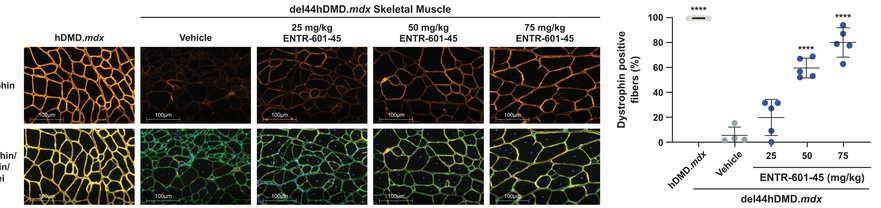
Figure 3. Efficacy of Q6W ENTR-601-45 in del44hDMD.*mdx* Mice.



del44hDMD.*mdx* mice were treated with three Q6W IV injections of ENTR-601-45 or vehicle. Human *DMD* exon 45 skipping **(A)** and dystrophin protein expression **(B)** were analyzed in the gastrocnemius, diaphragm, and heart 6 weeks after the final 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≤0.05, **p≤0.01, ****p≤0.0001 vs. vehicle. ANOVA, analysis of variance; hDMD, human dystrophin transgene; IV, intravenous; Q6W, every 6 weeks.

- Three Q6W doses of ENTR-601-45 produced dose-dependent increase in dystrophin-positive muscle fibers showing localization in the sarcolemma in the gastrocnemius of del44hDMD.*mdx* mice 6 weeks following the final dose (**Figure 4**). Significant increases in dystrophin-positive muscle fibers that were evenly distributed across the tissue were observed with 50 and 75 mg/kg ENTR-601-45 compared with vehicle.

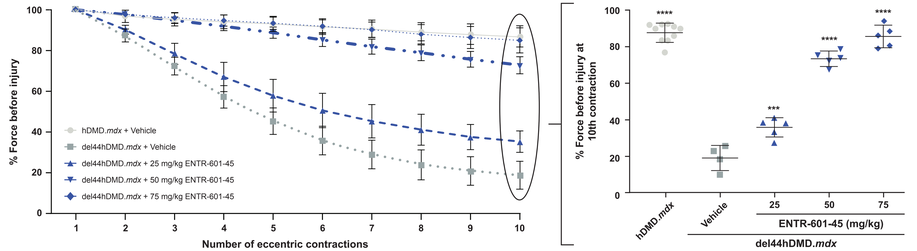
Figure 4. Dystrophin Distribution and Localization in Skeletal Muscle of del44hDMD.*mdx* Mice With Q6W ENTR-601-45.



del44hDMD.*mdx* mice were treated with three Q6W IV injections of ENTR-601-45 or vehicle. Dystrophin protein distribution and cellular localization (red) was analyzed by immunofluorescence in the gastrocnemius 6 weeks after the final dose. 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; Q6W, every 6 weeks.

ENTR-601-45 Improved Skeletal Muscle Function in Mice With an Exon 45 Skip–Amenable Mutation

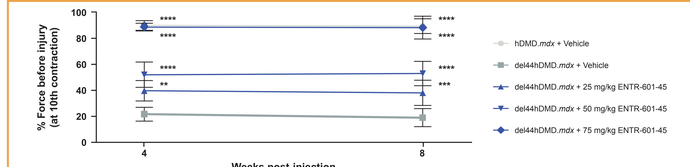
Figure 5. Improved Skeletal Muscle Membrane Stability With Q6W ENTR-601-45 in del44hDMD.*mdx* Mice.



del44hDMD.*mdx* mice were treated with three Q6W IV injections of ENTR-601-45 at 25, 50, or 75 mg/kg or vehicle. ECC-induced muscle force loss generated by repeated ECC contraction of the gastrocnemius muscle was assessed 5 weeks after the third dose. Data (mean ± standard deviation) shown across 10 ECC contractions normalized into 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 del44hDMD.*mdx* mice; **p≤0.01, ****p≤0.0001. ANOVA, analysis of variance; ECC, eccentric force; hDMD, human dystrophin transgene; IV, intravenous; Q6W, every 6 weeks.

- A significant and dose-dependent increase in resistance to membrane damage was observed following the tenth contraction with all doses of ENTR-601-45 five weeks after the third Q6W dose (**Figure 5**). The 75 mg/kg treatment group was indistinguishable from the hDMD.*mdx* controls.
- In a washout study, resistance to membrane damage was maintained until at least 8 weeks after the third dose with all 3 doses of ENTR-601-45 (**Figure 6**).

Figure 6. Retention of Skeletal Muscle Membrane Stability With Q6W ENTR-601-45 in del44hDMD.*mdx* Mice.



del44hDMD.*mdx* mice were treated with three Q6W IV injections of ENTR-601-45 at 25, 50, or 75 mg/kg or vehicle. Eccentric (ECC)-induced muscle force loss generated by repeated ECC contraction of the gastrocnemius muscle was assessed 4 and 8 weeks after the third dose. Data (mean ± standard deviation) shown as the percentage of force retained after the 10th contraction. Vehicle-treated hDMD.*mdx* mice were used as a control group for normal muscle function. Two-way ANOVA was used for statistical comparison to vehicle-treated del44hDMD.*mdx* mice within each timepoint; **p≤0.01, ***p≤0.001, ****p≤0.0001 vs. vehicle. ANOVA, analysis of variance; hDMD, human dystrophin transgene; IV, intravenous; Q6W, every 6 weeks.