Preclinical Efficacy of ENTR-601-51 for the Treatment of Exon 51 Skip–Amenable Duchenne Muscular Dystrophy

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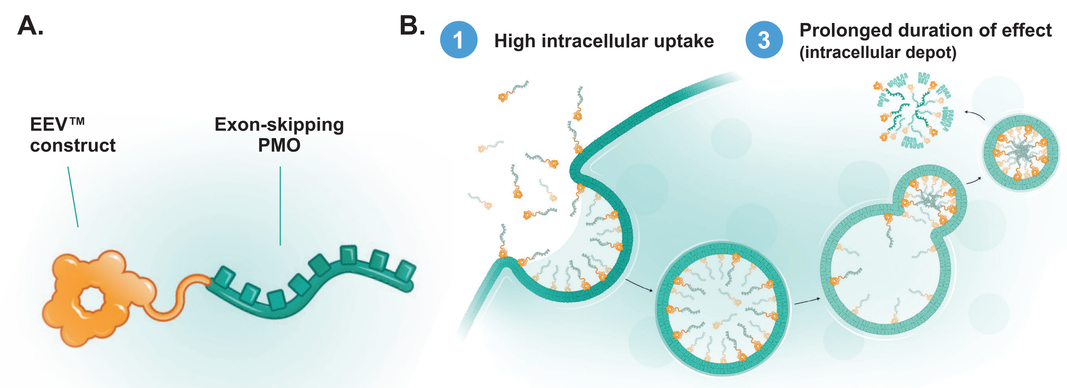
BACKGROUND

- 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-601-51, an exon 51 skipping PMO conjugated to an EEV cyclic peptide, in development for the treatment of DMD amenable to exon 51 skipping.

MATERIALS AND METHODS

- ENTR-601-51 is a *DMD* exon 51 skipping PMO conjugated to an EEV cyclic peptide and is in development for the treatment of exon 51 skip–amenable DMD.
- DMDΔ52 induced pluripotent stem cells (iPSC) were genetically engineered to contain a deletion of DMD exon 52 from a normal control iPSC line. These cell lines also contain a doxycycline-inducible myogenic differentiation 1 gene to allow differentiation into skeletal muscle cells.
- Skeletal muscle cells were derived from patients with exon 51 skip–amenable *DMD* harboring a deletion of *DMD* exons 48-50 (DMDΔ48-50), exons 45-50 (DMDΔ45-50), or exon 50 (DMDΔ50).
- Exon skipping efficiency was analyzed by digital droplet polymerase chain reaction (ddPCR) (Bio-Rad, Hercules, CA). Dystrophin restoration was evaluated by Simple Western Jess (Bio-Techne, Minneapolis, MN).
- Del52hDMD.*mdx* are human dystrophin (hDMD)–expressing mice engineered with a deletion in the hDMD exon 52 transgene on the *mdx* background, resulting in an exon 51 skip–amenable mouse line.[6] hDMD.*mdx* mice were used as healthy controls for dystrophin quantification and muscle function, as they contain a normal hDMD transgene on the *mdx* background.
- 20X immunohistochemistry images were acquired with an Olympus VS200 Slide Scanner System and quantified with Halo Image Analysis Platform Software (Indica Labs, Albuquerque, NM).
- In vivo specific tetanic torque and resistance toward eccentric (ECC)-induced muscle damage via repeated ECC contractions was measured in the gastrocnemius muscle bundle using a 3-in-1 Whole Animal Muscle Physiology system (Aurora Scientific, Aurora, ON).

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

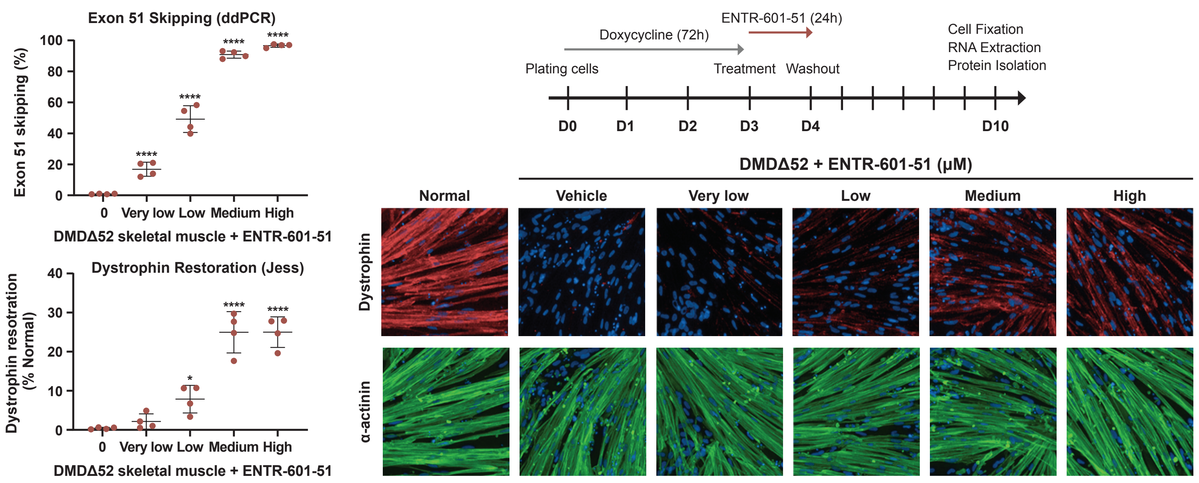
- To assess the efficacy and therapeutic potential of ENTR-601-51 in human cell and mouse models of DMD amenable to exon 51 skipping.

RESULTS

Exon Skipping and Dystrophin Restoration With ENTR-601-51 in iPSC-Derived Muscle Cells

- Treatment of iPSC-derived muscle cells with ENTR-601-51 resulted in dose-dependent *DMD* exon 51 skipping and dystrophin protein expression.

Figure 2. Efficacy of ENTR-601-51 in Engineered DMDΔ52 iPSC-Derived Myotubes



DMDΔ52 engineered skeletal muscle cells were incubated with ENTR-601-51 for 24 hours after 3 days of differentiation and analyzed after an additional 6 days of differentiation. Dystrophin protein is shown in red, α-actinin in green, and nuclei in blue. Exon skipping was calculated as the concentration of the skipped product divided by the sum of the concentration of the skipped and unskipped products. Dystrophin protein restoration as measured by Simple Western Jess was calculated as the percent of the dystrophin protein observed in the isogenic normal control after normalizing to total protein loaded. Each data point represents the mean of 4 replicates performed in independent experiments. Data are shown as mean ± standard deviation; one-way ANOVA with Dunnett's multiple comparisons test, compared to untreated DMDΔ52 cells was used for exon skipping and dystrophin restoration (n=4 per cohort); **p*<0.05, ****p*<0.001, *****p*<0.0001. ANOVA, analysis of variance; DMD, Duchenne muscular dystrophy; iPSC, induced pluripotent stem cells.

Exon Skipping and Dystrophin Protein Restoration Across Multiple Exon 51 Skip–Amenable Genotypes

- ENTR-601-51 drives exon 51 skipping and promotes dystrophin restoration in DMDΔ45-50, DMDΔ48-50, DMDΔ50, and DMDΔ52 cell lines (**Table 1**).

Table 1. ENTR-601-51 in Multiple Exon 51 Skip–Amenable Myotubes

Dose response	Myotube genotype			
	Patient-derived			iPSC-engineered
	DMDΔ45-50	DMDΔ48-50	DMDΔ50	DMDΔ52
Exon 51 skipping	✓	✓	✓	✓
Dystrophin protein restoration	✓	✓	✓	✓

DMD, Duchenne muscular dystrophy; iPSC, induced pluripotent stem cells.

Exon Skipping and Dystrophin Production With ENTR-601-51 in Mice With an Exon 51 Skip–Amenable Mutation

- Three Q6W doses of ENTR-601-51 lead to a significant, dose-dependent increase in exon 51 skipping (A) and dystrophin protein restoration (B) in skeletal and cardiac muscles in del52hDMD.*mdx* mice (**Figure 3**).

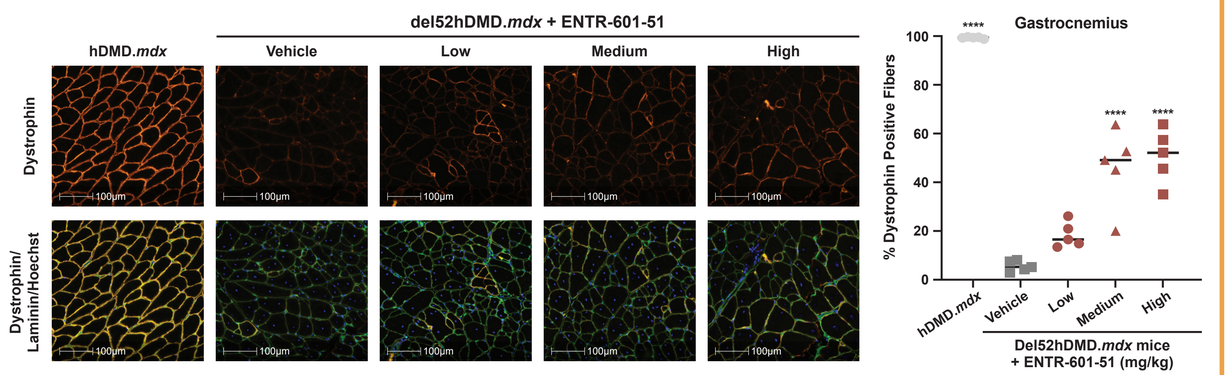
Figure 3. Efficacy of ENTR-601-51 in Del52hDMD.*mdx* Mice

del52hDMD.*mdx* mice were treated with 3 IV injections of ENTR-601-51 or vehicle 6 weeks apart. Human DMD exon 51 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 with Dunnett's post-test was used for statistical comparison; **p*≤0.01, ***p*≤0.01, *****p*≤0.0001 vs. vehicle. ANOVA, analysis of variance; hDMD, human dystrophin transgene; IV, intravenous.

Quantification of Dystrophin-Positive Fibers in Skeletal Muscles With ENTR-601-51 in Mice With an Exon 51 Skip–Amenable Mutation

- Three Q6W doses of ENTR-601-51 produced significant, dose-dependent increases in dystrophin-positive muscle fibers in the gastrocnemius. Dystrophin was localized at the sarcolemma and evenly distributed throughout the muscle (**Figure 4**).

Figure 4. Dystrophin Distribution and Localization in Gastrocnemius Muscle of del52hDMD.*mdx* Mice

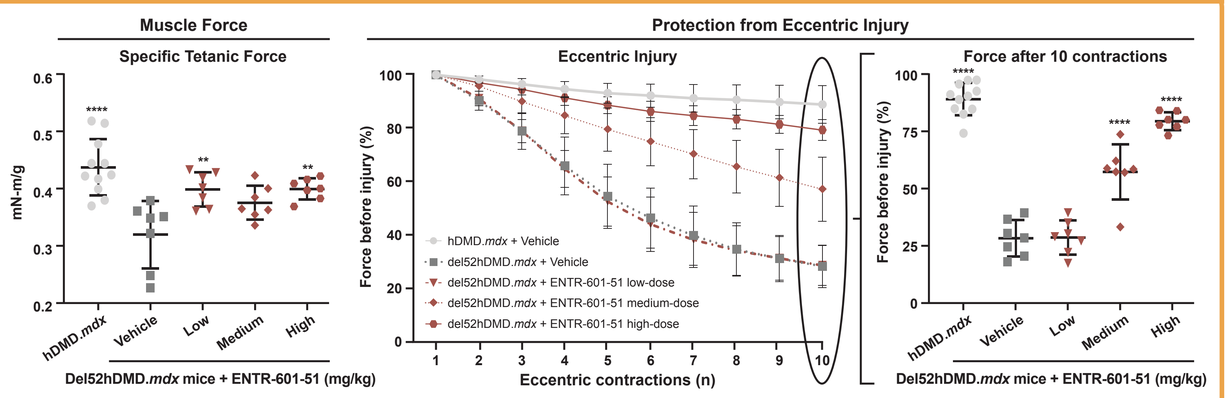


del52hDMD.*mdx* mice were treated with 3 IV injections of ENTR-601-51 or vehicle 6 weeks apart. Tissues were collected 6 weeks after the final dose. Quantification via Halo Image Analysis Software is shown as the percentage of dystrophin-positive muscle fibers relative to the total number of muscle fibers, as determined by laminin staining (green) and dystrophin staining (red); co-localization to the sarcolemma appears yellow, and nuclei appear blue. Representative images are shown. Data shown as mean ± standard deviation. One-way ANOVA with Dunnett's post-test was used for statistical comparison; *****p*≤0.0001 vs. vehicle. ANOVA, analysis of variance; hDMD, human dystrophin transgene; IV, intravenous.

Treatment With ENTR-601-51 Improves Muscle Function in Exon 51 Skip–Amendable Mice

- ENTR-601-51 produced significant, dose-dependent increases in specific tetanic force and provides protection from force loss following repeated ECC contractions (**Figure 5**).

Figure 5. Improved Skeletal Muscle Function With ENTR-601-51 in del52hDMD.*mdx* Mice



del52hDMD.*mdx* mice were treated with 3 IV injections of ENTR-601-51 or vehicle 6 weeks apart. Functional assessments were measured 5 weeks after the final dose. Tetanic force was measured at 150 Hz. A series of 10 ECC contractions were performed by lengthening the muscle 20% while under tetanic contraction. ECC contractions were normalized to the peak force at the first contraction. All measurements were normalized to body weight. One-way ANOVA with Dunnett's post-test was used for statistical comparison; ***p*≤0.01, *****p*≤0.0001 vs. vehicle. ANOVA, analysis of variance; ECC, eccentric; hDMD, human dystrophin transgene; IV, intravenous.

CONCLUSIONS

- ENTR-601-51 demonstrated significant dose-dependent exon skipping and dystrophin production in both in vitro (cell-based) and in vivo (mouse) models of DMD amenable to exon 51 skipping.
- Cell lines with different exon 51 skip–amenable mutations all demonstrated exon 51 skipping and dystrophin protein production upon exposure to ENTR-601-51.
- In mice treated with ENTR-601-51, dystrophin protein was correctly localized at the sarcolemma of muscle fibers and evenly distributed throughout the muscle.
- Treatment with ENTR-601-51 resulted in the expression of functional dystrophin, as evidenced by enhanced skeletal muscle function in a mouse model amenable to human exon 51 skipping.
- These findings demonstrate the therapeutic potential of ENTR-601-51 and support further studies in patients with DMD amenable to exon 51 skipping.

ACKNOWLEDGMENTS

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