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

PP01.37

Michael St. Andre, Nick Long, Xiang Li, Mahasweta Girgenrath, Mary Lou Beermann, Jia Qi Cheng-Zhang, Mohanraj Dhanabal, Haoming Liu, **Christopher M. Brennan**, Pauline Tan, Yongchao Mou, Terrance A. Stadheim, Wenlong Lian

Entrada Therapeutics, Boston, MA

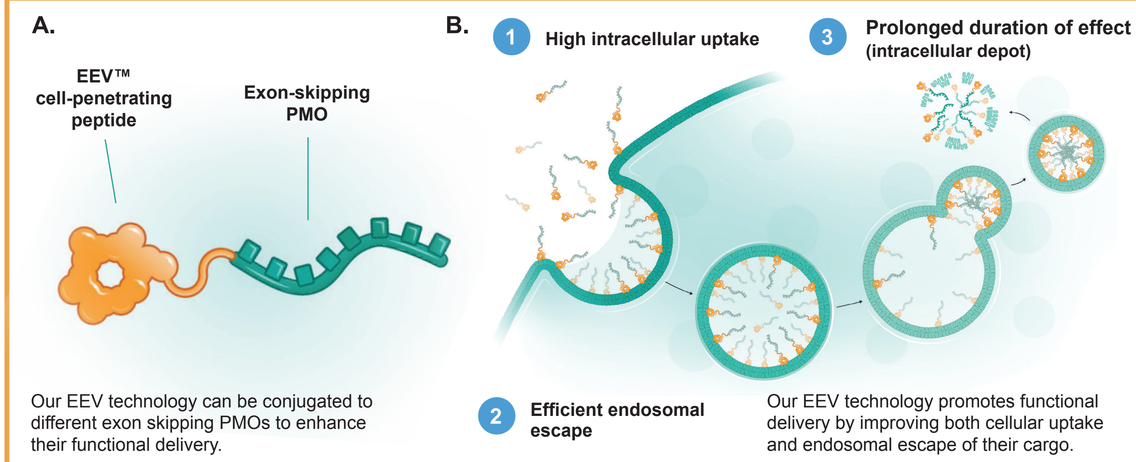
BACKGROUND

- 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]

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 patients with exon 51 skip–amenable DMD.
- DMDΔ52 induced pluripotent stem cells (iPSC) were genetically engineered from a normal control iPSC line to include a deletion of DMD exon 52. These cell lines also contain a doxycycline-inducible myogenic differentiation 1 gene to allow differentiation into skeletal muscle cells.
- Additional myogenic cells were derived from primary myogenic cells isolated 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 on the *mdx* background, resulting in an exon 51 skip–amenable mouse line.[6] hDMD.*mdx* mice carrying the full-length hDMD were used as healthy controls for dystrophin quantification and muscle function.
- 20× or 40× 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 force and protection from eccentric (ECC)-induced muscle damage in response to repeated ECC contractions force and protection were measured in the gastrocnemius muscle 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

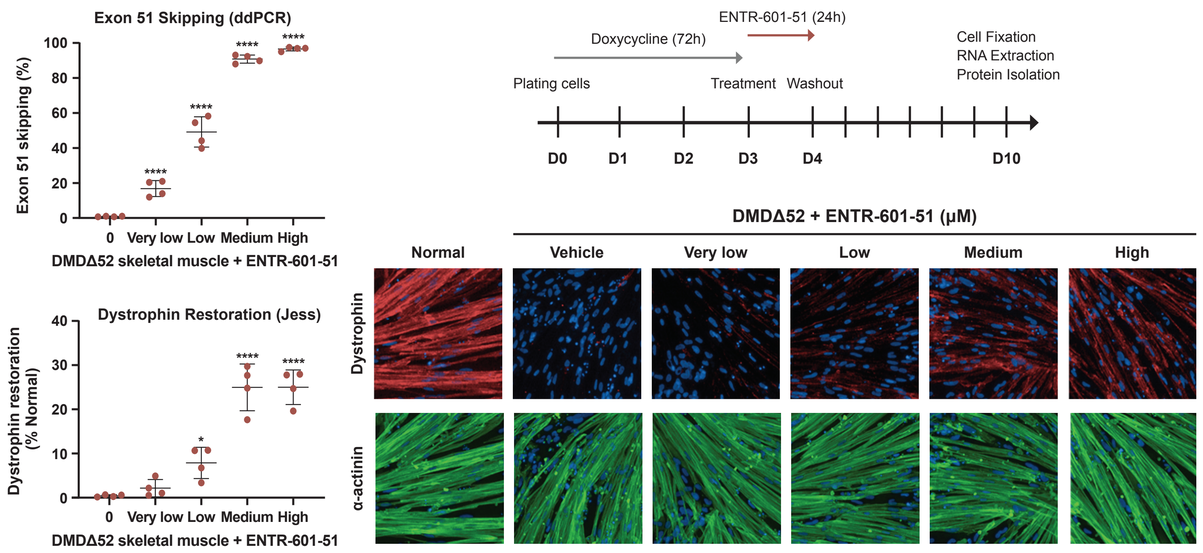
- To assess the efficacy and therapeutic potential of ENTR-601-51 in human cell and mouse models of DMD amenable to exon 51 skipping.

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**).

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

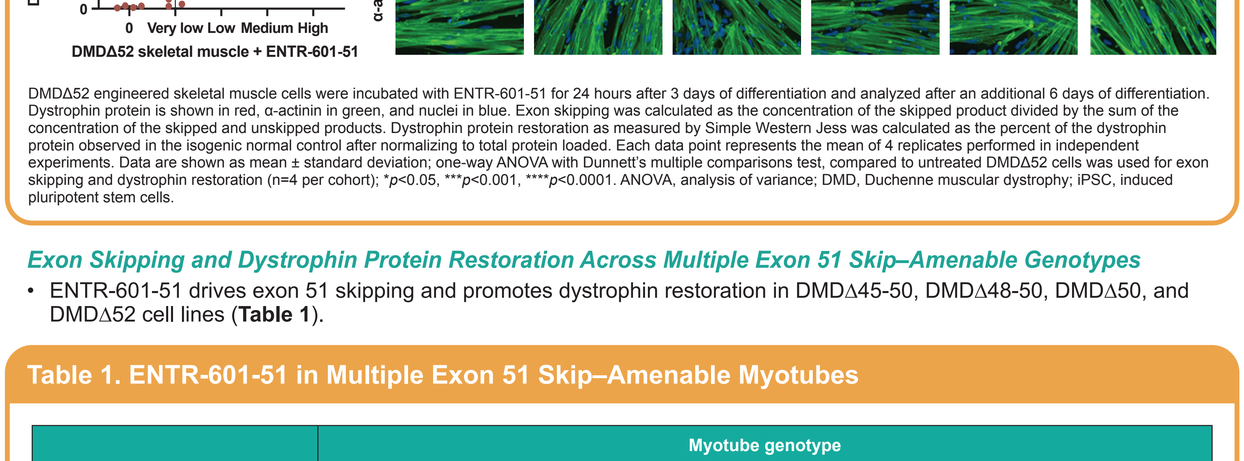
Dose response	Myotube genotype			
	Patient-derived			iPSC-engineered
	DMDΔ45-50	DMDΔ48-50	DMDΔ50	DMDΔ52
Exon 51 skipping	✓	✓	✓	✓
Dystrophin protein restoration	✓	✓	✓	✓

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

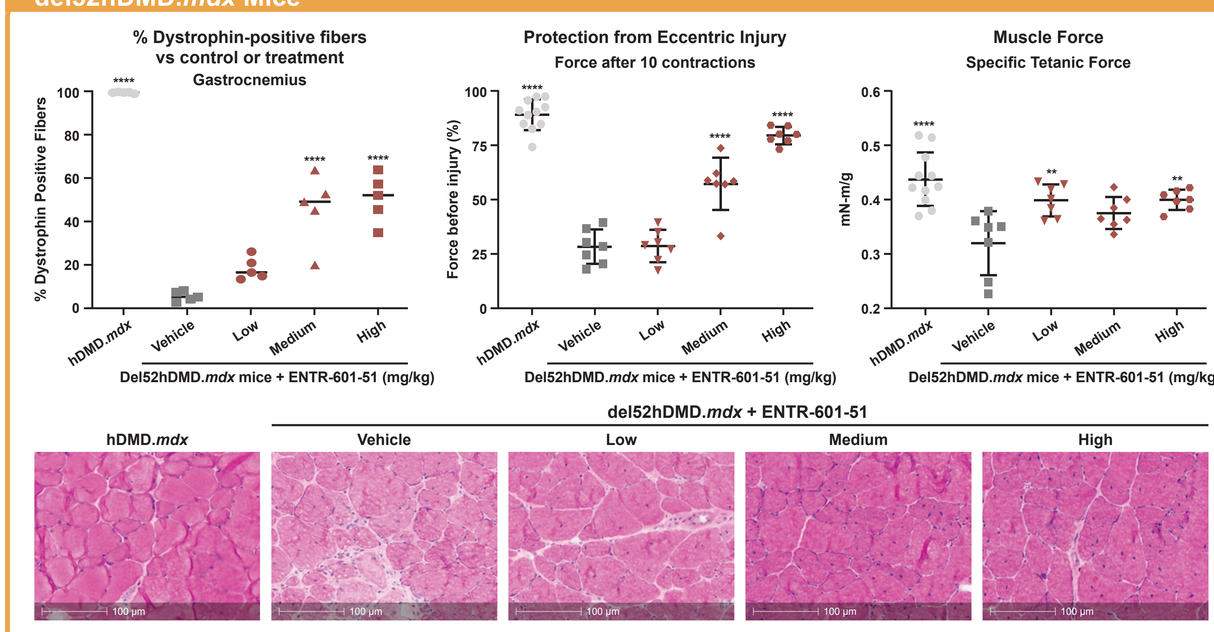


del52hDMD.*mdx* mice were treated with 3 IV injections of ENTR-601-51 or vehicle 6 weeks apart. Human DMD exon 51 skipping (A) and dystrophin protein expression (B) were analyzed in the gastrocnemius, diaphragm, and heart 6 weeks after the final dose. Percent dystrophin protein restoration is normalized 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.

Treatment With ENTR-601-51 Increases Dystrophin-Positive Fibers and Improves Muscle Function in Exon 51 Skip–Amendable Mice

- Three Q6W doses of ENTR-601-51 produced significant, dose-dependent increases in dystrophin-positive muscle fibers in the gastrocnemius.
- ENTR-601-51 also produced significant, dose-dependent increases in specific tetanic force and provides protection from force loss following repeated ECC contractions (**Figure 4**).
  - Histological staining of gastrocnemius muscle shows reductions in interstitial spacing and decreased numbers of infiltrating cells in del52hDMD.*mdx* mice treated with medium and high doses of ENTR-601-51.

Figure 4. Improved Skeletal Muscle Morphology and Function With ENTR-601-51 in del52hDMD.*mdx* Mice

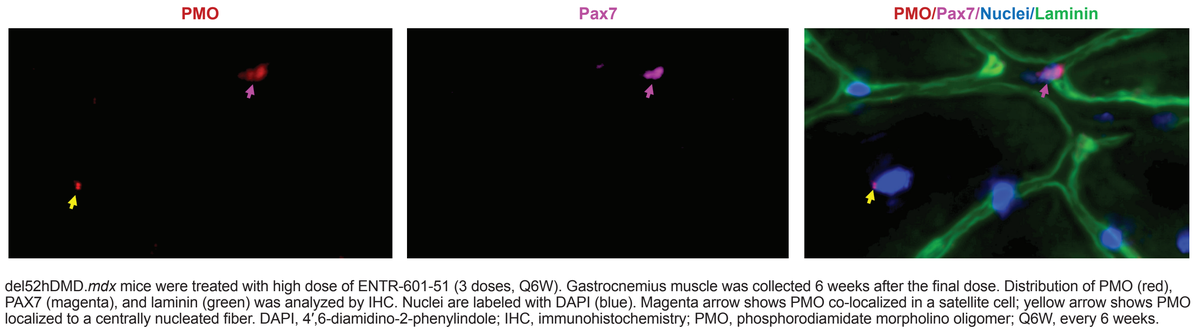


del52hDMD.*mdx* mice were treated with 3 IV injections of ENTR-601-51 or vehicle 6 weeks apart. Tissues were collected 6 weeks after the final dose. Quantification via Halo Image Analysis Software is shown as the percentage of dystrophin-positive muscle fibers relative to the total number of muscle fibers. 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. [a]H&E staining of gastrocnemius muscle sections shows reductions in interstitial spacing and infiltrating cells in del52hDMD.*mdx* mice treated with medium and high doses of ENTR-601-561. One-way ANOVA with Dunnett's post-test was used for statistical comparisons; ***p*≤0.01, *****p*≤0.0001 vs. vehicle. ANOVA, analysis of variance; ECC, eccentric; hDMD, human dystrophin transgene; H&E, hematoxylin and eosin; IV, intravenous.

ENTR-601-51 PMO Localization in Mice With an Exon 51 Skip–Amenable Mutation

- After administration of ENTR-601-51 (3 doses, Q6W) to del52hDMD.*mdx* mice, PMO localized in nucleated fibers and satellite cells (Pax7 positive) in gastrocnemius muscle (**Figure 5**).

Figure 5. PMO Localization in Centrally Nucleated Fibers and Satellite Cells in Gastrocnemius Muscle of del52hDMD.*mdx* Mice



del52hDMD.*mdx* mice were treated with high dose of ENTR-601-51 (3 doses, Q6W). Gastrocnemius muscle was collected 6 weeks after the final dose. Distribution of PMO (red), PAX7 (magenta), and laminin (green) was analyzed by IHC. Nuclei are labeled with DAPI (blue). Magenta arrow shows PMO co-localized in a satellite cell; yellow arrow shows PMO localized to a centrally nucleated fiber. DAPI, 4′,6-diamidino-2-phenylindole; IHC, immunohistochemistry; PMO, phosphorodiamidate morpholino oligomer; Q6W, every 6 weeks.

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.
- All cell lines with different mutations amenable to 51 skipping demonstrated efficient exon 51 skipping and dystrophin protein production upon exposure to ENTR-601-51.
- Treatment with ENTR-601-51 resulted in the expression of partially functional dystrophin, as evidenced by restoration of tissue architecture and enhanced skeletal muscle function in the deletion 52 mouse model.
- ENTR-601-51 PMO were co-localized in the satellite cells and in the centrally nucleated fibers.
- These findings demonstrate the therapeutic potential of ENTR-601-51 and support further studies in patients with DMD amenable to exon 51 skipping.

ACKNOWLEDGMENTS

Conflicts of interest: all authors are employees of Entrada Therapeutics, Inc. This research was funded by Entrada Therapeutics, Inc (Boston, MA). Editorial and studio support for this poster were provided by Ashfield MedComms (US), an Inizio company, and were funded by Entrada Therapeutics, Inc. 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, et al. MDA 2022. Poster 126. 6. Yavas A, et al. *PLoS One*, 2020.