A Novel EEV-Conjugated PMO, ENTR-701, Reduces Nuclear Foci and Corrects Aberrant Splicing in Myotonic Dystrophy Type 1 Preclinical Models

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INTRODUCTION

- Myotonic dystrophy type 1 (DM1) is a multi-systemic disease that affects over 40,000 individuals in the US and 50,000 in Europe.
  - Pathology manifests as myotonia, muscle weakness and atrophy, cardiac conduction abnormalities, pulmonary complications, cataracts, and endocrine dysfunction.[1]
  - Currently there are no approved therapies for DM1.
- DM1 is caused by a CUG trinucleotide repeat expansion in the *DMPK* (dystrophia myotonica protein kinase) mRNA that sequesters splicing regulatory proteins such as MBNL (muscleblind-like).[2]
  - Mutant *DMPK* mRNA and MBNL proteins aggregate to form nuclear foci.[3] MBNL splicing activity is decreased as a result of nuclear sequestration, thereby inhibiting splicing and expression of many downstream transcripts.[4,5]
- One therapeutic approach for treatment of DM1 is the use of oligonucleotide therapeutics to sterically block CUG repeat expansions.
  - There are significant barriers to the development of oligonucleotide-based therapies such as limited exposure and poor endosomal escape in target tissue after systemic administration.
- We developed an Endosomal Escape Vehicle (EEV™) delivery platform based on cyclic cell-penetrating peptides (cCPPs) to improve cellular uptake and enhance endosomal escape of oligonucleotides and other biologics.[6,7]
  - ENTR-701 is our lead clinical candidate for the treatment of DM1.

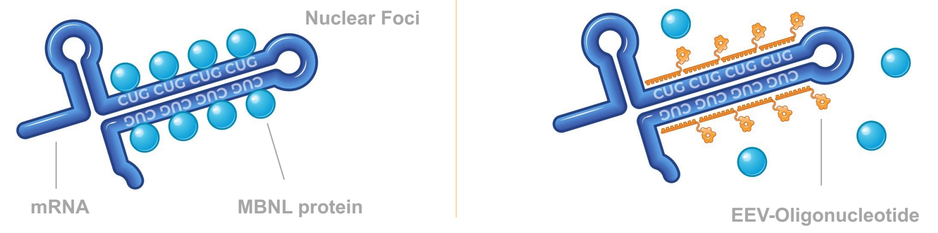
OBJECTIVES

- To examine the therapeutic potential of ENTR-701 in preclinical models of DM1.

MATERIALS AND METHODS

- ENTR-701 consists of a phosphorodiamidate morpholino oligomer (PMO) that blocks CUG repeats in an allele-specific manner, conjugated to our EEV platform (**Figure 1**).
- Nuclear foci reduction, mutant transcript levels, and correction of aberrant splicing were assessed in cellular models and HSA-LR mice.
  - HeLa480 cells stably expressing either (CTG)480 or (CTG)0 DMPK transgenes showed MBNL1-dependent aberrant splicing.[8]
  - Immortalized myoblasts were derived from DM1 patient primary skeletal muscle cells and contain 2,600 CUG repeats within the 3'UTR of *DMPK*.[9]
  - HSA-LR[10] mice carry a transgene with a (CTG)220 repeat expansion in the 3'-UTR of the human skeletal actin gene (ACTA1) which recapitulates molecular pathology and results in a myotonia phenotype.

Figure 1. Mechanism of EEV-PMO Conjugate in DM1.



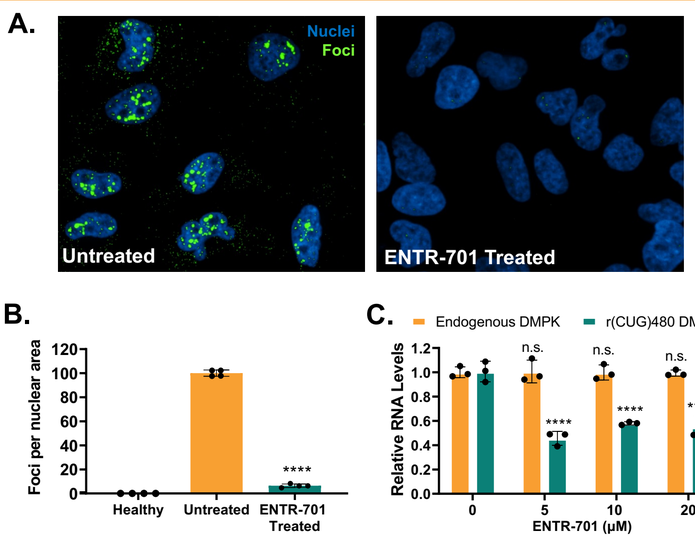
Steric blockade of CUG repeats on mutant *DMPK* mRNA leads to reduced nuclear foci, selective mRNA reduction, normal MBNL function, and corrected spliceopathy.

RESULTS

ENTR-701 in HeLa480 Mutant DMPK Cell Line

ENTR-701 reduced nuclear foci in HeLa480 cells (**Figures 2A, 2B**). In addition, free uptake of ENTR-701 selectively reduced (CUG)480 containing *DMPK* mRNA (**Figure 2C**).

Figure 2. ENTR-701 Reduces Nuclear Foci and Mutant mRNA in HeLa480 Cells.

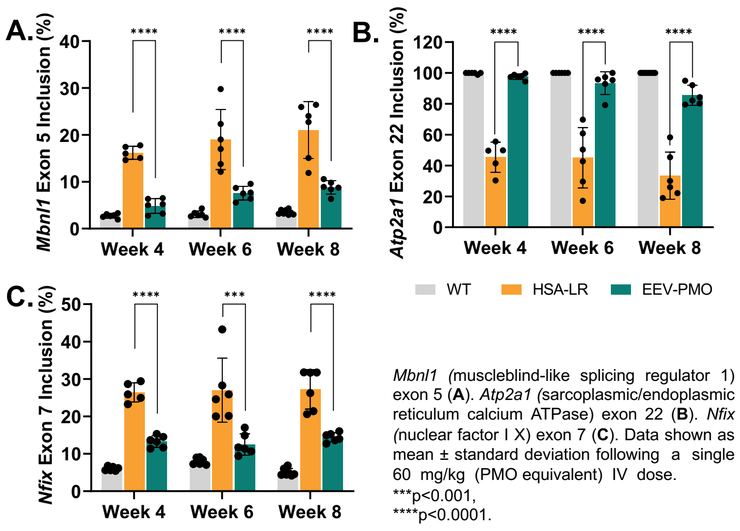


HeLa480 cells were treated with ENTR-701 and number of nuclear foci were analyzed by were quantitated relative to nuclear area and normalized to untreated cells (**A, B**). *DMPK* transcript levels were measured by qPCR. Mutant r(CUG)480 levels were normalized to untreated samples (**C**). Data shown as mean ± standard deviation. ****p<0.0001 vs. untreated (**B**) and vs. 0 μM (**C**), n.s., not significant compared to 0 μM (panel C).

Durable Efficacy of ENTR-701 in HSA-LR Mice

A single dose of ENTR-701 corrected aberrant splicing of *Mbnl1* (**Figure 5A**), *Atp2a1* (**Figure 5B**), and *Nfix* (**Figure 5C**) for at least 8 weeks post-dose.

Figure 5. Durable Splicing Correction in HSA-LR Mouse Tibialis Anterior Muscle With a Single Dose of ENTR-701.



Mbnl1 (muscleblind-like splicing regulator 1) exon 5 (**A**). *Atp2a1 (*sarcoplasmic/endoplasmic reticulum calcium ATPase) exon 22 (**B**). *Nfix (*nuclear factor I X) exon 7 (**C**). Data shown as mean ± standard deviation following a single 60 mg/kg (PMO equivalent) IV dose.
***p<0.001,
****p<0.0001.

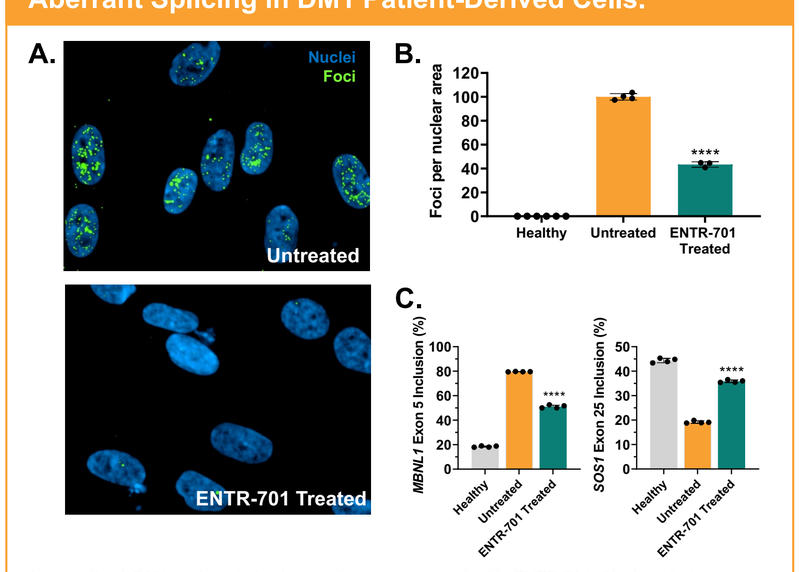
ENTR-701 Rescues the Myotonia Phenotype in HSA-LR mice

A single 60 mpk (PMO equivalent) dose of ENTR-701 ameliorated observable pinch-induced myotonia symptoms for at least 8 weeks post-dose.

Treatment of DM1 Patient-Derived Myotubes with ENTR-701

ENTR-701 treatment in DM1 patient-derived muscle cells resulted in significant nuclear foci reduction (**Figures 3A, 3B**) and correction of aberrant splicing (**Figure 3C**).

Figure 3. ENTR-701 Reduces Nuclear Foci and Correct Aberrant Splicing in DM1 Patient-Derived Cells.

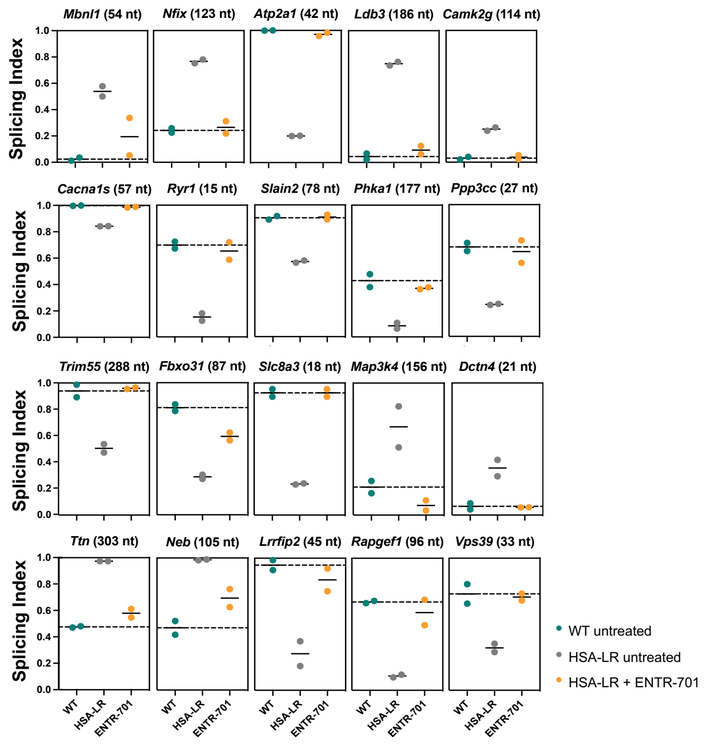


Immortalized DM1 patient-derived myotubes were treated with ENTR-701. Nuclear foci area was quantitated relative to total nuclear area and normalized to untreated cells (**A, B**). *MBNL1* exon 5 and *SOS1* exon 25 inclusion were analyzed by RT- PCR and Labchip (**C**). Data shown as mean ± standard deviation. ****p<0.0001 vs. untreated.

Spliceopathy in HSA-LR mice corrected with ENTR-701

RNAseq data illustrates that HSA-LR splicing defects are corrected after treatment with ENTR-701 (**Figure 6**).

Figure 6. Splicing Correction in HSA-LR Mouse Gastrocnemius Muscle With a Single IV Dose of ENTR-701.

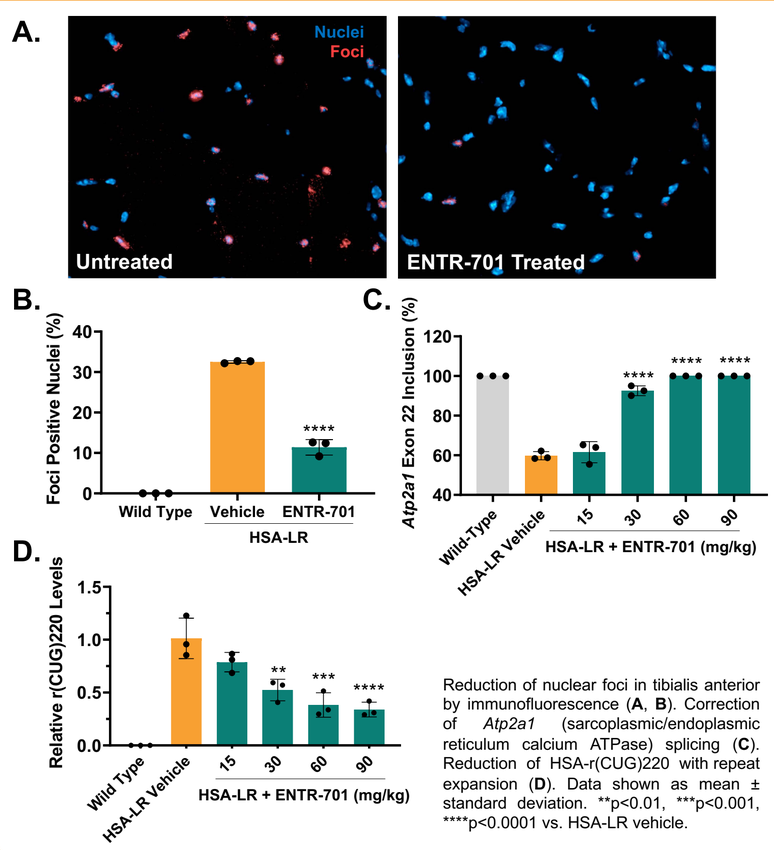


HSA-LR mice administered 60 mg/kg PMO equivalent ENTR-701 IV. RNAseq data was normalized to transcriptome.

Efficacy of ENTR-701 in HSA-LR Mouse Model of DM1

ENTR-701 treatment reduced the number of nuclear foci (**Figure 4A, 4B**), corrected aberrant splicing of *Atp2a1* (**Figure 4C**) and reduced HSA-LR(CUG)220 mRNA in HSA-LR mice (**Figure 4D**).

Figure 4. Efficacy of ENTR-701 in HSA-LR Mice.



Reduction of nuclear foci in tibialis anterior by immunofluorescence (**A**, **B**). Correction of *Atp2a1* (sarcoplasmic/endoplasmic reticulum calcium ATPase) splicing (**C**). Reduction of HSA-r(CUG)220 with repeat expansion (**D**). Data shown as mean ± standard deviation. **p<0.01, ***p<0.001, ****p<0.0001 vs. HSA-LR vehicle.

CONCLUSIONS

- Our DM1 clinical candidate, ENTR-701, reduces nuclear foci and CUG-repeat expansion containing transcript levels in the HeLa480 cell model and the HSA-LR mouse model of DM1.
- DM1 patient-derived muscle cells with 2600 CUG repeats also showed a reduction in nuclear foci when treated with ENTR-701.
- ENTR-701 corrected aberrant downstream splicing in all three models.
- A single dose of ENTR-701 demonstrates durable splicing correction and ameliorates myotonia for at least 8 weeks post-dose.
- These results illustrate the therapeutic potential of the EEV-oligonucleotide approach for DM1 and support further study of ENTR-701 in patients with DM1.

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