Development of Endosomal Escape Vehicle Constructs for Enhanced Delivery of Antisense Oligonucleotides to Muscle Tissues

P286

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INTRODUCTION

- Intracellular delivery of biologics (such as oligonucleotides, peptides, and proteins) is challenging because of poor cellular entry and limited endosomal escape. Because ≈75% of disease-causing drug targets are located inside the cell,[1] the development of an efficient delivery vehicle is critical to the clinical success of intracellular therapeutics.
- To address these challenges, we developed a family of cyclic cell-penetrating peptides that form the core of our Endosomal Escape Vehicle (EEV™) platform (**Figure 1**).
- EEV peptides have been shown to efficiently deliver exon skipping phosphorodiamidate morpholino oligomers (PMOs) to skeletal and cardiac muscle cells in animal models of Duchenne muscular dystrophy (DMD).[2]
- A phase 1 clinical trial of ENTR-601-44, an EEV-*DMD* exon 44 skipping antisense oligonucleotide construct, in healthy volunteers demonstrated favorable target exposure and engagement in skeletal muscle with no adverse events related to treatment.[3]
- Here, we used a medicinal chemistry approach to identify an optimized EEV construct by investigating the role of different residues in the cyclic peptide ring. The optimized EEV construct demonstrated enhanced functional delivery of PMOs, as quantified by robust exon skipping and dystrophin protein restoration to muscles of *mdx* mice, a mouse model of DMD.

OBJECTIVES

- To design and evaluate EEV analogs to optimize oligonucleotide delivery.
- To demonstrate proof-of-concept functional delivery in a murine disease model.

MATERIALS AND METHODS

EEV Library Design and Screening

- Alanine scanning was performed to identify key residues that contributed to the cell-penetration activity of a first-generation EEV construct, EEV1[2,4] (**Figure 2**).
- EEV constructs were screened using the Chloroalkane Penetration Assay (CAPA),[5] in HEK293 cells stably expressing HaloTag-Actin fusion protein (**Figure 2**). High content imaging was used to quantify fluorescence and calculate CP_{50} values (**Tables 1** and **2**). Further optimizations were carried out to achieve rationally designed EEV peptides with desirable properties (**Figure 3**).

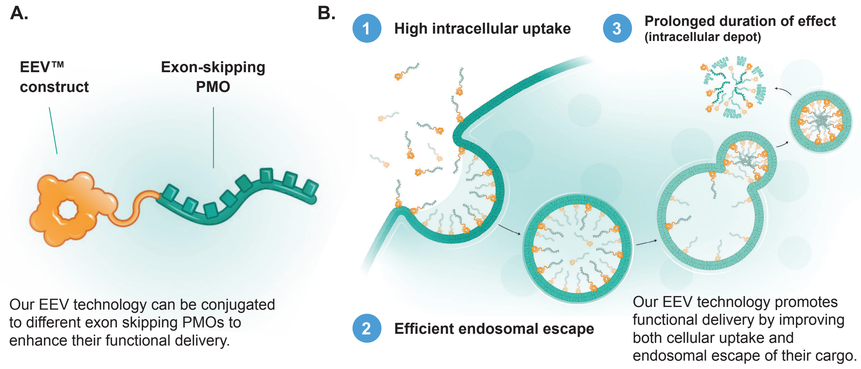
Functional Delivery of Cargo

- To assess functional cargo delivery in vitro, a HeLa-EGFP654 reporter cell line was used, which expresses a non-fluorescent enhanced green fluorescent protein (EGFP) due to the inclusion of a mutated intron of β-globin (IVS2-654) in the *EGFP* gene. Delivery of PMO654—which targets the cryptic splice site in EGFP654 pre-messenger RNA (mRNA)—to the nucleus results in splice correction and restoration of fluorescent EGFP expression. EEV3 was conjugated to PMO654 and assessed for functional nuclear delivery by quantifying EGFP fluorescence (high content imaging) (**Figure 4**).
- EEV3-PMO654 was administered to mice that stably and ubiquitously express the interrupted EGFP-654 gene, to assess functional delivery and tissue accumulation in vivo. The percentage of splice-corrected *EGFP* mRNA transcripts was quantified by reverse transcription–polymerase chain reaction (RT-PCR), and EGFP protein was quantified by capillary electrophoresis and immunodetection (**Figure 5**).

PMO Delivery in mdx *Mice*

- The efficacy of EEV3 was tested in a disease-relevant mouse model, *mdx*, a murine model of DMD. These mice possess a nonsense mutation in exon 23 of the *Dmd* gene, resulting in arrested dystrophin protein production and progressive muscle degeneration. EEV3 was conjugated to PMO23, which binds to the *DMD* pre-mRNA to skip exon 23. Nuclear delivery of the PMO results in a restored reading frame and subsequent expression of a truncated, but functional, dystrophin protein. The delivery efficiency of EEV3-PMO23 was assessed by calculating exon 23 skipping efficacy in different tissues (RT-PCR), and duration of effect of dystrophin protein level restoration was determined by capillary electrophoresis and immunodetection (**Figure 6**).

Figure 1. EEV-PMO Structure and Mechanism of Action.



(A) Illustration of EEV-PMO structure consisting of a cyclic cell-penetrating peptide-based EEV and an exon-skipping PMO. **(B)** Mechanism of EEV-mediated PMO delivery. EEV, Endosomal Escape Vehicle; PMO, phosphorodiamidate morpholino oligomer.

RESULTS

EEV Alanine Scanning Revealed Residues Important for Delivery

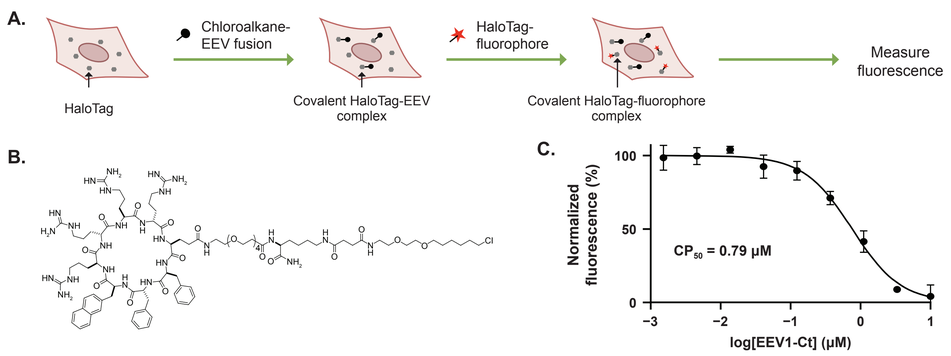
- Alanine analogs of EEV1, an efficacious first-generation EEV,[2,4] were screened using the Chloroalkane Penetration Assay (CAPA)[5] (**Figure 2**).
- Phenylalanine was important for efficacy (**Table 1**), while changes to the four arginine residues were well tolerated.
- One or more arginine residues could be omitted from an EEV1 derivative, which may potentially improve the in vivo tolerability of the resulting compound.

Table 1. Alanine Analogs of EEV1.

Compound	EEV sequence	CP_{50} (μM)
EEV1	*cyclo*(FfΦRrRrQ)	0.79±0.06
EEV1-F1A	*cyclo*(AfΦRrRrQ)	0.87±0.098
EEV1-f2a	*cyclo*(FaΦRrRrQ)	0.90±0.23
EEV1-Φ3A	*cyclo*(FfARrRrQ)	0.44±0.060
EEV1-R4A	*cyclo*(FfΦArRrQ)	0.88±0.27
EEV1-r5a	*cyclo*(FfΦRaRrQ)	0.76±0.17
EEV1-R6A	*cyclo*(FfΦRrArQ)	0.63±0.18
EEV1-r7a	*cyclo*(FfΦRrRaQ)	0.69±0.11

CP_{50}, the concentration at which 50% cell penetration is observed; Φ, L-2-naphthylalanine; a, D-alanine; EEV, Endosomal Escape Vehicle; f, D-phenylalanine; r, D-arginine.

Figure 2. Evaluation of Cellular Uptake Efficiency by Chloroalkane Penetration Assay.



(A) Schematic overview of the chloroalkane penetration assay (CAPA). **(B)** Structure of EEV1-Ct. **(C)** Dose-dependent reduction in fluorescent signal observed with EEV1-Ct in HaloTag-expressing HEK293 cells in the chloroalkane penetration assay and its corresponding CP_{50}. CP_{50}, the concentration at which 50% cell penetration is observed; EEV, Endosomal Escape Vehicle; EEV1-Ct, chloroalkane-tagged conjugated EEV1 peptide.

Evaluation of Arginine Analogs

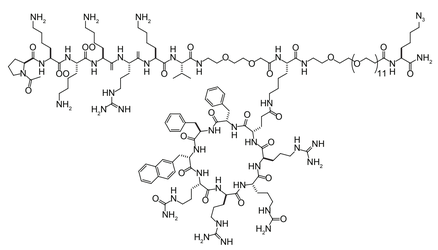
- Arginine residues were modified with a pair of alternative residues to determine whether side-chain elongation, truncation, or substitution of arginine would improve the activity of EEV1 (**Table 2**).
- Citrulline-containing cyclic cell-penetrating peptide (CPP), EEV1-E, retained efficacy while eliminating two arginine residues (thereby reducing overall cationic charge on the EEV).
- Previously, the addition of an exocyclic sequence (PKKKRKV) enhanced the delivery efficiency of EEV1.[2] We engineered the citrulline-containing analog (EEV1-E) with the same exocyclic sequence, resulting in EEV3 (**Figure 3**).

Table 2. Arginine Analogs of EEV1.

Compound	EEV sequence	CP_{50} (μM)
EEV1-A	*cyclo*(FfΦ-Agp-r-Agp-rQ)	0.63±0.19
EEV1-B	*cyclo*(FfΦ-Agb-r-Agb-rQ)	0.70±0.061
EEV1-C	*cyclo*(FfΦ-hR-r-hR-rQ)	0.79±0.26
EEV1-D	*cyclo*(FfΦ-4gp-r-4gp-rQ)	0.70±0.093
EEV1-E	*cyclo*(FfΦ-Cit-r-Cit-rQ)	0.85±0.060
EEV1-F	*cyclo*(FfΦ-Pia-r-Pia-rQ)	0.88±0.088
EEV1-G	*cyclo*(FfΦ-Dml-r-Dml-rQ)	0.93±0.10

CP_{50}, the concentration at which 50% cell penetration is observed; Φ, L-2-naphthylalanine; 4gp, L-4-guanidinophenylalanine; Agb, L-4-guanidinobutanoic acid; Agp, L-4-guanidinopropionic acid; Cit, L-citrulline; EEV, Endosomal Escape Vehicle; hR, L-homoarginine; Pia, 3-(4-pyridyl)-L-alanine; r, D-arginine.

Figure 3. Structure of EEV3.

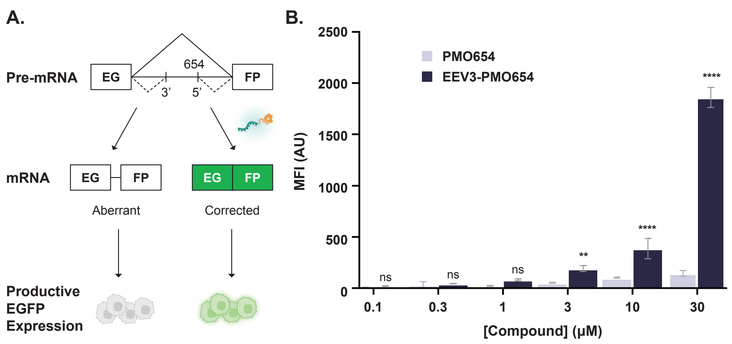


Structure of EEV3 with azide linker for PMO conjugation. EEV, Endosomal Escape Vehicle; PMO, phosphorodiamidate morpholino oligomer.

EEV3 Enabled Robust Functional Oligonucleotide Delivery in Vitro

- PMO654 delivery to HeLa-EGFP654 cells restores functional (fluorescent) EGFP protein expression (**Figure 4**).
- At 3, 10, and 30 μM, EEV3-PMO654 led to respective 3-, 4-, and 13-fold increases in EGFP levels relative to unconjugated PMO654.

Figure 4. Functional Delivery of PMO654 by EEV3 in HeLa-EGFP-654 Cells.

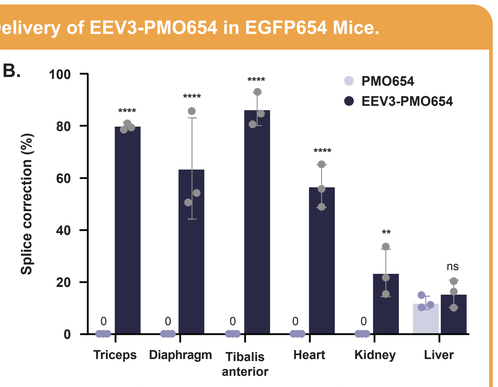


(A) Schematic overview of the EGFP654 transgene design and the mechanism of action of intracellularly delivered EEV-oligonucleotide constructs. **(B)** Comparison of the internalization efficiency at the indicated doses of EEV3-PMO and unconjugated PMO in HeLa-EGFP-654 cells quantified by the upregulation of EGFP expression as the functional readout. HeLa-EGFP-654 cells were treated with unconjugated PMO654 or conjugated EEV3-PMO654 for 24 hours before being analyzed by high content imaging for EGFP upregulation. N=3; a statistical analysis was performed using one-way ANOVA with significance given in relation to the PMO654 group; **p<0.05; ****p<0.0001. ANOVA, analysis of variance; AU, arbitrary units; EEV, Endosomal Escape Vehicle; EGFP, enhanced green fluorescent protein; MFI, mean fluorescence intensity; mRNA, messenger RNA; ns, not significant; PMO, phosphorodiamidate morpholino oligomer.

EEV3 Enabled Robust Functional Delivery of PMO654 to Skeletal and Cardiac Muscle Cells

- Intravenous (IV) administration of EEV3-PMO654 in EGFP654 mice led to significantly enhanced functional delivery in a selective manner to skeletal, cardiac, and respiratory (diaphragm/abdominal) muscles (**Figure 5**).
- Expressed EGFP protein was also quantified to confirm functional delivery (data not shown); in the triceps and tibialis anterior, the mean level of expression of EGFP induced by EEV3-PMO654 was approximately 17-fold higher than that observed with unconjugated PMO654, whereas the mean expression level in the diaphragm was 35-fold higher.

Figure 5. Functional Delivery of EEV3-PMO654 in EGFP654 Mice.

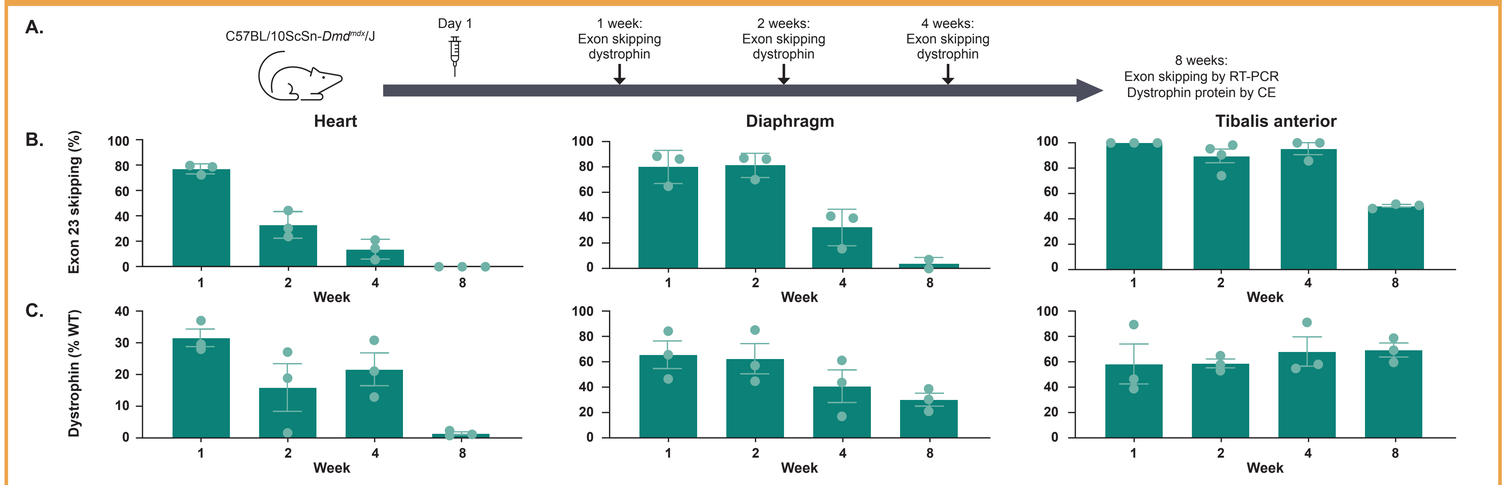


Comparison of EEV3-PMO construct and unconjugated PMO after IV injections in transgenic EGFP-654 mice as quantified by EGFP mRNA splice correction. **(A)** Study design and injection timeline; all injections administered using 10 mg/kg of the indicated compound. **(B)** Splice correction detected in the indicated tissues. Data represent the mean (SD) percentage of splice-corrected transcript as determined by RT-PCR. N=3; a statistical analysis was performed using one-way ANOVA with significance given in relation to PMO654; **p<0.01; ****p<0.0001. ANOVA, analysis of variance; CE, capillary electrophoresis; EEV, Endosomal Escape Vehicle; EGFP, enhanced green fluorescent protein; IV, intravenous; mRNA, messenger RNA; ns, not significant; PMO, phosphorodiamidate morpholino oligomer; RT-PCR, reverse transcription–polymerase chain reaction.

EEV3 Enabled Robust Functional Oligonucleotide Delivery in mdx *Mice*

- EEV3-PMO23 showed robust exon skipping and dystrophin protein expression after a single IV dose in the *mdx* mouse model for DMD (**Figure 6**).
- The sustained duration of effect of dystrophin protein production by EEV-PMO over 8 weeks supports the opportunity for less frequent dosing than currently approved unconjugated exon skipping therapies for DMD.

Figure 6. Efficacy of EEV3-PMO23 in *mdx* Mice.



Efficacy of EEV3-PMO23 in *mdx* mice after intravenous administration. **(A)** Study design and collection time points. **(B)** Duration of effect of EEV3-PMO23 induced *Dmd* mRNA exon 23 skipping in *mdx* mice after a single 40-mg/kg IV administration. Data represent the mean (SD) percentage of *Dmd* mRNA exon 23 skipping as determined by RT-PCR. **(C)** Duration of effect of dystrophin protein level restoration as determined by capillary electrophoresis and immunodetection. CE, capillary electrophoresis; EEV, Endosomal Escape Vehicle; IV, intravenous; mRNA, messenger RNA; PMO, phosphorodiamidate morpholino oligomer; RT-PCR, reverse transcription–polymerase chain reaction; WT, wild type.

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CONCLUSIONS

- Our rational medicinal chemistry approach for optimizing EEV peptides resulted in the discovery of an EEV with enhanced functional delivery of oligonucleotide cargoes to muscle tissues.
- Our findings highlight that the precise medicinal chemistry of CPPs should be an important consideration for therapeutic cargo delivery.
- The preclinical efficacy of EEV-PMO conjugates demonstrated here and in earlier work[2] supports the development of EEV-PMO constructs that are being evaluated in clinical trials for the treatment of exon 44 and 45 skip amenable DMD (ENTR-601-44 and ENTR-601-45, respectively)[6] and myotonic dystrophy type 1 (VX670).[7]