Cell-penetrating peptide–morpholino conjugates alter pre-mRNA splicing of DMD (Duchenne muscular dystrophy) and inhibit murine coronavirus replication in vivo

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Abstract

The cellular uptake of PMOs (phosphorodiamidate morpholino oligomers) can be enhanced by their conjugation to arginine-rich CPPs (cell-penetrating peptides). Here, we discuss our recent findings regarding (R-Ahx-R)4AhxB (Ahx is 6-aminohexanoic acid and B is β-alanine) CPP–PMO conjugates in DMD (Duchenne muscular dystrophy) and murine coronavirus research. An (R-Ahx-R)4AhxB–PMO conjugate was the most effective compound in inducing the correction of mutant dystrophin transcripts in myoblasts derived from a canine model of DMD. Similarly, normal levels of dystrophin expression were restored in the diaphragms of mdx mice, with treatment starting at the neonatal stage, and protein was still detectable 22 weeks after the last dose of an (R-Ahx-R)4AhxB–PMO conjugate. Effects of length, linkage and carbohydrate modification of this CPP on the delivery of a PMO were investigated in a coronavirus mouse model. An (R-Ahx-R)4AhxB–PMO conjugate effectively inhibited viral replication, in comparison with other peptides conjugated to the same PMO. Shortening the CPP length, modifying it with a mannosylated serine moiety or replacing it with the R9F2 CPP significantly decreased the efficacy of the resulting PPMO (CPP–PMO conjugate). We attribute the success of this CPP to its stability in serum and its capacity to transport PMO to RNA targets in a manner superior to that of poly-arginine CPPs.

Introduction

PMOs (phosphorodiamidate morpholino oligomers) are uncharged antisense compounds [1]. By binding to complementary RNA target sequences, PMOs can stericly block access of other biomolecules to specific motifs, thereby altering pre-mRNA splicing or inhibiting mRNA translation. Conjugation of PMOs to arginine-rich CPPs (cell-penetrating peptides) enhances the cellular delivery of PMOs. Here, we highlight our recent findings on PPMOs (CPP–PMO conjugates) in DMD (Duchenne muscular dystrophy) models and against a coronavirus, MHV (murine hepatitis virus).

DMD

DMD is a serious X-linked disease resulting from mutations in the human dystrophin gene, most commonly resulting in premature termination of translation, so that no functional dystrophin is produced. Progressive muscle degeneration leads to a decrease in the quality of life and predicted lifespan. Modification of splicing was observed in the presence of (R-Ahx-R)4AhxB (Ahx is 6-aminohexanoic acid and B is β-alanine) PPMOs in a canine model of DMD. Similarly, normal levels of dystrophin expression were restored in the diaphragms of mdx mice, with treatment starting at the neonatal stage, and protein was still detectable 22 weeks after the last dose of an (R-Ahx-R)4AhxB–PMO conjugate. Effects of length, linkage and carbohydrate modification of this CPP on the delivery of a PMO were investigated in a coronavirus mouse model. An (R-Ahx-R)4AhxB–PMO conjugate effectively inhibited viral replication, in comparison with other peptides conjugated to the same PMO. Shortening the CPP length, modifying it with a mannosylated serine moiety or replacing it with the R9F2 CPP significantly decreased the efficacy of the resulting PPMO (CPP–PMO conjugate). We attribute the success of this CPP to its stability in serum and its capacity to transport PMO to RNA targets in a manner superior to that of poly-arginine CPPs.

Key words: cell-penetrating peptide (CPP), Duchenne muscular dystrophy (DMD), exon skipping, morpholino, poly-arginine, toxicity.

Abbreviations used: Ahx, 6-aminohexanoic acid; B, β-alanine; CPP, cell-penetrating peptide; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy; MHV, murine hepatitis virus; PMO, phosphorodiamidate morpholino oligomer; PPMO, CPP–PMO conjugate; Tat, transactivator of transcription.

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Figure 1 | (R-Ahx-R)₄AhxB-PMO induced exon 23 skipping in cardiomyocytes isolated from neonatal mdx mice
The cells were treated with 300–1000 nM PPMO. Control is vehicle-treated cells only.


Immunofluorescence detection of dystrophin revealed that normal levels were present in the diaphragms of mdx mice, treated as neonates, 2 weeks after a single i.p. (intraperitoneal) injection of 10 mg/kg PPMO. Four doses of the PPMO at 5 mg/kg per dose, administered once a week for 4 weeks, resulted in normal levels of dystrophin being present in the diaphragms of 6-week-old mice. The PPMO effect was more modest in the tibialis anterior, with a significant reduction in dystrophin present in the heart. Levels of dystrophin measured in different muscles by Western blotting co-ordinated well with the immunofluorescence of dystrophin measured in different muscles by Western blotting co-ordinated well with the immunofluorescence data. At 22 weeks after the fourth injection, dystrophin was still detectable in the diaphragm, but was discontinuous with some disruption of muscle architecture. Influence of the age of mdx mice on the effectiveness of PPMO treatment was also determined. Treatment of animals was started 1 day after birth, 4 weeks after birth or 1 year after birth. While splice-modified dystrophin expression increased for all ages treated, treatment at a younger age was clearly more beneficial, as the older animals had more established pathology. Peptide-related toxicity was not observed in the 5 mg/kg dose regime [6]. Absence of exon skipping in the heart is a concern for the therapeutic use of the conjugate. Application of the PPMO to neonatal mdx cardiomyocytes ex vivo induced pronounced exon 23 skipping (Figure 1), suggesting in vivo limitations on the uptake of PPMO by cardiac muscle.

Conclusions and perspectives
In vivo efficacy of the (R-Ahx-R)₄AhxB CPP has been demonstrated in both muscular dystrophy and MHV in vivo models. Although this CPP is more effective in delivering PMO than poly-arginine and Tat–CPPs, other studies have shown that it has dose-dependent cellular toxicity [10,11] and its PMO delivery efficiency is still limited by its endosomal entrapment [9], both of which may limit its therapeutic use. Optimization of the CPP structure may reduce cellular toxicity and endosomal trapping.

We thank Dr Jon Moulton for editing this manuscript pre-submission and Dr Joshua Steinhaus for cardiomyocyte data.
References

Received 18 May 2007