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)$_4$AhxB (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)$_4$AhxB–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)$_4$AhxB–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)$_4$AhxB–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 R$_9$F$_2$ 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 sterically 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)$_4$AhxB (Ahx is 6-aminohexanoic acid and B is β-alanine) PPMOs in a canine DMD model *in vitro* and the *mdx* mouse model *in vivo*. The PPMO caused exclusion of a specific exon, in order to bypass the truncation mutation, resulting in expression of partially functional dystrophin.

GRMD (golden retriever muscular dystrophy) is caused by a mutation in intron 6 that causes altered splicing and exclusion of exon 7, resulting in a disruption of the reading frame. Excision of exons 6 and 8 would restore the reading frame and produce dystrophin which, although containing internal deletions, is still partially functional. Among the several antisense structural types tested, PPMO was the most effective at inducing the desired exon-skipping effect in GRMD myoblasts. At concentrations as low as 300 nM, high levels of corrected transcript was observed along with almost no mutant transcript. The PPMO resulted in a more complete splice modification than 2′-*O*-methylphosphorothioate cationic lipoplexes. At 10 days after a single treatment, PPMO-treated cells continued to show abundant induced in-frame transcripts [2].

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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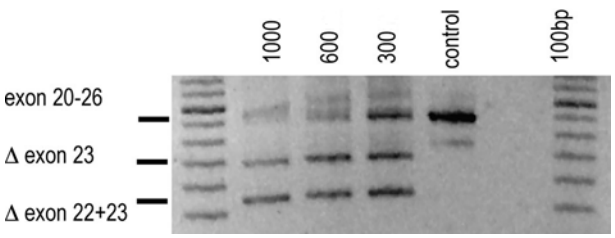
Figure 1 | (R-Ahx-R)$_4$AhxB–PMO induced exon 23 skipping in cardiomyocytes isolated from neonatal *mdx* mice

The cells were treated with 300–1000 nM PPMO. Control is vehicle-treated cells only.



A mouse model of muscular dystrophy (*mdx*) carries a nonsense mutation in exon 23 [3]. Excision of exon 23 in *mdx* mouse cells restores functional dystrophin expression [4,5]. A PPMO induced specific exon skipping and successfully restored dystrophin expression in *mdx* mice [6] at lower dosages than PMO alone [4,5].

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, gluteus maximus and triceps brachialis, with a weak effect in the colon and stomach and no dystrophin present in the heart. Levels 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.

Coronavirus

The recently emerged SARS (severe acute respiratory syndrome) coronavirus, has made clear the need for effective therapies against coronaviruses. Effects of the length, linkage, sequences and carbohydrate modification of (R-Ahx-R)$_4$AhxB on the efficacy of the resulting PPMOs were determined against MHV. Experiments in MHV-infected cells showed that a PPMO targeting the 5′-terminal nucleotides of the viral genome was highly efficacious. By using the effective PMO sequence, the effect of CPP length was determined by comparing (R-Ahx-R)$_4$C–, (R-Ahx-R)$_3$C– and (R-Ahx-R)$_2$C–PPMOs. To test the effect of linkage type, the (R-Ahx-R)$_4$AhxB–PPMOs (amide) were compared with the (R-Ahx-R)$_4$C–PPMOs (maleimide). (R-Ahx-R)$_4$–PPMOs were compared with R_9F_2C–PPMOs, to determine the effect of incorporating Ahx into a poly-arginine CPP. The effect of mannose modification on the efficacy of PPMO was determined by comparing (R-Ahx-R)$_4$AhxB–PPMOs with (mannose-serine)$_3$-(R-Ahx-R)$_4$AhxB–PPMOs, in which the peptide had three O-linked mannosylated serine residues at its N-terminus [7].

Both cell culture and mouse models were used to compare efficacy of the above PPMOs at inhibiting MHV replication. The most effective conjugate in mice was (R-Ahx-R)$_4$AhxB, which significantly reduced viral titres and protected tissue from virus-induced damage of MHV-infected mice. Shortening the peptide length from four repeats to three or two repeats of (R-Ahx-R) abolished the activity of the resulting PPMOs in mice and significantly decreased their activity in cell culture. The R_9F_2C–PPMO was significantly less effective than (R-Ahx-R)$_4$AhxB–PPMO in mice, indicating the potential benefit of Ahx. We have shown previously that (R-Ahx-R)$_4$AhxB was more stable in serum [8] and has a greater capability to deliver PMO to the target RNA than the R_9F_2C and Tat (transactivator of transcription)–PPMO [9]. The serum stability of (R-Ahx-R)$_4$AhxB–PPMO probably increased its liver availability in the MHV–infected livers.

The (mannose-serine)$_3$–(R-Ahx-R)$_4$AhxB–PPMO did not inhibit MHV replication in mice or in cultured cells [7]. We hypothesize that the bulky mannosylated serine moiety may reduce the flexibility of the CPP and result in reduced cellular uptake. Thus positioning the carbohydrate moiety at the opposite end of a PMO conjugated to a CPP may be a more appropriate configuration to target the mannose receptors of MHV-infected liver cells. (R-Ahx-R)$_4$AhxB and (R-Ahx-R)$_4$C had similar efficacies in cell culture, indicating that the amide and maleimide linkage worked equally well.

Conclusions and perspectives

In vivo efficacy of the (R-Ahx-R)$_4$AhxB 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.

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