A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient \textit{mdx} mice

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Duchenne muscular dystrophy (DMD) is caused by mutations in the \textit{DMD} gene that abolish the synthesis of dystrophin protein. Antisense oligonucleotides (AOs) targeted to trigger excision of an exon bearing a mutant premature stop codon in the \textit{DMD} transcript have been shown to skip the mutated exon and partially restore functional dystrophin protein in dystrophin-deficient \textit{mdx} mice. To fully exploit the therapeutic potential of this method requires highly efficient systemic AO delivery to multiple muscle groups, to modify the disease process and restore muscle function. While systemic delivery of naked AOs in DMD animal models requires high doses and is of relatively poor efficiency, we and others have recently shown that short arginine-rich peptide-AO conjugates can dramatically improve \textit{in vivo} DMD splice correction. Here we report for the first time that a chimeric fusion peptide (B-MSP-PMO) consisting of a muscle-targeting heptapeptide (MSP) fused to an arginine-rich cell-penetrating peptide (B-peptide) and conjugated to a morpholino oligomer (PMO) AO directs highly efficient systemic dystrophin splice correction in \textit{mdx} mice. With very low systemic doses, we demonstrate that B-MSP-PMO restores high-level, uniform dystrophin protein expression in multiple peripheral muscle groups, yielding functional correction and improvement of the \textit{mdx} dystrophic phenotype. Our data demonstrate proof-of-concept for this chimeric peptide approach in DMD splice correction therapy and is likely to have broad application.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe muscle degenerative disorder characterized by mutations that disrupt the reading frame in the dystrophin (\textit{DMD}) gene leading to the absence of functional protein (1). Antisense oligonucleotide (AO)-mediated exon skipping offers a potential therapy for DMD by restoring the open reading frame of mutant \textit{DMD} transcripts (2–12), yielding the production of shorter functional forms of dystrophin protein that retain the critical amino terminal, cysteine rich and carboxy terminal domains necessary for function (13,14). The therapeutic potential of this method has now been successfully shown in human subjects via local intramuscular AO injection (10).

To fully exploit AO-mediated splice correction as an effective therapy in DMD patients will require systemic correction of the DMD phenotype with increased potency. Systemic intravenous delivery of 2'-O-methyl phosphorothioate RNA and phosphorodiamidate morpholino oligomer (PMO) AOs have been shown to restore dystrophin expression in multiple peripheral muscles in \textit{mdx} mice. However, correction was of low efficiency for both AO types, and for the latter required a multiple dosing regimen comprising seven weekly doses of PMO at 100 mg/kg (3) to achieve a moderate restoration of dystrophin protein. Recently, we and others have reported that PMO conjugated to short arginine-rich cell-penetrating peptides (CPPs) can induce effective systemic dystrophin exon skipping, including in cardiac muscle (15–18),

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showing the potential of PMO-peptide conjugates as therapeutic agents for DMD.

Few studies to date have investigated the possibility that cell-targeting peptides might permit enhanced in vivo tissue-specific nucleic acid delivery and activity. Although a recent report demonstrated successful transvascular nucleic acid delivery to brain using a neuronal targeting peptide derived from rabies virus glycoprotein complexed with double-stranded siRNA (19). We hypothesize that such a cell-targeting approach may enhance AO delivery to muscle for DMD. In the present study, we test this hypothesis by conjugating a muscle-specific heptapeptide (MSP) (20) or a chimeric fusion peptide comprising MSP and a CPP (B peptide) to PMO, and evaluate these peptide-PMO conjugates in mdx mice. Our study shows for the first time that the chimeric peptide conjugate (B-MSP-PMO) induces highly effective systemic dystrophin exon skipping in mdx mice at doses as low as 6 mg/kg, with body-wide restoration of dystrophin protein and improvement of muscle pathology and function with no evidence of toxicity. This study demonstrates that such a chimeric peptide approach provides a safe and effective method for systemic AO delivery for DMD splice correction therapy and is likely to have broad utility.

RESULTS

MSP-PMO conjugate is much less effective than B-PMO for dystrophin splice correction in mdx mice

To test the ability of cell-targeting peptides to enhance systemic dystrophin correction in mdx mice, we investigated a muscle-specific heptapeptide (MSP), previously identified by in vivo phage display as having increased muscle- and cardiactargeting properties (20), for its ability to enhance PMO splice correcting activity in muscle. We compared the MSP-PMO conjugate directly with the previously studied B-PMO conjugate (see Fig. 1A for the oligonucleotide and peptide sequences) in mdx mice at a 25 mg/kg single intravenous dose as B-PMO had been previously shown to restore expression of dystrophin in the tibialis anterior (TA) muscle by a single intramuscular injection (16). Three weeks following the single injection, all skeletal muscle groups analysed demonstrated near normal levels of dystrophin protein by immunostaining following treatment with the B-PMO conjugate (Fig. 1B), consistent with previous reports (18). Surprisingly, the activity of MSP-PMO was found to be low, although more effective than PMO alone at the same dose (data not shown). High levels of dystrophin exon skipping and protein restoration were detected in hind limb, forelimb, abdominal wall and diaphragm muscles and also in cardiac tissue in mdx mice treated with the B-PMO conjugate, shown by RT–PCR and western blot (Supplementary Material, Fig. S1a and b). Increased levels of cardiac dystrophin restoration with the B-PMO conjugate were seen with higher intravenous doses of 30 and 40 mg/kg (Supplementary Material, Fig. S2a), which showed about 20 and 50% of normal levels, respectively, as indicated by western blot (Supplementary Material, Fig. S2b). Moreover, B-PMO also restored components of the dystrophin-associated protein complex (DAPC) (21,22), which in the absence of functional dystrophin fail to localize accurately to the muscle sarcolemma (Supplementary Material, Fig. S1c). As a result, using a functional test of grip force strength (23,24), mdx mice treated with B-PMO were found to have significantly improved grip strength within the normal range compared with untreated mdx mice (Supplementary Material, Fig. S1d).

Chimeric B-MSP-PMO induces efficient dystrophin splice correction

Since MSP has a high affinity for skeletal and cardiac muscle (20), we hypothesized that the poor activity of the MSP-PMO conjugate might be due to its weak ability to facilitate PMO internalization following tissue localization. We therefore tested whether fusion of the MSP motif to the B-peptide to generate a chimeric fusion peptide could improve its activity following systemic delivery. We tested two conjugated forms of this chimeric peptide, B-MSP-PMO, in which the MSP domain was positioned between the B and PMO sequences, and MSP-B-PMO in which the MSP domain was positioned away from PMO (Fig. 1A). In order to discover whether either of these conjugates provided enhanced activity over the B-PMO conjugate, we investigated a low dose multiple injection protocol of 3 mg/kg in six weekly intravenous injections, reasoning that differences in efficacy would be most apparent at lower doses. Surprisingly, B-MSP-PMO, not MSP-B-PMO, proved highly effective in its ability to restore dystrophin expression in multiple skeletal muscle groups at this low dose compared with B-PMO. Widespread, uniform dystrophin expression was found throughout muscle cross-sections with the B-MSP-PMO conjugate, whereas fewer dystrophin-positive fibres were detected following B-PMO treatment at this dose (Supplementary Material, Table S1). Virtually no dystrophin expression was detected with the alternative chimeric peptide PMO conjugate (MSP-B-PMO) (Fig. 2A). No detectable dystrophin expression in heart was found with all three conjugates at this dose. The most striking difference between B-MSP-PMO and B-PMO conjugates was seen in abdominal and diaphragm muscles; no detectable exon skipping products were found with B-PMO in these two tissues, whereas ~20% of exon 23 transcripts were skipped with B-MSP-PMO as shown by RT–PCR (Fig. 2B) and confirmed by sequence analysis (Fig. 2C). It should be noted that RT–PCR is likely to overestimate the proportion of skipped transcripts given that full-length transcripts containing the nonsense mutation will be subject to nonsense-mediated decay. Western blot analysis showed that ~5% of the normal level of dystrophin was restored in TA and quadriceps muscles with B-MSP-PMO, whereas only ~1% was observed in the same tissues with B-PMO (Fig. 2D). Consistent with the immunostaining data, minimal exon skipping activity and protein restoration were found with the MSP-B-PMO conjugate (data not shown).

Enhanced systemic exon skipping efficiency with B-MSP-PMO in body-wide skeletal muscles

To fully explore the splice-correcting potential of the B-MSP-PMO conjugate harbouring both muscle-targeting heptapeptide and arginine-rich CPP domains, we optimized
Figure 1. Systemic administration of MSP-PMO and B-PMO conjugates in mdx mice. Dystrophin expression following single 25 mg/kg intravenous injections of the B-PMO and MSP-PMO AO conjugates in adult mdx mice. (A) Schematic figure illustrating the four different AO constructs utilized. PMO contains the sequence of GGCCAAACCTCGGCTTACCTGAAAT (5’–3’). Peptides are written from N to C orientation using the standard one letter amino acid code except for X and B, which are un-natural amino acids (X = 6-aminohexanoic acid, B = beta-alanine). (B) Immunostaining of muscle tissue cross-sections to detect dystrophin protein expression and localization in C57BL6 normal control (top panel), untreated mdx mice (middle panel), B-PMO treated (third panel) and MSP-PMO treated mdx mice (bottom panel). Muscle tissues analysed were from tibialis anterior (TA), gastrocnemius, quadriceps, biceps, abdominal wall (abdominal), diaphragm and heart muscles (scale bar = 200 μm).
Figure 2. Investigation of muscle-specific chimeric peptide PMO conjugates at low systemic doses. Dystrophin exon-skipping and protein expression following systemic administration of muscle-specific fusion peptide PMO conjugates in adult mdx mice. (A) Immunohistochemistry to detect dystrophin expression in muscle cross-sections from mdx mice treated with B-PMO (upper panel), B-MSP-PMO (second panel) and MSP-B-PMO (lower panel) conjugates at the low 3 mg/kg dose. Data from control normal C57BL6 and untreated mdx mice not shown. Muscle tissues analysed were from tibialis anterior (TA), gastrocnemius, quadriceps, biceps, abdominal wall (abdominal), diaphragm and heart muscles (scale bar = 200 μm). Dystrophin expression was not found in heart with all three conjugates at this dose. (B) RT–PCR to detect the dystrophin exon skipping products in treated mdx mouse muscle groups as shown (exon-skipped bands indicated by Δexon23—for exon 23 deleted; Δexon22 + 23—for exons 22 and 23 deleted). (C) Sequence analysis confirming precise skipping of exon 23 and another RT–PCR product with both exon 22 and 23 skipped. (D) Western blot for detection of dystrophin protein in the indicated muscle groups from treated mdx mice compared with C57BL6 and untreated mdx control mice. One hundred microgram protein was loaded for each sample except for C57BL6 control lane where 1 μg of protein was loaded. α-actinin was used as loading control.
the dosing regimen by administering the same total dose of 18 mg/kg over three weekly intravenous injections of 6 mg/kg each. When compared directly with B-PMO, B-MSP-PMO proved highly efficacious at this dose giving high-level body-wide correction of dystrophin protein expression in multiple peripheral skeletal muscles, although only at low levels in heart (Fig. 3A and Supplementary Material, Table S2). Little variation in dystrophin exon skipping efficiency was observed between different muscle groups treated with B-MSP-PMO as has been reported previously following naked PMO treatment (3). Enhanced exon skipping efficiency of the B-MSP-PMO conjugate was seen by RT–PCR, with negligible full-length uncorrected dystrophin transcripts detectable in biceps, abdominal and diaphragm muscles (Fig. 3B). Up to 25% of the normal level of dystrophin protein was restored in skeletal muscles of mdx mice treated with B-MSP-PMO compared with the B-PMO conjugate, which showed ∼10% of normal levels as indicated by western blot (Fig. 3C and D). These results clearly demonstrated that the B-MSP-PMO conjugate facilitated enhanced dystrophin splice correction compared with B-PMO lacking the MSP domain.

**Functional and phenotypic improvement of the mdx mouse with B-MSP-PMO treatment**

Given the high activity of the B-MSP-PMO conjugate, we next examined its ability to restore function and correct disease pathology in mdx mice. First, we evaluated DAPC expression in mdx mice treated with the 6 mg/kg dose regimen. Serial immunostaining showed restored expression and correct localization of DAPC component proteins β-dystroglycan, α-sarcoglycan and β-sarcoglycan in B-MSP-PMO and B-PMO treated mdx mouse TA muscles compared with untreated mdx mice (Fig. 4A). The DAPC also has important signalling functions via nNOS (21) and its restoration and correct localization was also detected following B-MSP-PMO treatment (Fig. 4A). Physically functional improvement was measured using grip strength tests, which test predominantly but not exclusively forelimb functional restoration (23,24). B-MSP-PMO treated animals showed significant strength improvement to within the normal range compared with untreated age-matched mdx controls, indicating a degree of functional recovery and close correlation with the percentage of dystrophin-positive fibres in treated biceps (Fig. 4B). Routine H&E and Azan Mollary histology of B-MSP-PMO treated muscles showed no overt evidence of toxicity and fibrosis (Supplementary Material, Fig. S3) and analysis of the number of centrally nucleated myofibres, an index of ongoing degeneration/regeneration cycles (25,26), revealed a significantly decreased level of degeneration and regeneration in TA, quadriceps and gastrocnemius muscles in mdx mice treated with the B-MSP-PMO conjugate (P < 0.001) compared with untreated age-matched control mice (Fig. 4C). Finally, we analysed serum biochemistry indices including creatinine kinase (CK), an index of ongoing muscle injury (25). This demonstrated significantly lower CK levels following B-MSP-PMO treatment than in untreated control mice (Fig. 4D), demonstrating the protective effects of systemic dystrophin restoration on myofibre integrity. Serum biochemistry including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme levels as indices of liver function also showed significant decreases compared with untreated controls and fell within the normal range in B-MSP-PMO treated animals (Fig. 4E). No change was observed in the levels of urea and creatinine in the B-MSP-PMO treated mdx mice, indicating no obvious renal toxicity (data not shown).

**DISCUSSION**

In this report, we demonstrate for the first time that a PMO oligomer conjugated to a chimeric fusion peptide (B-MSP-PMO) comprising a muscle-targeting domain and an arginine-rich cell penetrating peptide domain, directs highly effective dystrophin protein restoration, muscle function restoration and correction of the dystrophic phenotype in mdx mice. Our data show that the B-MSP-PMO conjugate has significant potential for enhanced restoration of dystrophin expression and arresting DMD pathology at very low systemic doses, compatible with successful application in human subjects. A previous study reported use of a fusion peptide comprising cell-targeting and arginine-rich peptide domains for siRNA delivery to brain (19). The present study is the first to show such chimeric peptide approach to AOs can permit enhanced systemic correction of a genetic defect in an animal model of human disease.

We and others have recently reported that short arginine-rich CPPs directly conjugated to PMO can induce efficient systemic splice correction in mdx mice (15–18), providing a significant advance on previous studies using systemic naked AO delivery for DMD (3,8). In the present study, the hypothesis that PMO conjugation to a cell-targeting peptide domain can induce enhanced muscle delivery and further improve the efficacy of systemic DMD splice correction has been tested. The MSP-PMO conjugate proved surprisingly ineffective. A possible explanation for this is that this cell-targeting peptide alone may direct the AO conjugate to the targeted cells in the absence of efficient internalization. Further studies will be needed to understand the delivery pathway and mechanism. However, the chimeric peptide with the B-MSP combination proved highly effective in inducing dystrophin splice correction and restoring the expression of dystrophin protein in body-wide skeletal muscles compared with the conjugate lacking the MSP domain. Utilizing very low B-MSP-PMO doses of 6 mg/kg in mdx mice, we have now shown highly efficient correction of dystrophin protein in multiple skeletal muscles (Fig. 3C and D), restoration of DAPC structural integrity (Fig. 4A), significant improvement in muscle strength which correlated closely with the percentage of dystrophin-positive fibres (Fig. 4B) and correction of the mdx dystrophic phenotype (Fig. 4C–E). The superior activity of the B-MSP-PMO conjugate to B-PMO alone in enhancing systemic dystrophin splice correction in mdx mice was also shown in a lower dose study (3 mg/kg dose). Overall, these findings indicate that the MSP cell-targeting peptide fails to augment systemic splice correction in the absence of an arginine-rich transduction domain, but that when coupled together in a chimeric fusion peptide the MSP peptide significantly enhances systemic PMO activity.

Surprisingly, the chimeric peptide with the MSP-B combination showed little activity in restoring the expression of...
Figure 3. Systemic administration of the B-MSP-PMO conjugate restores dystrophin expression in body-wide skeletal muscles. Dystrophin exon-skipping and protein expression following systemic administration of the B-MSP-PMO conjugate in adult mdx mice at a dose of 6 mg/kg. (A) Immunohistochemistry to detect dystrophin expression in muscle cross-sections from mdx mice treated with B-PMO (top panel), B-MSP-PMO (bottom panel) conjugates at the 6 mg/kg dose. Data from control normal C57BL6 and untreated mdx mice are not shown. Muscle tissues analysed were from tibialis anterior (TA), gastrocnemius, quadriceps, biceps, abdominal wall (abdominal), diaphragm and heart muscles (scale bar = 200 μm). Widespread, uniform dystrophin expression detected in all skeletal muscles treated with the B-MSP-PMO conjugate; however, low level of dystrophin expression was found in heart. (B) RT-PCR to detect dystrophin exon skipping products in treated mdx muscle groups as shown (Δexon23 indicates exon 23 deleted; Δexon22 + 23—exons 22 and 23 deleted). (C) Western blot detection of dystrophin protein in the indicated muscle groups from treated mdx mice compared with C57BL6 and untreated mdx control. Equal loading of 25 μg protein is shown for each sample except for C57BL6 control lane where 6.25 μg of protein was loaded and α-actinin as a loading control. (D) Quantification of dystrophin protein levels relative to normal controls in differently treated muscles. The mean percentage of dystrophin protein relative to normal controls restored in different muscles treated with B-MSP-PMO was 24.3, 20.1, 15.7, 19.3, 17.2, 1.7 and 14.5% in TA, quadriceps, gastrocnemius, biceps, diaphragm, heart and abdominal muscle, respectively, in comparison with the 9.9, 6.9, 4.2, 5.9, 4, 2.5 and 6.9% in the B-PMO treated mice (the percentage is shown as mean ± SEM, n = 4 mice).
Figure 4. Functional and phenotypic correction in *mdx* mice following treatment with the B-MSP-PMO conjugate. (A) Restoration of the dystrophin-associated protein complex (DAPC) in *mdx* mice treated with B-MSP-PMO at 6 mg/kg was studied to assess dystrophin function and recovery of normal myoarchitecture. DAPC protein components β-dystroglycan, α and β-sarcoglycan and nNOS were detected by immunostaining in serial tissue cross-sections of TA muscles from treated *mdx* mice compared with B-PMO treated *mdx* mice (arrowhead indicated identical fibres). (B) Muscle function was assessed using a functional grip strength test to determine the physical improvement of B-MSP-PMO treated *mdx* mice showing close correlation with the percentage of dystrophin-positive fibres in biceps muscles ($R^2 = 0.8007$). (C) Evaluation of the numbers of centrally nucleated myofibres in TA, gastrocnemius and quadriceps muscles following B-MSP-PMO treatment compared with the corresponding untreated *mdx* muscles. Data show a significant decrease in the number of centrally nucleated myofibres in treated *mdx* mice compared with untreated controls ($P < 0.001$). (D) Measurement of serum creatine kinase (CK) levels as an index of ongoing muscle membrane instability in treated *mdx* mice compared with *mdx* control mice. Data show a significant fall in the serum CK levels in *mdx* mice treated with B-MSP-PMO compared with untreated age-matched *mdx* controls ($P < 0.05$). (E) Measurement of serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes in treated *mdx* mice compared with untreated *mdx* mice. Data show improved pathological parameters in B-MSP-PMO treated *mdx* mice compared with untreated controls with significantly lower serum levels of both enzymes.
dystrophin (Fig. 2A). Subsequent studies with fluorescein-labelled PMO AO conjugates both in vitro and in vivo have shown that internalization of PMO was facilitated by the B-MSP fusion peptide, whereas the alternative MSP-B peptide failed to provide efficient cell uptake (Yin et al., in preparation). Therefore, although the mechanism is unclear, the location of an MSP domain within the chimeric fusion peptide is position-dependent in order to facilitate the effective internalization of AO-peptide conjugates.

In order to verify the cell-targeting role of MSP, we quantified the PMO concentration in muscles from the mdx mice treated with B-MSP-PMO and B-PMO at the 6 mg/kg dose. The tissue distribution data demonstrated higher tissue uptake for B-MSP-PMO compared with B-PMO in most muscle groups although the difference in uptake was not statistically significant except for the diaphragm (Supplementary Material, Fig. S4). No significant differences were observed in non-muscle tissues such as liver and kidney between these two constructs (data not shown). Our hypothesis therefore is that the role of the fusion peptide is to allow greater internalization of AO into muscle cells. This is supported by in vitro data showing that B-MSP-PMO had the greatest efficacy in inducing exon skipping in mdx primary muscle cells compared with B-PMO and MSP-B-PMO over a range of concentrations (Wang et al., submitted for publication).

This little evidence for correction of cardiac dystrophin expression was found (for B-PMO as well as the B-MSP-PMO conjugate), is most likely due to the low doses utilized in this study and the 2–3-fold lower binding affinity that the MSP peptide has for cardiac compared with skeletal muscle (20). This is supported by the finding that approximately 15–20% of normal dystrophin protein was detected in heart when a single 25 mg/kg dose B-MSP-PMO was administered to mdx mice intravenously when compared with 10% for B-PMO (data not shown). Nevertheless, cardiac dystrophin correction by peptide-PMOs (B-MSP-PMO as well as B-PMO), even at higher doses, is clearly less efficient than that seen in peripheral muscles. While it is possible that exon skipping of the DMD pre-mRNA is less efficient in heart, efficient dystrophin correction is seen in primary cardiomyocytes in culture (Wang and Yin, submitted for publication), and therefore the most likely explanation at present is that differences in the cardiac microvasculature and endothelial barrier prevent less efficient PMO access than occurs in peripheral muscle groups. Given the significant potential of the B-MSP-PMO conjugate, detailed toxicological analysis and long-term studies will now determine whether it is suitable for clinical evaluation in DMD patients. Further studies of the B-MSP chimeric peptide, including investigation of the lack of efficacy of the MSP-B-PMO conjugate, will yield improved versions of this fusion peptide likely to have broad experimental and clinical utility.

**MATERIAL AND METHODS**

**Animals**

Six- to 8-week-old mdx mice were used in all experiments (four mice each in the test and control groups). The experiments were carried out in the Animal unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK according to procedures authorized by the UK Home Office. Mice were killed by CO₂ inhalation or cervical dislocation at desired time points, and muscles and other tissues were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C.

**PMO and PMO-peptide conjugates**

Four peptide-conjugated PMOs were synthesized and purified to >90% purity by AVI Biopharma Inc. (Corvallis, OR, USA). The nomenclature and sequences of these constructs are shown in Figure 1A. The PMO AO was targeted to the murine dystrophin exon23/intron 23 boundary site. The four peptides are named as MSP, B, B-MSP and MSP-B. The PMO was conjugated to the carboxyl groups at the C-terminus of the four peptides using a method described elsewhere (27).

**RNA extraction and nested RT–PCR analysis**

Total RNA was extracted with Trizol (Invitrogen, UK) and 200 ng of RNA template was used for 20 μl RT–PCR with OneStep RT–PCR kit (Qiagen, UK). The primer sequences were used as previously reported (16). The products were examined by electrophoresis on a 2% agarose gel.

**Systemic injections of peptide-PMO conjugates**

Various amounts of PMO-peptide conjugates in 80 μl saline buffer were injected into tail vein of mdx mice at the final dose of 25, 30, 40, 3 and 6 mg/kg, respectively. The animals were killed at various time points after injection by CO₂ inhalation and tissues were removed and snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C.

**Immunohistochemistry and histology**

Series of 8 μm sections were examined for dystrophin expression and dystrophin-associated protein complex (DAPC) with a series of polyclonal antibodies and monoclonal antibodies as described (16). Routine haematoxylin and eosin and Azan Mallory staining was used to examine overall muscle morphology and assess the level of infiltrating mononuclear cells and fibrosis.

**Centrally nucleated fibre counts**

TA, quadriceps and gastrocnemius muscles from mdx mice treated with PMO-peptide conjugates were examined. To ascertain the number of centrally nucleated muscle fibres, sections were stained for dystrophin with rabbit polyclonal antibody 2166 and counter-stained with DAPI for cell nuclei (Sigma, UK). About 500 dystrophin positive fibres for each tissue sample were counted and assessed for the presence of central nuclei using a Zeiss AxioVision fluorescence microscope. Fibres were judged centrally nucleated if one or more nuclei were not located at the periphery of the fibre. Untreated age-matched mdx mice were used as controls.
Protein extraction and western blot

Protein extraction and western blot were carried out as previously described (16). Various amounts protein from normal C57BL6 mice as a positive control and corresponding amounts of protein from muscles of treated or untreated mdx mice were used. The membrane was probed with DYS1 (monoclonal antibody against dystrophin R8 repeat, 1:200, NovoCastra, UK) for the detection of dystrophin protein and α-actinin as a loading control (mouse monoclonal antibody, 1:3000, Sigma, UK). The bound primary antibody was detected by horseradish peroxidase-conjugated goat anti-mouse IgGs and the ECL Western Blotting Analysis system (Amersham Pharmacia Biosciences, UK). The intensity of the bands obtained from treated muscles was measured by Image J software; the quantification is based on band intensity and area, and is compared with that from normal muscles of C57BL6 mice.

Functional grip strength analysis

Treated mice and control mice were tested using a commercial grip strength monitor (Chatillon, UK). Each mouse was held 2 cm from the base of the tail, allowed to grip a protruding metal triangle bar attached to the apparatus with their forepaws and pulled gently until they released their grip. The force exerted was recorded and five sequential tests were carried out for each mouse, averaged at 30 s apart.

Clinical biochemistry

Serum and plasma were taken from the mouse jugular vein immediately after the killing with CO₂ inhalation. Analysis of serum CK, AST, ALT and urea and creatinine levels was performed by the clinical pathology laboratory (Mary Lyon Centre, Medical Research Council, Harwell, Oxfordshire, UK).

Tissues biodistribution analysis

Tissues were thawed at room temperature and then pre-weighed into individual 1.5 ml eppendorf tubes. Lysis buffer containing trypsin and proteinase K was added to pre-weighed tissue. Samples were placed into a shaking incubator temperature controlled at 60°C overnight. After incubation, samples were centrifuged at 14000 g for 10 min and the supernatant was collected. Lysates were extracted 3:1 in acetonitrile, frozen on dry ice and lyophilized. Lyophilized samples were reconstituted in HBS-P buffer (BIACore, Piscataway, NJ, USA) and transferred to a 96 well plate. Plates were spun down (1000g, 10 min) to pellet any particulate matter. Surface performance resonance (SPR) detection was performed on a Biacore T100 (GE/ BIAcore) instrument operated at 25°C. A CM dextran matrix pre-immobilized streptavidin sensor chip was bound with a biotin-labelled cDNA (Integrated DNA technologies, USA) and transferred to a 96 well plate. Plates were spun for 10 min and the supernatant was collected. Lysates were extracted 3:1 in acetonitrile, frozen on dry ice and lyophilized. Lyophilized samples were reconstituted in HBS-P buffer (BIACore, Piscataway, NJ, USA) and transferred to a 96 well plate. Plates were spun down (1000g, 10 min) to pellet any particulate matter. Surface performance resonance (SPR) detection was performed on a Biacore T100 (GE/ BIAcore) instrument operating at 25°C. A CM dextran matrix pre-immobilized streptavidin sensor chip was bound with a biotin-labelled cDNA (Integrated DNA technologies) complementary to the PMO sequence. Target immobilization level for SA chip was set to maximum. Ligand was immobilized in a flow of 10 μl/min. The chip was fully saturated in a single 10 min pulse and resulting in 1345 RU immobilized on the surface. The contact time during the concentration measurements was 120 s at a flow 30 μl/min followed by a dissociation time of 15 s. The DNA surface was regenerated with a single pulse (5 s, 50 μl/min) of 10 mM glycine-HCl at pH 1.75. Biacore concentration analysis was performed as follows: a direct binding assay was used to determine tissue concentrations. Calibration was performed by spiking blank matrix with known concentrations of PMO at 100, 50, 25, 12.5, 6.25, 3.125 and 0 nM. Three controls, 1, 10 and 50 nM, were run every 15 cycles to assess integrity of calibration over time. Blank tissues were used to establish the limits of detection.

Statistical analysis

All data are reported as mean ± SEM. Statistical differences between treatment groups and control groups were evaluated by SigmaStat (Systat Software, UK) and student’s t-test was applied.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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