Refinement of antisense oligonucleotide mediated exon skipping as therapy for Duchenne muscular dystrophy

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Chapter 2

In vivo comparison of 2'-*O*-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping

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RESEARCH ARTICLE

In vivo comparison of 2′-*O*-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping

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Abstract

Background Antisense-mediated exon skipping is a putative treatment for Duchenne muscular dystrophy (DMD). Using antisense oligonucleotides (AONs), the disrupted DMD reading frame is restored, allowing generation of partially functional dystrophin and conversion of a severe Duchenne into a milder Becker muscular dystrophy phenotype. *In vivo* studies are mainly performed using 2′-*O*-methyl phosphorothioate (2OMePS) or morpholino (PMO) AONs. These compounds were never directly compared.

Methods *mdx* and humanized (h)DMD mice were injected intramuscularly and intravenously with short versus long 2OMePS and PMO for mouse exon 23 and human exons 44, 45, 46 and 51.

Results Intramuscular injection showed that increasing the length of 2OMePS AONs enhanced skipping efficiencies of human exon 45, but decreased efficiency for mouse exon 23. Although PMO induced more mouse exon 23 skipping, PMO and 2OMePS were more comparable for human exons. After intravenous administration, exon skipping and novel protein was shown in the heart with both chemistries. Furthermore, PMO showed lower intramuscular concentrations with higher exon 23 skipping levels compared to 2OMePS, which may be due to sequestration in the extracellular matrix. Finally, two mismatches rendered 2OMePS but not PMO AONs nearly ineffective.

Conclusions The results obtained in the present study indicate that increasing AON length improves skipping efficiency in some but not all cases. It is feasible to induce exon skipping and dystrophin restoration in the heart after injection of 2OMePS and unconjugated PMO. Furthermore, differences in efficiency between PMO and 2OMePS appear to be sequence and not chemistry dependent. Finally, the results indicate that PMOs may be less sequence specific than 2OMePS. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords antisense oligonucleotides; Duchenne muscular dystrophy; exon skipping; 2′-*O*-methyl phosphorothioate; phosphorodiamidate morpholino oligomer

Introduction

During the last decade, specific modification of splicing to restore the disrupted reading frame of the dystrophin transcript has emerged as a promising

therapy for Duchenne muscular dystrophy (DMD) [1,2]. This lethal muscle-wasting disease is the result of the absence of functional dystrophin due to out-of-frame mutations in the *DMD* gene [3–5]. By contrast, mutations that maintain the reading frame and give rise to internally deleted, partially functional dystrophin are associated with the milder Becker muscular dystrophy (BMD) [4,5]. Using antisense oligonucleotides (AONs) interfering with splicing signals, the skipping of specific exons can be induced in the DMD pre-mRNA, thus restoring the open reading frame and converting the severe DMD into a milder BMD phenotype [6]. The broad therapeutic applicability of exon skipping has been confirmed in various patient-derived cell cultures carrying different mutations [6–10]. For each patient, the targeted exon was specifically skipped and dystrophin synthesis restored after treatment with 2′-*O*-methyl phosphorothioate (2OMePS) RNA AONs. *In vivo* proof-of-concept was first obtained in the *mdx* mouse model, dystrophin-deficient due to a nonsense mutation in the in-frame exon 23. Intramuscular injections of 2OMePS AONs targeting the mutated exon 23 restored dystrophin expression for at least 3 months [11]. This was accompanied by restoration of dystrophin-associated proteins at the fiber membrane as well as functional improvement of the treated muscle. *In vivo* skipping of human exons has also been achieved in the humanized (h)DMD mouse model, which contains a complete copy of the human *DMD* gene integrated in chromosome 5 of the mouse [12,13]. After intramuscular injection of 2OMePS AONs targeting the human exons 44, 46 and 49, the human but not the murine exons were skipped, emphasizing the specificity of this strategy [12]. We recently successfully completed a first in-man study where 2OMePS AON targeting exon 51 was injected into a small area of the tibialis anterior muscle of four DMD patients. Novel dystrophin expression was observed in the majority of muscle fibers, and the AON was safe and well tolerated [14].

Besides the 2OMePS backbone, alternative AON chemistries with distinct biological, biochemical and biophysical properties have also been under investigation. Recently, the PMO backbone has shown to be another promising analog for the exon skipping approach [15–18]. PMOs contain a six-membered morpholine moiety instead of the sugar ribose, and phosphorodiamidate linkages [19]. PMOs have a non-ionic backbone at physiological pH, making them notoriously hard to transfect in tissue culture experiments [20]. However, *in vivo* their non-ionic nature results in higher tissue concentrations, probably due to the lack of nonspecific interactions with cellular components [20]. PMOs are typically synthesized with 25 or more nucleotides, whereas most 2OMePS AONs studied contain up to 20 nucleotides. Compared to their 2OMePS counterparts, PMOs have been demonstrated to be more efficient in healthy human explants [21], and in *mdx* mice after intramuscular injections compared to 2OMePS targeting the same sequence [16].

Because over 30% of the body consists of muscle tissue, research is now focusing on full body treatment. An initial study by Lu *et al*. [22] in *mdx* mice showed that a weekly tail vein injection for three weeks with 100 mg/kg of 2OMePS AONs was sufficient to restore dystrophin in different skeletal muscle groups at levels of up to 5% of wild-type levels. However, no detectable dystrophin levels were observed in the heart [22]. One intravenous injection of a PMO per week for seven weeks at a dose of approximately 100 mg/kg resulted in restoration of dystrophin at 10–50% of wild-type levels in skeletal muscle groups in *mdx* mice [15]. However, dystrophin levels varied both within and between different muscle groups and no dystrophin expression could be observed in the heart. Injecting 12 mg/kg of a peptide PMO conjugate on four consecutive days resulted in high exon skipping and protein levels, including the heart [23].

Due to different experimental designs with regard to AON length, sequence, dose and injection frequency, it is difficult to draw conclusions on the relative efficiencies of 2OMePS and PMO AONs. Recent evidence shows that increasing the length of 2OMePS AONs may improve efficiency for some, but not all AONs [18,24]. In the present study, we directly compared 2OMePS of different lengths with 25-mer PMO AONs targeting mouse exon 23 and human exons 44, 45, 46 and 51. AONs targeting mouse exon 23 were tested intramuscularly and systemically in the *mdx* mouse model. AONs targeting the human exons were tested intramuscularly in the hDMD mouse model.

Materials and methods

Oligoribonucleotides, animals and delivery methods

AONs were 2′-*O*-methyl RNA oligonucleotides with a full-length phosphorothioate backbone (Eurogentec, Liege, Belgium) or morpholinos (Gene Tools, Philomath, USA). AON sequences are given in Figure 1A; AONs targeting exon 23 in the *mdx* mouse have previously been described [17,25], whereas the short AONs targeting human exon 44, 45, 46 and 51 were identified by our own group [8,26,27]. The long AONs were based on these AONs and designed to target as many exonic splicing enhancer sites as possible [27,28]. For the intramuscular experiments, 4–5-week-old *mdx* or hDMD mice were injected in the gastrocnemius muscle on two consecutive days with 2.9 nmol AON (approximately 20 μg) in 50 μl physiologic salt, or saline (negative control). AONs were injected in four to eight muscles for each AON (exon 23: $n = 6$, 4 and 7 for short 2OMePS, long2OMePS and PMO, respectively; exon 44 and 46: $n = 4$ for each AON; exon 45: $n = 4$ for short 2OMePS, $n = 6$ for long 2OMePS and PMO; exon 51: $n = 8$ for both 2OMePS and 7 for PMO). Gastrocnemius muscles were pretreated to induce local muscle damage and regeneration with a toxic reagent, which should lead

Figure 1. Comparison of 2OMePS and PMO AONs after intramuscular injections of *mdx* and hDMD mice. (A) Relative location of the AONs with respect to to their target sequences. For the AONs tested in hDMD mice, both human and murine sequences are depicted. Mismatches between the human and mouse are underlined in the mouse sequence and are indicated by a gray box in the AONs. Note that AONs are depicted from 3′ to 5′. (B) An example of RT-PCR analysis of hDMD muscle injected with 2OMePS and MPO AONs targeting exon 45. In the human transcript, exon 45 skipping can be observed for all AONs. The target sequence of the short 2OMePS AON is completely homologous with the mouse sequence and this AON indeed appears equally efficient in skipping the mouse and the human exon 45. The long 2OMePS and PMO AON have two mismatches with the murine transcript. This greatly reduces the efficacy for the 2OMePS, whereas the PMO still induces considerable exon skipping. M, 100 bp size marker; NT, nontreated; PMO, morpholino; 2O, 2OMePS AON, –RT-PCR, negative control. (C–G) Quantification of the exon skipping efficiencies at the RNA level. For the AONs targeting the human exons, the figure shows two RT-PCR replicates for every sample. Each quantified value is indicated by a dot. No exon skipping was observed in nontreated muscles (data not shown). (C) Exon 23 skipping in *mdx* muscle. The PMO induces significantly higher levels of exon skipping than both 2OMePS AONs. The long 2OMePS is significantly less efficient than the short version. (D) Exon 44 skipping in hDMD muscle. There is no significant difference between the skipping levels in the human transcript. No exon 44 skipping could be observed in the murine transcript. (E) Exon 45 skipping in hDMD muscle. The long 2OMePS and PMO are equally efficient in skipping of the human transcript. Despite two mismatches with the mouse exon, the PMO induces exon 45 skipping at levels similar to those found in the human transcript. The long 2OMePS AON on the other hand is almost ineffective in the mouse transcript. The short 2OMePS is homologous to both the human and mouse target sequence and induces similar levels of exon 45 skipping in both transcripts, albeit at significantly lower levels than obtained with the long 2OMePS and PMO AONs. (F) Exon 46 skipping in hDMD muscle. On average, the PMO induces the highest levels of skipping, followed by the long 2OMePS and the short 2OMePS AONs. The PMO is significantly more efficient than both the short and long 2OMePS AON. These AONs contain at least eight mismatches with the mouse sequences and, indeed, none of them induces exon 46 skipping in the mouse transcript. (G) Exon 51 skipping in hDMD muscle. The only significant difference is between the short 2OMePS and the PMO AONs. All AONs contain two mismatches with the mouse sequences, which renders the 2OMePS AONs ineffective, whereas low levels of exon 51 skipping were observed for the PMO. *$P < 0.05$; **$P < 0.01$

to enhanced AON uptake as observed for dystrophic muscles [12]. Prior to intramuscular injections, mice were anaesthetized by intraperitoneal injection of a 1 : 1 (v/v) Hypnorm/Dormicum solution (Janssen Pharmaceutica, Beerse, Belgium/Roche Diagnostics, Almere, The Netherlands). Ten days after the last injection, mice were sacrificed by cervical dislocation and treated muscles were isolated.

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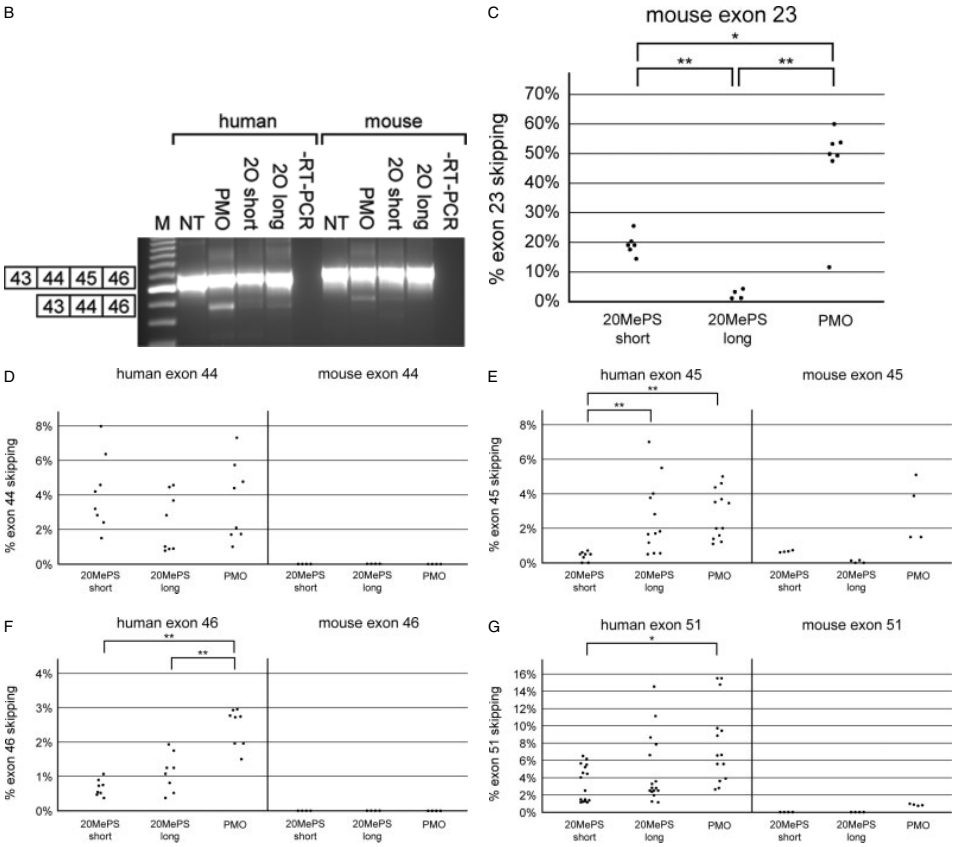

Figure 1. (*Continued*)

For intravenous experiments 4-week-old *mdx* mice were intravenously injected three times a week with equal molar amounts (14.52 μmol/kg) of the respective AONs (compared to approximately 100 mg/kg) or saline, for 3 weeks ($n = 4$ for short 2OMePS and $n = 2$ for long 2OMePS and PMO). Four days after the last injection, mice were sacrificed, and muscles and heart, kidneys and liver were snap frozen in liquid nitrogen-cooled 2-methylbutane and stored at −80 °C. Experiments were approved by the local Animal Ethics Committee of the Leiden University Medical Center.

Hybridization-ligation assay

The assay for measuring the concentration of 2OMePS and PMO AONs in plasma and tissue samples is based on a previously published hybridization ligation assay [29]. Calibration curves of the analysed AON prepared in 1% control mouse plasma in phosphate-buffered saline (PBS) were included. For liver and kidney samples all dilutions (at least 200 times) were performed in PBS and the absorption was read against a calibration curve in PBS. Heart and muscle samples were first diluted 60 times in PBS; subsequent dilutions and the calibration curves were performed in 60 × control heart or muscle in PBS. All analyses were performed in duplicate.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Muscles were minced in RNA-Bee (Campro Scientific, Veenendaal, The Netherlands) using MagNa Lyser green beads (Roche Diagnostics) and the MagNa Lyser (Roche Diagnostics) according to the manufacturer's instructions. Total RNA was extracted, and 400 ng of RNA was used for RT-PCR analysis using Transcriptor Reverse Transcriptase polymerase (Roche Diagnostics) in 20 μl at 55 °C for

30 min with an appropriate primer (primer sequences on request). Primary and nested PCRs were described previously [26]. Primary PCRs were performed by 20 cycles of 94 °C for 40 s, 60 °C for 40 s and 72 °C for 120 s. One microliter of these reactions was then reamplified in nested PCRs by 32 cycles of 94 °C for 40 s, 60 °C for 40 s and 72 °C for 100 s. PCR products were visualized on 2% agarose gels and quantified with the Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA, USA).

Statistical analysis

The results of the intramuscular experiment were statistically analysed. Short 2OMePS, long 2OMePS and PMO exon skipping levels were compared using the independent samples *t*-test with SPSS, version 14.0 (SPSS, Chicago, IL, USA). RT-PCR replicates were averaged.

Protein extraction and western blotting

Western blotting was performed as described [7]. Briefly, muscles were homogenized to a concentration of 25 mg/ml in treatment buffer (75 mM Tris-HCl pH 6.8, 15% SDS, 5% β-mercaptoethanol, 2% glycerol, 0,001% bromophenol blue) using MagNa Lyser green beads in the MagNa Lyser (Roche Diagnostics). Samples were boiled for 5 min, loaded on a 4–7% gradient polyacrylamide gel and run overnight at 4 °C. Gels were blotted to nitrocellulose BA83 (Whatman, Schleicher & Schuell, Dassel, Germany) for 6 h at 4 °C. Blots were blocked with 5% nonfat dried milk (Campina Melkunie, Zaltbommel, The Netherlands) in Tris-buffered saline (TBS) followed by an overnight incubation with NCL-DYS1 (dilution 1 : 125; NovaCastra, Newcastle, UK) in TBS plus 0.05% Tween20 to detect dystrophin. The fluorescent IRDye 800CW goat-anti-(mouse IgG) (dilution 1 : 5000; Li-Cor, Lincoln, NE, USA) was used as a secondary antibody. Blots were visualized and quantified with the Odyssey system and software (Li-Cor, NE, USA).

Antibodies and immunohistochemistry

Sections of 10 µm were cut from the gastrocnemius, quadriceps, triceps, tibialis anterior, heart and diaphragm at 100 µm intervals using a Shandon Cryotome (Thermo Fisher Scientific Co., Pittsburgh, PA, USA). Sections were fixed for 1 min with ice-cold acetone and analysed as described previously [12]. Monoclonal mouse antibody NCL-DYS2 (dilution 1 : 30; NovaCastra) was used to detect dystrophin. To detect the mouse antibody on mouse tissue, the MOM-kit fluorescein (Vector Laboratories, Inc., Burlingame, CA, USA) was used according to the manufacturer's instructions. Sections were mounted in Vectashield hard set mounting medium (Vector Laboratories, Inc.). Slides were analysed using a fluorescence microscope (DM RA2; Leica Microsystems, Wetzlar, Germany), and digital images were taken using a CCD camera (CTR MIC; Leica Microsystems).

Results

Comparison of 2OMePS and PMO AONs after intramuscular injection

Whereas PMO AONs generally are 25-mers, the 2OMePS AONs used so far are typically 17- to 20-mers (referred to as 'short' 2OMePS). We now also included 25-mer 2OMePS AONs (referred to as 'long' 2OMePS), completely similar to the PMO sequence with replacement of U for T nucleotides (Figure 1A). The AONs were injected at equal molar concentrations in the gastrocnemius muscles of either *mdx* or hDMD mice for mouse exon 23 or human exons 44, 45, 46 and 51, respectively. RNA was analysed by RT-PCR (see exon 45 example in Figure 1B). Skipping levels were quantified with the Agilent 2100 Bioanalyser (Figures 1C and 1G). The PMO targeting the murine exon 23 was significantly more effective than both the short and long 2OMePS in *mdx* muscle (over 50% versus 20% and 2.5% skipping levels, $P = 0.016$ and 0.003, respectively; Figure 1C), which is in accordance with earlier observations [17]. The 25 nt 2OMePS AON was less effective than its shorter counterpart (20 nt) ($P < 0.001$).

For hDMD muscle, both the (targeted) human and (nontargeted) murine DMD transcripts were analysed. Skipping efficiencies varied, even between samples treated with the same AON (Figures 1C and 1G). This is inherent to the intramuscular injection procedure. For exon 44, no significant difference between the long and short 2OMePS and PMO AONs was observed (Figure 1D). For exon 45 (Figures 1B and 1E), the long 2OMePS and the PMO AONs were more efficient than the short 2OMePS AONs ($P < 0.001$). With a similar range, the PMO was more efficient than both 2OMePS AONs for exon 46. For exon 51 (Figure 1G), the only difference was between the PMO AON and the short 2OMePS AON, which was marginally significant ($P = 0.021$). Analysis of the murine transcript was performed to determine AON specificity because there are several mismatches between the murine and human sequences in the targeted regions (Figure 1A). The short 2OMePS AON targeting exon 45 is completely homologous to both the human and the murine sequence and, indeed, induced equal levels of exon 45 skipping in both transcripts (Figures 1B and 1E). By contrast, the long 2OMePS and PMO contain two mismatches with the target sequence. For 2OMePS, this greatly decreased skipping, whereas PMO skipping remained equally efficient in the murine and human transcript. This suggests a reduced specificity of the PMO agent. No exon 46 skipping was observed in the murine transcript for any of the human AONs, which was expected because the murine sequence differs by approximately 50% from the human target in this area. Surprisingly, we did not observe any mouse exon

44 skipping for any of the AONs, even though the short 2OMePS AON is completely homologous to the mouse sequence (Figure 1A). For exon 51, two mismatches rendered both 2OMePS AONs ineffective for the mouse sequence, whereas low levels of skipping remained again for the PMO, emphasizing the reduced specificity of the PMO agent.

Systemic delivery of 2OMePS and PMO AONs in the *mdx* mouse

To directly compare biodistribution and efficiencies, 20-mer ('short', $n = 4$) and 25-mer ('long', $n = 2$) 2OMePS and 25-mer PMOs ($n = 2$) targeting exon 23 (Figure 1A) were injected at equal molar concentrations intravenously every other day for 3 weeks in *mdx* mice. Four days after the last injection, mice were sacrificed and different muscles, kidney and liver were isolated. Using a hybridization-ligation enzyme-linked immunosorbent assay method, AON concentrations were determined (Figure 2). The highest AON levels were observed in kidney, especially for PMO. In the liver, 2OMePS AON levels were approximately 1.5- to two-fold lower than in the kidney, but higher than the PMO levels. For each skeletal muscle analysed, PMO levels were lower than 2OMePS levels. With the exception of the tibialis anterior, the amount of long 2OMePS AON exceeded that of the short AON in all tissues analysed. Levels in the heart were comparable to levels observed in skeletal muscle for each AON.

Exon 23 skipping levels were analysed by RT-PCR analysis. By contrast to the biodistribution data, long 2OMePS were least effective (<5% exon 23 skipping), followed by the short 2OMePS (approximately 10% skipping) and PMO (approximately 40% skipping) (Figure 3A). The only exception is the diaphragm, where the long and short 2OMePS were equally efficient (approximately 7% skipping), whereas PMO was approximately 40%. The levels of exon skipping in different skeletal muscles were comparable per compound. Notably, we observed exon skipping in the heart for each AON, albeit at low levels (Figure 3B). This shows, for the first time that, at sufficient doses, it is feasible to induce exon skipping in the heart after systemic treatment using unconjugated PMO or 2OMePS. Interestingly, in contrast to skeletal muscle, skipping levels were comparable for long and short 2OMePS AONs in the heart (approximately 1.5%), whereas PMO skipping levels were only slightly higher (approximately 2.5%).

Western blot analysis confirmed dystrophin restoration at varying levels for the different AONs (Figure 4). In correlation with the RT-PCR data, the highest levels of dystrophin were found after PMO treatment (7–27% of wild-type), whereas lower levels were found for the long (0.5–2%) and short (1–2%) 2OMePS AONs. Interestingly, in contrast to exon skipping at the RNA level, protein levels did vary between different muscles; for each AON, dystrophin levels after treatment were highest in the tibialis anterior muscle and lowest in the gastrocnemius muscle. Moreover, the low levels of exon skipping in the heart appeared sufficient to result in detectable dystrophin levels (0.3–1.7%). Interestingly, these levels are comparable for the different compounds, despite the two- to five-fold higher exon 23 skipping levels observed after PMO treatment.

Using immunohistochemical analysis (Figure 5) approximately 10–20% of muscle fibers stained brightly for dystrophin in the gastrocnemius, tibialis anterior and quadriceps muscles after morpholino treatment, whereas a much lower amount of fibers (approximately 5%) was dystrophin-positive in the triceps and diaphragm. The number of positive fibers after short 2OMePS AON treatment was similar to the number of revertant fibers observed in saline-treated muscles for all skeletal muscles and the diaphragm. The only exception was the triceps muscle, where the number of dystrophin positive fibers was slightly enhanced (3% versus approximately 1%). Only occasional positive fibers were observed in the heart (indicated by asterisks in Figure 5) after 2OMePS and morpholino treatment. This corresponded to the number of revertant fibers found in saline-treated samples.

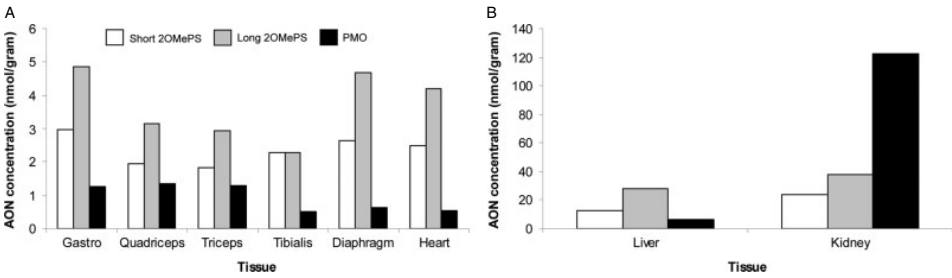

Figure 2. Biodistribution of short and long 2OMePS AONs and PMOs targeting mouse exon 23. Each sample was analysed twice. Average values of these replicates are shown. (A) The highest levels were observed in each skeletal muscle tissue for the long 2OMePS AON compared to the short 2OMePS AON, whereas PMO concentrations were lower. (B) 2OMePS AONs were found in comparable levels in the kidney and liver, whereas PMOs were almost exclusively found in kidney. Gastro is gastrocnemius

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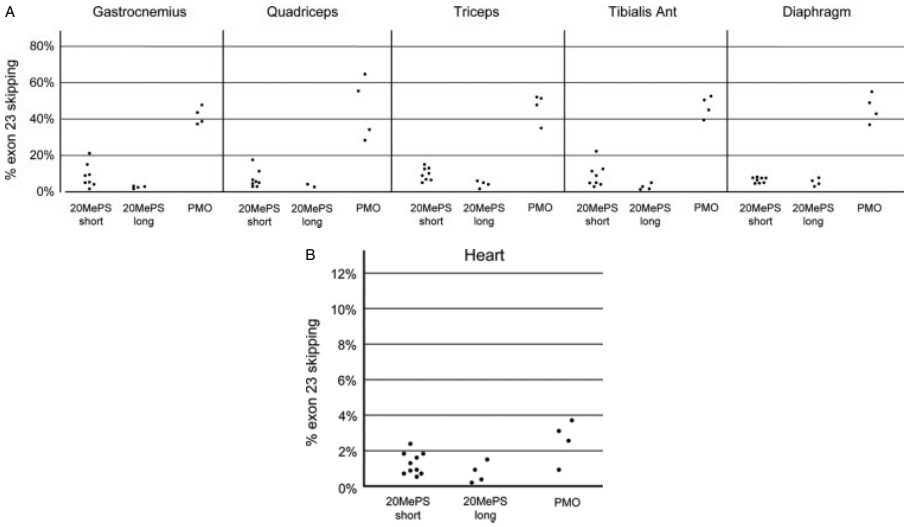

Figure 3. Quantification of exon 23 skipping levels analysed by RT-PCR for skeletal and heart muscle of *mdx* mice after intravenous injection of 2OMePS and PMO AONs. RT-PCR analysis was performed twice for each RNA sample and each replicate is shown. The quantified values are shown as separate dots for each sample. No exon 23 skipping was observed in saline-treated muscles (data not shown). (A) For all skeletal muscles analysed, PMOs induced the highest levels of exon skipping (approximately 40%), followed by the short 2OMePS AONs (approximately 10%) and the long 2OMePS AONs (approximately 5%). We did not observe a difference in skipping levels between the muscles analysed, with the exception of the diaphragm, where skipping levels for the short 2OMePS AON were somewhat lower than in other muscles. (B) Low levels of exon skipping were detectable in the heart for each AON. By contrast to skeletal muscle, the levels of skipping where only slightly higher for the PMO compared to the 2OMePS AONs (approximately 2.5% versus 1.5%)

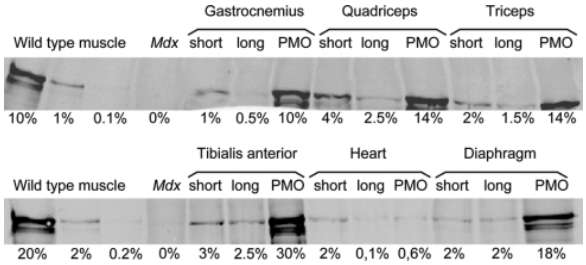

Figure 4. Western blot analysis of skeletal and heart muscle of *mdx* mice systemically treated with the long and short 2OMePS and PMO AONs targeting mouse exon 23. NCL-DYS1 was used to detect dystrophin. Dystrophin levels were quantified as percentages of wild type gastrocnemius dystrophin and are shown underneath the western blot. The results obtained were comparable for the different mice within each treatment group and therefore only a single sample per muscle is shown per group. No protein was detected in nontreated *mdx* mouse muscle (*Mdx*). After treatment, dystrophin was detectable in each skeletal muscle analysed. Dystrophin levels of PMO treated animals were higher than those treated with either the short or long 2OMePS AON for the skeletal muscles, which corresponds to the RT-PCR data. In heart, protein levels found are low for each AON, again in accordance with the low skipping levels found at the RNA level

Discussion

In the present study, we directly compared the effect and biodistribution of 2OMePS and PMO chemistries after intramuscular or systemic injections of equal molar amounts and identical dosing regimes. The difference in efficiency found for exon 23 PMO and 2OMePS AONs in *mdx* mice was less prominent or absent for AONs targeting human exons in hDMD mice. In addition, for one out of four tested sequences, the longer 2OMePS AON was significantly more efficient than the shorter (i.e. the AON targeting exon 45). This is in accordance with a study by Harding *et al*. [24] reporting that longer

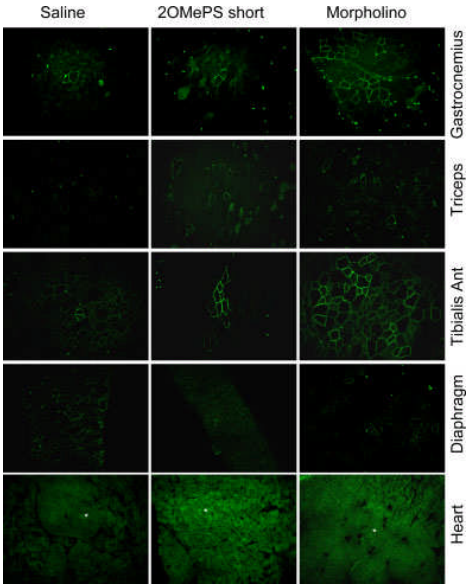

Figure 5. Immunohistochemical analysis of skeletal and heart muscle of *mdx* mouse treated with long and short 2OMePS and morpholino AONs. NCL-Dys2 was used to detect dystrophin. In the gastrocnemius, quadriceps and tibialis anterior muscles, approximately 10–20% of fibers were dystrophin positive after systemic delivery of morpholinos. Lower levels of positive fibers were found in the triceps and diaphragm (approximately 5–10%). The number of dystrophin positive fibers was similar for saline and 2OMePS treated animals (<1%). Only in the triceps muscle, somewhat higher levels were observed (approximately 3%). For the heart, the number of dystrophin positive fibers after either 2OMePS or morpholino treatment did not differ from saline-injected animals (<0.5%)

AONs are more effective for some but not all target sequences. The endogenous murine *DMD* gene in the hDMD mouse allowed us to test AON specificity in parallel. For two of the short 2OMePS AONs (targeting exon 44 and 45), the mouse sequence was completely homologous to the human sequence. For the exon 45 AON, we indeed observed similar levels of exon 45 skipping in human and mouse transcripts. However, for exon 44, we only observed skipping of the human exon and not of the mouse exon. The mouse sequence was checked for polymorphisms, but none were found. Another explanation for this unexpected finding is that the predicted secondary structure of human and mouse exon 44, as predicted by the m-fold program [30], are very dissimilar. The predicted target site of the AON is an open, accessible structure in humans, whereas it is located in a closed structure in the mouse exon. For the longer 2OMePS and PMO AONs, there were at least two mismatches with the mouse sequence. For the 2OmePS, these mismatches almost completely abolished AON efficacy. By contrast, two of the four PMOs tested induced skipping of the murine exon. For exon 51, this aspecific skipping was at a lower level than that observed for human exon 51. However, for exon 45, the skipping levels of the mouse and human exons were similar. AONs are screened with BLAST to confirm that they only hybridize to their intended target exon. However, if AONs are unable to discriminate between their target sequence and a sequence containing two mismatches, the chance of mistargeting will increase significantly. Thus far, there is no report of such off target effects, but it is expected that, in future applications of this therapy, patients will have to be dosed repeatedly and chronically, which may increase the chance of detrimental side-effects.

hDMD mice contain the full-length murine and human *DMD* genes that both produce functional dystrophin. Therefore, they do not have a dystrophic phenotype [12,13]. Thus, AON uptake by the muscle after systemic delivery is expected to be very low. We have performed a pilot experiment where h51AON1 was injected intravenously for 100 mg/kg for several weeks in hDMD mice. As anticipated we only observed very low levels of exon 51 skipping (<0.5%) in some and no exon skipping in other muscles (Heemskerk *et al.*, unpublished data). The healthy nature of the fibers also explains why exon skipping levels after intramuscular injection of AONs are much lower than those observed in the *mdx* mouse (Figure 1C) and in previous tissue culture experiments [27].

After systemic injection, PMO AONs were present in lower amounts in skeletal muscle than 2OMePS AONs, whereas PMOs were more efficient in inducing DMD exon 23 skipping in the *mdx* mouse model. This higher efficiency has been noted previously [15,16]. In addition, Sazani *et al.* [31] reported that, in skeletal muscle, PMO AONs outperformed 2′-*O*-methoxyethyl PS AONs (2OMOEPS) AONs, with a chemistry that is very similar to 2OMePS [31]. The discrepancy may be explained by the fact the hybridization-ligation assay gives the total amount of AON in the entire tissue, without any specification as to whether AONs are present in muscle fibers or in interstitial spaces. It is possible that, for the PMO, the relative amount located within the muscle was high, whereas part of the 2OMePS AONs was sequestered in the interstitial space. Similarly, it is not unlikely that the long AON is sequestered at higher levels in the extracellular matrix in interstitial spaces due to its increased length. The high PMO levels in the kidney may be explained by the fact that the uncharged PMO binds less plasma proteins nonspecifically. This leads to a higher amount of PMO being filtered and reabsorbed in the kidney and, subsequently, to higher concentrations in the kidneys and lower concentrations in muscle and liver [32]. Because the long 2OMePS will bind many plasma proteins, the serum half-life will increase [33] and more will end up in muscle and liver. Finally, although similar AON levels were found in the heart and skeletal muscles, exon skipping levels were much lower in the heart. This might also be explained by AONs being captured in the interstitial space and therefore unable to induce exon skipping.

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Despite similar percentages of exon skipping at the RNA level, the amount of dystrophin protein varied between different muscle types. This difference might be caused by the level of fibrosis per muscle. The exon skipping approach utilizes dystrophin transcripts and dystrophin is not expressed in fibrotic tissue. The tibialis anterior generally has a low level of fibrosis and, as a result, more fibers that produce dystrophin. A similar difference was seen between patients with a high level of fibrosis and a low level of fibrosis in the clinical trial by van Deutekom *et al*. [14]. However, within a certain muscle, different levels of exon skipping did correlate with protein levels; for example, in the triceps, the short 2OMePS was more efficient than the long 2OMePS at the RNA level and, accordingly, more dystrophin was detected by western blotting, whereas long and short AONs induced similar levels of exon skipping and dystrophin protein in the diaphragm. This possibly reflects different protein and RNA turnover in the different muscles. In the heart on the other hand, the levels of exon skipping did not correlate with the levels of dystrophin restoration as, at the RNA level, the PMO was the most efficient, whereas efficiencies are comparable on protein levels. We have no explanation for the fact that, within the same tissue, exon skipping levels and protein levels do not correlate. Furthermore, heart dystrophin levels were comparable to skeletal muscle levels after short 2OMePS treatment, despite two- to five-fold lower exon skipping levels. This finding was confirmed for four individual mice (data not shown). An explanation might be that more dystrophin is present in the heart, or that dystrophin is more stable in heart tissue. Notably, this is the first time that exon skipping and protein production in the heart has been described after systemic delivery of unconjugated PMO or 2OMePS. Compared to other studies with unconjugated AONs [15,16], we applied a higher total dose over a shorter time period, which might explain why other studies have failed to detect exon skipping and protein production in the heart to date. In addition, we assessed protein levels with the Odyssey system, which may be more sensitive than traditional methods. The amount of dystrophin after morpholino treatment determined by western blotting does not correlate with the number of dystrophin positive fibers assessed by immunohistochemical analysis. The triceps and diaphragm show relatively high levels of dystrophin by western blotting, whereas the number of dystrophin-positive fibers was notably lower than those of the quadriceps and gastrocnemius muscles. However, immunohistochemical analysis is not quantitative and it is possible that either a small number of fibers expressed high levels of dystrophin or that the majority of fibers expressed low (undetectable) levels of dystrophin.

In conclusion, the difference in efficiency observed for PMOs and 2OMePS targeting exon 23 in the *mdx* mouse was confirmed in the present study, but was less pronounced or even absent for human target sequences. In addition, our data show that PMOs may be less sequence-specific than 2OMePS AONs. Finally, we show that systemic administration of both 2OMePS and PMO can induce skipping and novel protein production in the heart.

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