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The dynamics of compound, transcript and protein effects after treatment with 2OMePS antisense oligonucleotides in *mdx* mice

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Chapter 4

Abstract

Antisense-mediated exon skipping is currently in clinical development for Duchenne muscular dystrophy (DMD) to amend the consequences of the underlying genetic defect and restore dystrophin expression. Due to turnover of compound, transcript and protein, chronic treatment with effector molecules (antisense oligonucleotides) will be required. To investigate the dynamics and persistence of antisense 2'-*O*-methyl phosphorothioate oligonucleotides, exon skipping, and dystrophin expression after dosing was concluded, *mdx* mice were treated subcutaneously for eight weeks with 100 mg/kg oligonucleotides twice weekly. Thereafter, mice were sacrificed at different time points after the final injection (36 hours-24 weeks). Oligonucleotide half-life was longer in heart (~65 days) compared with that in skeletal muscle, liver and kidney (~35 days). Exon skipping half-lives varied between 33 and 53 days, whereas dystrophin protein showed a long half-life (>100 days). Oligonucleotide and exon skipping levels peaked in the first week and declined thereafter. By contrast, dystrophin expression peaked after three to eight weeks and then slowly declined, remaining detectable after 24 weeks. Concordance between levels of oligonucleotides, exon skipping and proteins was observed, except in heart, wherein high oligonucleotide levels, but low exon skipping and dystrophin expression were seen. Overall these results enhance our understanding of the pharmacokinetics and pharmacodynamics of 2'-*O*-methyl phosphorothioate oligos used for the treatment of DMD.

Introduction

Duchenne muscular dystrophy (DMD) is the most prevalent form of inherited muscular dystrophies affecting around one in 5 000 newborn boys.[1,2] Patients display severe progressive muscular weakness due to the absence of the dystrophin protein, which functions as mechanical stabiliser during muscle contraction. In the absence of dystrophin the muscle fibres are easily damaged and are gradually replaced by fibrotic and adipose tissues. First symptoms generally become apparent at two or three years of age, after which, pathology develops rapidly. Patients often die before the age of 30 years due to respiratory and/or cardiac failure.[5]

In humans, lack of dystrophin is generally caused by out-of-frame deletions or small mutations that introduce premature stop codons in the *DMD* gene.[670] Several animal models are available for DMD, among which, the *mdx* mouse (C57Bl/10ScSn-DMD*mdx*/J) -which does not express dystrophin due to a premature stop codon in exon 23- is the most widely used.[79] However, the lack of dystrophin is less disastrous in *mdx* mice than in humans. These mice have a nearly normal life expectancy, only slightly impaired muscle function and a better muscle quality compared with DMD patients.[671] Nevertheless, this mouse model displays several features of the DMD pathology, *i.e.* muscle degeneration and leaky fibres due to the absence of dystrophin, and is very useful for preclinical studies.[672]

During the past several years a lot of progress has been made toward the development of antisense oligonucleotide (AON)-mediated exon skipping as a potential therapy targeting the underlying genetic defect of DMD. This aims to restore the reading frame or bypass a small mutation by skipping one or more exons. Thereby translation can continue and a largely functional protein can be formed, as is found in the related, but much milder Becker muscular dystrophy. After obtaining proof-of-principle *in vitro* in cultured cells and *in vivo* in animal models, clinical trials for AONs with two different backbone chemistries, 2'-*O*-methyl phosphorothioate RNA (2OMePS) and phosphorodiamidate morpholino oligomers (PMO), are currently ongoing.[673] Systemic (subcutaneous) treatment with drisapersen (2OMePS AON, targeting exon 51) for five weeks induced dystrophin expression in ten of 12 patients up to 15.6% of levels found in healthy persons.[229] Treatment with eteplirsen (PMO AON, targeting exon 51) by intravenous infusion resulted in dystrophin restoration in seven of 19 patients at highly variable percentages up to 18% of control levels.[231] Placebo-controlled phase 2 and 3 trials have been conducted for drisapersen. Encouraging results were reported for the phase 2 trials, suggesting improved walking distance in six minutes for treated patients compared with placebo administered after 24 and 48 weeks of treatment. However, it was recently reported that the primary outcome measure (distance walked in six minutes) in the phase 3 trial,[233] although slightly improved in the treated patients, did not differ significantly from the placebo treated patients. More detailed analysis is pending. In addition to drisapersen (targeting exon 51), AONs targeting other exons, *i.e.* exon 44, 45 and 53, are under clinical evaluation.

Because RNA-mediated therapies are subject to clearance and turnover of AONs and the dystrophin transcripts and proteins, repeated, life-long injections will be required to maintain therapeutic effects. Therefore, insight in the pharmacokinetic (PK) and pharmacodynamic (PD) properties of these compounds is essential to determine how long the effects persist and to assess dosing frequencies and regimens. Previous studies have revealed a plasma half-life for 2OMePS AONs of around four weeks in patients[229] and a tissue half-life of two to six weeks in *mdx* mouse muscle.[200] It has previously been observed that uptake of AONs is better in dystrophic muscle than in healthy muscle, probably due to the dystrophic "leaky" nature of the muscle fibres. Both subcutaneous and intravenous routes of administration resulted

in muscle uptake and exon skipping, but intravenous injections led to much higher AON levels in liver and kidneys, although AON levels in muscle were comparable with those adter subcutaneous treatment.[200] Furthermore, for 2OMePS AONs, exon skipping and dystrophin protein levels vary among different muscle tissues. Generally exon skipping levels in heart are lower, whereas the levels are comparable in limb muscle and diaphragm.[161,200,201,219,240,259] Increase in exon skipping and accumulation of dystrophin protein were seen up to 12 weeks of treatment and long term treatment for six months has been shown to be feasible.[201,219] Comparison of various maintenance regimens revealed that eight weeks after the last dose, the decline in AON levels was faster than the decline in exon skipping and protein levels, indicating differences in turnover.[240] However, the relation between clearance and turnover of the compound, the induced skipped transcript, and restored dystrophin protein and long term analysis of the persistence of different effects after subcutaneous treatment have not been studied. In order to expand our understanding of the PK/PD relationship of 2OMePS AONs, *mdx* mice, in which the mutation can be bypassed by skipping of exon 23, were dosed with 100 mg/kg twice weekly for eight weeks via the subcutaneous route, after which, tissues were harvested at different time points for the analysis of 23AON, exon skipping and protein levels.

Materials and methods

All experiments were approved by the local ethical committee for animal experiments of the Leiden University Medical Center. Mice were housed in individually ventilated cages in the animal facility of the Leiden University Medical Center and received food and drink *ad libitum*. *Mdx* mice (C57Bl/10ScSn-DMD*mdx*/J) were obtained from our own breeding facility.

Treatment of mdx mice with 23AON and sample preparation

Starting at an age of 3-4 weeks, 36 *mdx* mice were treated subcutaneously with 100 mg of 23AON/kg body weight in 100 μL saline twice weekly for 8 weeks. The 23AON molecule, previously described as M23D(+2-18), is a 2'-*O*-methyl phosphorothioate RNA oligonucleotide with a full-length phosphorothioate backbone, specifically targeting exon 23[197] (Prosensa Therapeutics, Leiden, the Netherlands). Mice were sacrificed by cervical dislocation at different time points after the last injection: t=36 hours and 1, 3, 8, 12, and 24 weeks (6 mice (3 males, 3 females) per time point). Before sacrifice, blood samples were taken for plasma PK analysis (see below). Plasma was generated by centrifuging at 18 000 *g* for 5 min and it was stored at -80 ºC until analysis. After sacrifice, muscles (triceps, tibialis anterior, quadriceps, heart and diaphragm) and liver, kidney and spleen were isolated, snap frozen in liquid nitrogen cooled 2-methylbutane and stored at -80 °C.

Assessment of 23AON levels with a hybridization-ligation assay

An assay based on a previously published hybridization-ligation assay was used for the determination of the 23AON level in different tissues at Prosensa Therapeutics.[664] Tissues were homogenized in 100 mM Tris-HCl (pH 8.5), 200 mM NaCl, 0.2% sodium dodecyl sulfate, 5 mM ethylenediaminetetraacetic, and 2 mg/mL protK using zirconium beads (1.4 mm; OPS Diagnostics, Lebanon, NJ) in a MagNA Lyser (Roche Diagnostics, Almere, the Netherlands)

according to manufacturer's protocol. Plasma samples were diluted tenfold, muscle samples 500 and 1 000 times and organs 1 000 and 5 000 in pooled control *mdx* tissue in phosphate-buffered saline. A signal probe (containing the peptide for antibody recognition) and a template (complementary to 23AON and the probe) were added to homogenized samples. Only when both 23AON and probe are bound to the template, a ligation step will take place. After this step and washing away of the unbound probe, enzyme-linked antibodies were used to detect the amount of probe-23AON. Calibration curves of the analysed 23AON prepared in 10% pooled plasma or in pooled control mouse *mdx* tissue in phosphate-buffered saline were used to quantify 23AON. All analyses were performed in duplicate.

RNA extraction and analysis of exon skipping by RT-PCR

Muscles were homogenized in TriPure isolation reagent (Roche Diagnostics) using zirconium beads (1.4 mm; OPS Diagnostics) by grinding in a MagNA Lyser (Roche Diagnostics). Total RNA was extracted and 600 ng was used for reverse transcription–polymerase chain reaction analysis. Complementary DNA was generated by incubating at 42 °C for 45 min with random hexamer primers (20 ng/µL) and Transcriptor reverse transcriptase polymerase (Roche Diagnostics) in 20 µL. Subsequently, 2 µL of cDNA was amplified in a 50 µL polymerase chain reaction reaction with 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, as previously described.[663] PCR products were visualized on 1.5% agarose gels and quantified using a DNA high sensitivity chip on the LabChip GX, in combination with the LabChip GX software (Caliper Life Sciences, Teralfene, Belgium).

Analysis of dystrophin protein expression by Western blot

Muscles were minced in treatment buffer containing 75 mM Tris-HCl pH 6.8-15% (w/v) sodium dodecyl sulphate using zirconium beads (1.4 mm; OPS Diagnostics) or MagNA Lyser green beads (Roche Diagnostics) in a BBY24M Bullet Blender Storm (Next Advance, Averill Park, NY). Protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Samples containing 30 µg of protein were made in treatment buffer with 20% (v/v) glycerol, 5% (v/v) β-Mercaptoethanol and 0.001% (w/v) Bromophenol blue and heated for 5 min at 95 °C. Wild type control samples containing 10%, 3.3%, 1.1% and 0.4% of protein were used as reference. Samples were loaded on 1.0 mm thick native PAGE Tris-acetate (polyacrylamide) gels, with a linear resolving gel gradient of 3-8% (BioRad, Veenendaal, the Netherlands) and run on the Trans-Blot Turbo system for 1 h at 75 V (0.07 A) and 2½ hrs at 150 V (0.12 A) in an ice container. Proteins were blotted on a nitrocellulose membrane using the Ready to use Trans-Blot Turbo transfer packs in combination with the Trans-Blot Turbo transfer system (BioRad) at 2.5 A and ~25 V for 10 min. Membranes were blocked in 10 mM Tris-HCl (pH 8) and 0.15 M NaCl Tris-buffered saline (TBS)-5% non-fat dried milk (Elk, Campina Melkunie, Zaltbommel, the Netherlands), washed in TBS-0.05% (v/v) Tween20 (TBST). Membranes were incubated overnight with 1:125 NCL-Dys1 (Dy4; NovoCastra, Newcastle Upon Tyne, UK) and α-actinin as loading control (1:7 500; AB72592; Abcam, Cambridge, UK) in TBS. The next day, membranes were washed in TBST, incubated 1 h with the fluorescent secondary antibodies IRDye 800CW goat-α-mouse IgG (1:5 000; Li-Cor; NE) and IRDye 680LT donkey-α-rabbit IgG (1:10 000; Li-Cor) in TBS, washed in TBST and TBS and analysed with the Odyssey system and software (Li-Cor) (M. Hulsker *et al.*, manuscript in preparation).

Statistical analysis

For 23AON levels, individual data are represented for each measurement and exponential curves were fitted for each tissue. For exon skipping and protein levels data are represented as mean ± SD for each tissue. PK plasma parameters (terminal half-lives of the molecule in plasma and tissue) were derived by non-compartmental analysis using Phoenix WinNonLin software (version 6.2; Pharsight Corporation, Mountain View, CA). For samples in which 23AON was not detectable, values were set at 50% of limit of quantification of mouse 23AON in neat plasma (*i.e.* 50% of 0.011 µg/ml). For statistical testing, SPSS version 20.0 (IBM Corporation, Armonk, NY) was used. A *p* value ≤0.05 was considered significant.

Results

Dynamics of 23AON levels in plasma, muscle and organs

To investigate the dynamics of the effects induced by treatment with 2OMePS AONs, mice were treated subcutaneously with 100 mg 23AON/kg body weight twice weekly for eight weeks. Six mice per group were sacrificed at different time points after the last injection, ranging from 36 hours up to 24 weeks. To assess the uptake, turnover and clearance of the AON in plasma and different tissues, 23AON levels were determined with an AON specific hybridization-ligation assay, and average results were calculated (Fig. 4.1; Table 4.1). For all tissues, this revealed a decline in AON levels after the final injection, and 23AON was still detectable in muscle, liver, and kidney after 24 weeks (on average around five to ten percent of the 23AON levels at 36 hours). Terminal half-lives ranged from 28 days in triceps
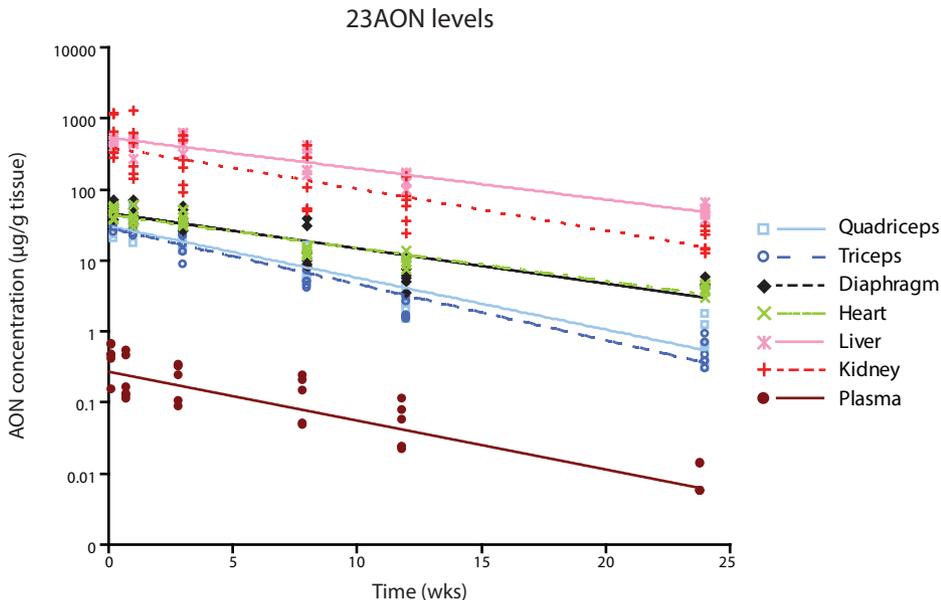

Fig. 4.1: 23AON levels in plasma, muscle and other organs over time

A hybridization-ligation assay was used to determine 23AON levels in plasma, muscle, and organs at different time points ranging from 36 hours up to 24 weeks after the final injection. Data are represented on a logarithmic scale. Each dot represents an individual measurement and lines are fitted through the averages for each tissue. AON, antisense oligonucleotide.

	AON levels[a]						
	Muscle					Other organs	
Time (wks)	Plasma	Quadriceps	Tibialis anterior	Diaphragm	Heart	Liver	Kidney
0.2	0.462 ± 0.205	32.3 ± 9.1	33.0 ± 5.6	54.3 ± 11.4	49.1 ± 9.0	477.0 ± 49.7	655.7 ± 419.9
1	0.245 ± 0.183	33.9 ± 16.3	28.2 ± 4.6	51.1 ± 16.3	40.7 ± 10.5	456.1 ± 101.3	479.7 ± 437.9
3	0.236 ± 0.119	18.8 ± 6.1	18.3 ± 6.6	40.5 ± 12.5	40.6 ± 9.2	510.9 ± 119.6	289.6 ± 200.3
8	0.137 ± 0.092	8.9 ± 5.1	5.7 ± 1.7	18.1 ± 12.9	13.3 ± 1.6	270.1 ± 109.3	160.1 ± 155.0
12	0.052 ± 0.041	2.9 ± 1.2	2.0 ± 0.7	6.4 ± 2.5	10.2 ± 2.0	142.7 ± 29.1	70.3 ± 44.5
24	0.008 ± 0.004	0.8 ± 0.6	0.5 ± 0.2	4.7 ± 0.7	4.0 ± 0.7	50.4 ± 10.2	20.1 ± 7.2
Terminal half-life (days)	30	32	28	45	65	48	38

Table 4.1: Average AON levels at each time point per tissue and terminal half-life for each tissue

[a]23AON levels are expressed as µg/mL for plasma and µg/g tissue for muscle and other organs (mean ± SD).

to 65 days in heart (Table 4.1). The half-life in heart was much longer than that in the other tissues. Although the terminal half-life in diaphragm was comparable to liver and kidney, it was slightly shorter in limb muscles (quadriceps and triceps) and comparable with that found in plasma. As observed previously,[200] AON levels were higher in liver and kidney than in muscle. When comparing the different muscles with each other, 23AON levels were higher in diaphragm and heart than in limb muscles ($p<0.05$). No differences were observed in 23AON levels when comparing liver and kidney. Oligonucleotide levels in plasma decreased rapidly after the final dose and by 24 weeks, 23AON was barely detectable when levels in only two out of six mice reached the limit of detection.

Dynamics of exon skipping levels in muscle

The exon 23 skipping levels were determined in several limb muscles, diaphragm and heart (Fig. 4.2; Table 4.2). Exon skipping levels were highest at the early time points (36 hours
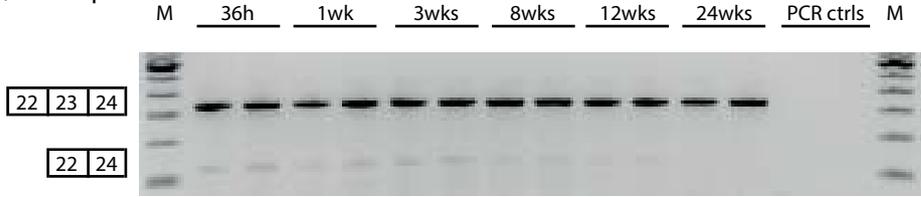
	Exon skipping levels[a]				Protein levels[b]	
Time (wks)	Quadriceps	Tibialis anterior	Diaphragm	Heart	Quadriceps	Diaphragm
0.2	11.3 ± 1.3	11.2 ± 2.6	11.3 ± 2.4	4.1 ± 0.5	0.61 ± 0.23	2.36 ± 0.49
1	9.7 ± 2.1	8.7 ± 1.9	11.7 ± 2.7	3.2 ± 0.7	0.95 ± 0.13	1.93 ± 0.52
3	6.9 ± 2.2	9.4 ± 1.6	10.6 ± 3.4	2.7 ± 0.5	1.31 ± 0.63	2.43 ± 0.85
8	5.7 ± 2.4	2.8 ± 1.0	8.6 ± 0.5	0.9 ± 0.7	0.91 ± 0.44	2.75 ± 1.21
12	3.1 ± 1.3	2.2 ± 1.7	3.7 ± 1.6	1.0 ± 0.3	0.56 ± 0.38	2.42 ± 0.99
24	ND	ND	ND	ND	0.39 ± 0.19	1.57 ± 0.63
Terminal half-life (days)	53	33	49	39	103	137

Table 4.2: Average exon skipping and protein levels at each time point per muscle and terminal half-life for each muscle

ND, not detectable.

[a]Exon 23 skipping levels are expressed as skipped transcript as percentage of total transcript (mean ± SD). [b]Dystrophin protein levels are expressed as percentage of wild type levels in the same muscle (mean ± SD).

A   Quadriceps

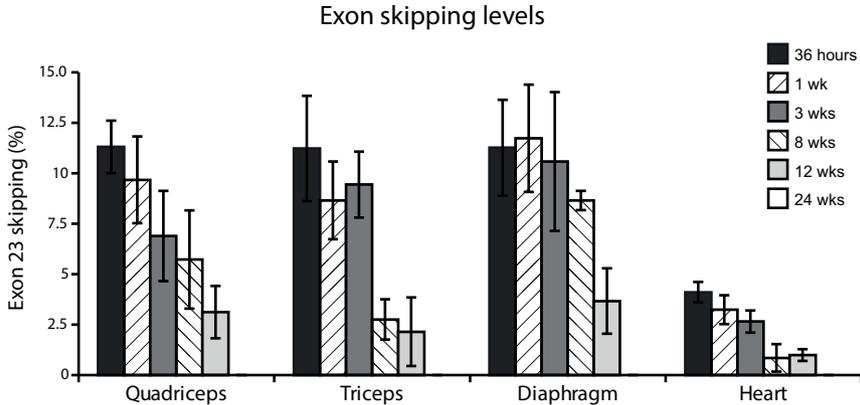B   Exon skipping levels

Fig. 4.2: Exon skipping levels in different muscles over time
a) Representative example of an RT-PCR analysis for the quadriceps. Unskipped product consists of 334 base pairs and exon 23 skipping results in a 121 base pair product. PCR ctrls indicates PCR controls, M is marker. b) Quantification of exon 23 skipping levels by RT-PCR analysis at several time points after the final 23AON injection. Data are represented as mean ± SD. AON, antisense oligonucleotide.

and one week after final AON injection) and declined thereafter. Exon skipping levels were significantly lower in heart compared to skeletal muscle ($p<0.01$). After 24 weeks skipping was undetectable in all samples. No large differences in rate of decline between individual muscles were observed, considering the interindividual variations within a tissue and the half-life varied between 33 and 53 days.

Dynamics of dystrophin protein expression

Restoration of dystrophin protein expression after AON treatment was detectable at each time point in quadriceps and diaphragm (Fig. 4.3; Table 4.2). No dystrophin was observed for untreated *mdx* mice (data not shown). In treated muscles dystrophin levels were initially low, peaked somewhere between three and eight weeks after the final injection for both diaphragm and quadriceps, and then slowly declined. Levels in diaphragm were and remained significantly ($p<0.01$) higher than those in quadriceps and also were slightly more stable, with half-lives of ~100 and 130 days, respectively. In heart no dystrophin protein levels >0.5% could be detected (data not shown).

Correlation among 23AON levels, exon skipping, and protein expression

For the majority of muscles analysed, a correlation among 23AON levels, exon skipping and protein expression was observed. The highest 23AON levels were observed in the diaphragm, resulting in highest exon skipping and dystrophin protein expression. Lower 23AON levels were observed in quadriceps and triceps, resulting in exon skipping levels that were at most
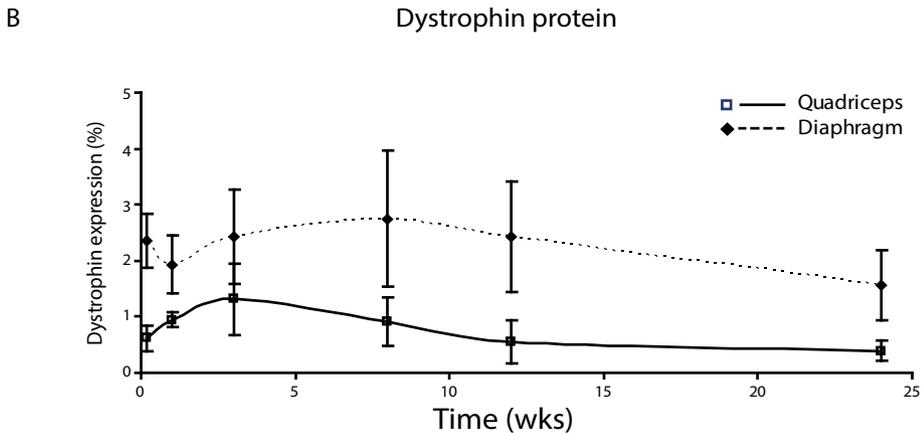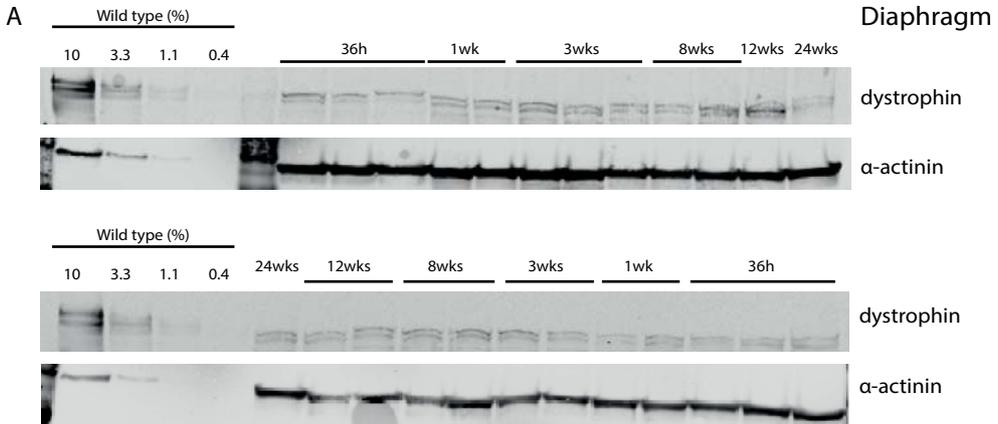
Fig. 4.3: Dystrophin protein levels in muscle over time

a) Representative examples of Western blot analyses for the diaphragm showing low levels of dystrophin protein (*top*) at all analysed time points. As loading control, α-actinin (*bottom*) was used. b) Quantification of dystrophin protein levels, detected by Western blot analysis. Data are represented as mean ± SD for each tissue.

time points analysed, slightly lower than or comparable with exon skipping levels in diaphragm. Furthermore, the level of dystrophin protein expression in quadriceps was lower than the corresponding values observed in diaphragm. By contrast, this correlation was not observed in heart, wherein 23AON levels were relatively high (comparable with diaphragm), while exon skipping levels were significantly lower than those in skeletal muscle; moreover dystrophin protein expression was very low.

When comparing patterns of the different parameters, both 23AON and exon skipping levels peaked shortly after treatment, within the first week, and thereafter showed a similar decline pattern, whereas the profile of dystrophin protein expression was shifted, peaking a few weeks after treatment and displaying a more prolonged effect (Fig. 4.4).
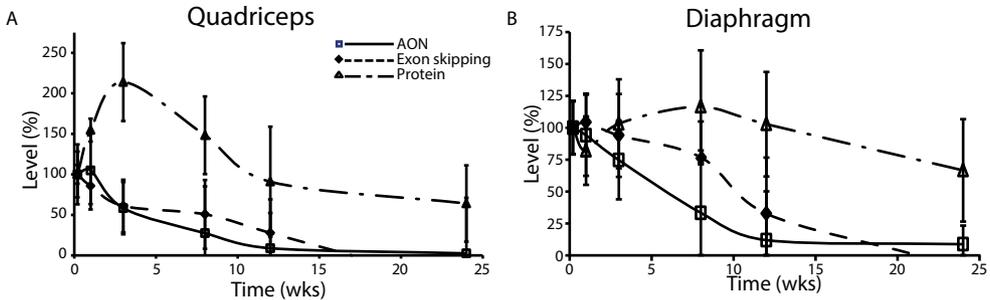
Fig. 4.4: Comparison of time effects at different levels
Comparison of time course of 23AON levels, exon skipping, and protein expression after the final injection for (a) quadriceps and (b) diaphragm. Data are represented as percentages of levels observed at time point t=36 hours (set at 100%) for 23AON, exon skipping ,and protein levels separately. Error bars represent the standard deviation. AON, antisense oligonucleotide

Discussion

AON-mediated exon skipping is currently in advanced stages of clinical development as a potential therapy for DMD aiming to partly correct consequences of the genetic defect. However, due to clearance of AONs, skipped transcripts, and restored dystrophin protein, chronic treatment will be required. One way to try and prolong the effect is to deliver antisense sequences using (viral) vectors in order to achieve a stabler expression of the AONs. Adeno-associated viruses (AAVs) expressing modified small nuclear ribonucleoprotein (snRNP) particles, *i.e.* U1 and U7 small nuclear ribonucleoprotein particles, in which the small nuclear RNA is replaced by the desired AON sequence, have shown to induce long term dystrophin rescue in the *mdx* mouse.[246] This approach has also been proved to result in long term cardiac expression of dystrophin in the GRMD dog,[327] harbouring a deletion of exon 7, requiring exon 6 and 8 skipping to restore the reading frame.[122] Drawbacks of using AAVs as vectors are toxicity concerns and the possibility of an immune response against the viral vector, resulting in the loss of transduced fibres.[300] A recent five year follow-up study in GRMD dogs did not show immune rejection; however after five years, disappearance of dystrophin protein expression was observed, probably due to instability of the newly formed protein.[251] The same was observed in a study in *mdx* mice, wherein it was also shown that AAV vectors and transgene expression were lost quicker from dystrophic muscle than from healthy muscle.[252] Therefore, repetition of treatment would still be required, which is not possible due to immunization against the AAV after the first injection. This makes it unlikely this approach will be clinically applicable in the near future. Thus, the AON approach is more viable in the short term, which involves repeated injections, and knowledge about the PK and PD effects is, therefore, valuable. The detailed longitudinal analysis of different muscles, kidney and liver is ethically and practically challenging in humans but more straightforward in mice. In this study, the clearance/turnover of AON, transcript, and protein over time in *mdx* mice after treatment with 2OMePS AON, one of the two background chemistries mainly used in clinical trials, was studied.

First of all the decline in 23AON levels over time was studied in plasma, muscle, and other organs following subcutaneous injections. After injection, AON levels in plasma decline rapidly due to uptake by organs and clearance, which occurs primarily through the kidney. For 2OMePS AONs this is partly modulated by the plasma protein binding properties of the phosphorothioate backbone of the AONs, but when high doses are injected exceeding the

binding capacity, unbound AONs will be cleared rapidly.[668,669] The first time point analysed was 36 hours after the final injection, the time point when previous studies indicated that the majority of the plasma clearance has already occurred.[240] The mean half-life determined for 23AONs in mice (30 days) was comparable with that observed for 51AON in patients (29 days).[229] When comparing different muscle types, higher 23AON levels in diaphragm and heart were observed, whrereas the longest terminal half-life was found in heart. This was probably due to reduced AON turnover because the turnover rate of cardiac muscle cells is much slower than that of skeletal muscle fibres (especially dystrophic skeletal muscle fibres). This is in line with previous observations of a longer half-life of AONs in heart compared with that in skeletal muscle. In the previous study, a markedly shorter half-life in triceps (~10 days) was observed compared with that in quadriceps (~33 days), whereas in the current studies half-lives are comparable (~30 days). Furthermore, the currently calculated half-life in heart is longer (65 days versus 46 days).[200] These discrepancies are probably due to the fact that in the previous study only four time points up to 14 days after the final injection were measured, consisting of two mice per time point, making the estimations less accurate. Furthermore, in the previous study intravenous injections were used, which show higher levels of AON uptake by the kidney and liver,[200] which can also have contributed to the observed differences. As expected, AON levels in liver and kidney were much higher than those in muscle. It has been noticed before that for 2OMePS AONs levels are similar in liver and kidney[161] and here is shown that the decline pattern is comparable too. Unfortunately, when comparing the terminal half-lives in the non-target organs (liver and kidney) to the targeted organ (muscle), half-lives were slightly shorter in limb muscles compared with those in liver and kidney. A longer muscle half-life would have been advantageous for planning off-treatment periods, *e.g.* to prevent accumulation of compounds in liver and kidney. Nevertheless, the absolute reduction of AON levels in liver and kidney is greater than that in muscles.

Second, exon skipping levels were determined in several skeletal muscles and the heart. Here, highest levels were also observed shortly after the final injection (within the first week), whereupon they declined in a similar pattern as the 23AON levels. For diaphragm, the higher 23AON levels were also accompanied by high exon skipping levels whereas by contrast, this correlation was not observed in heart, wherein, although 23AON levels were relatively high (comparable to diaphragm), exon skipping levels were significantly lower than those in skeletal muscle.

Third, dystrophin protein levels were determined. These showed a delayed pattern, peaking a few weeks later, compared with AON and exon skipping levels. Importantly, although levels were low in general, dystrophin protein expression was observed up to 24 weeks. Wu *et al.* determined the half-life of the dystrophin protein in *mdx* mice after a single intravenous injection with peptide-conjugated PMOs (pPMOs) targeting exon 23. This resulted in a protein half-life of ~2 months for skeletal muscles and levels had dropped to around 10% after five months.[206] The half-lives measured in our experiments were a bit longer (three to four months), resulting in a smaller decrease in dystrophin levels; 30-50% of initial levels were still observed after ~5 months (levels at 24 weeks compared with peak levels) in both muscles. A possible explanation is the difference in experimental set-up and backbone chemistry of the AONs between both studies. In the present study, 2OMePS AONs were used, whereas the study of Wu *et al.* used pPMOs. These two chemistries probably have a different biodistribution pattern, as was also seen between 2OMePS AONs and naked PMOs.[161] Because AON PKs were not measured in the study of Wu *et al.*, it is not known how much AON remained in the muscle to induce further exon skipping and dystrophin production,

resulting in differences over time. Furthermore Wu *et al.* only used a single AON injection, whereas mice were treated for a longer period in our study. A single injection is more likely to give variation in AON levels between mice, which influences the preciseness of the half-life estimations. Therefore, both results are not directly comparable. Unfortunately, dystrophin protein was barely detectable in heart. This was also seen in a study using PMO AONs wherein dystrophin expression could be detected in all analysed muscles, except for the heart, after seven weeks of treatment.[202] Probably, overall higher skipping levels and/or longer treatment times are needed for protein restoration. This is underlined by the fact that after six months of weekly treatment with 200 mg/kg, dystrophin was readily detectable in heart.[201]

Overall there was a good correlation between AON effects at different levels. In diaphragm, the higher 23AON levels were accompanied by higher exon skipping levels and protein expression. Only in heart, the higher 23AON levels did not result in higher exon skipping and protein levels. This can be explained by inherent differences between cardiac and skeletal muscle. As mentioned before, skeletal muscles become permeable in the absence of dystrophin, facilitating the uptake of AONs. The heart is built up of individual cardiomyocytes, which do not display this leakiness, thereby making it harder for AONs to get inside.[200,203] The assay used for determination of 23AON levels does not discriminate between AON inside the heart as whole organ and AON that is actually inside the cardiomyocytes. Therefore, the presence of higher 23AON levels, but lower exon skipping levels, suggest that in heart the majority of AON is probably located in the interstitium and therefore ineffective. This might also be the reason why, although low 23AON levels were still detectable after 24 weeks, no exon skipping could be observed at this time point. Other possibilities are that this is due to differences in sensitivity of the assay used for determination of AON levels versus those for determining exon skipping levels or that the low AON levels are insufficient to induce exon skipping. Expression percentages for dystrophin protein are much lower than those for exon skipping. This is usually seen for both 2OMePS[201,240] and PMO/pPMO AONs,[161,206,211,212] and this discrepancy can be explained by the fact that exon skipping levels are compared to the total transcript levels in the *mdx* mouse, whereas dystrophin protein levels are compared with wild type dystrophin levels. It has been reported that compared with wild type dystrophin transcripts, a 5' to 3' imbalance exists for *mdx* dystrophin transcripts. This means that not all *mdx* dystrophin transcripts are complete and cannot be translated into dystrophin protein.[674]

In conclusion, 23AON and exon skipping levels show a similar decline pattern over time after the final injection. Exon skipping is not detectable anymore after six months, but its effects on dystrophin protein restoration remain quite stable. After treatment AONs are taken up by muscles and other organs, partly leading to immediate excretion without inducing further effects and partly leading to exon skipping inside the muscle fibres. Thereafter, new dystrophin protein can be formed, which displays a long half-life. The differences in rates of turnover of the compound itself, RNA and protein influence these effects, in addition to differences in composition and amount of degeneration and regeneration between different muscle groups. Our results proved further insight in how these processes interact and are useful for studying the long term effects of AON treatment.

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