Dose-dependent pharmacokinetic profiles of 2'-*O*-methyl phosphorothioate antisense oligonucleotides in *mdx* mice

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Chapter 3

Abstract

Antisense-mediated exon skipping is a promising therapeutic approach for Duchenne muscular dystrophy. It aims to restore the dystrophin open reading frame by skipping exons with antisense oligonucleotides (AONs) to allow production of partly functional proteins. The approach is currently tested in phase III clinical trials, but dosing and maintenance regimens have not yet been well studied.

This study compared pharmacokinetic and pharmacodynamic effects of different 2'-*O*-methyl phosphorothioate RNA AON dosing and maintenance regimens in the preclinical *mdx* mouse model. When comparing different dosing regimens over a period of eight weeks, higher levels of AON, exon skipping, and protein were observed in muscle after low daily doses compared with weekly large doses. Secondly, after receiving a high loading dose (1 250 mg/kg) in the first week, mice treated with maintenance injections twice weekly for eight weeks showed higher preservation of therapeutic effects than mice receiving less or no maintenance injections.

In both cases, the regimen resulting in highest AON and exon skipping levels in muscle, also resulted in high AON levels in liver and kidneys. These studies underline the importance of balancing optimal AON efficacy and tolerable levels in non-target organs, which may be fine-tuned by further optimisation of AON treatment regimens.

Introduction

Duchenne muscular dystrophy (DMD) is a severe, progressive muscle-wasting disorder affecting around one in 5 000 newborn boys.[660] It is caused by mutations in the *DMD* gene, located on the X-chromosome, leading to a disruption of the open reading frame, thereby causing the complete absence of the encoded dystrophin protein. Dystrophin plays an important role in the stabilization of muscle fibres during contraction, by connecting the intracellular actin cytoskeleton to the extracellular matrix. In the absence of dystrophin, muscle fibres will be damaged with normal exercise, eventually leading to loss of muscle tissue and function and premature death in the third or fourth decade of life.[5]

The exon skipping approach aims to partly correct the underlying genetic defect on RNA level by restoring the dystrophin reading frame to allow production of a slightly shorter, but largely functional, dystrophin protein as is found in Becker muscular dystrophy (BMD), a much milder form of muscular dystrophy.[661] To achieve exon skipping, antisense oligonucleotides (AONs) are used. AONs are small pieces of RNA, reverse complementary to a specific sequence in the pre-messenger RNA (pre-mRNA). By binding to its target sequence in the pre-mRNA, the AON interferes with the splicing of a specific exon, thereby preventing its incorporation in the mRNA.[164] AONs can have different chemistries. For exon skipping the development with 2'-*O*-methyl phosphorothioate RNA (2OMePS) and phosphorodiamidate morpholino oligomers (PMO) or peptide-conjugated PMOs (pPMO) is most advanced.

Proof-of-principle for exon skipping-mediated restoration of dystrophin has first been shown *in vitro*, *i.e.* in DMD patient-derived myoblast cultures, and *in vivo* in dystrophic animal models (reviewed in Aartsma-Rus *et al.*[269]). The most widely used model for DMD is the *mdx* mouse (C57Bl/10ScSn-DMD^{mdx}/J). These mice have a premature stopcodon in exon 23, leading to the complete absence of dystrophin protein. Although these mice display a relatively mild phenotype compared to human DMD patients,[81,159] this model well facilitates preclinical pharmacokinetic (PK) and pharmacodynamic (PD) AON studies for DMD. Treatment of *mdx* mice with 2OMePS or PMO AONs targeting exon 23 showed dystrophin restoration and improved muscle function.[161,182,197,199,200] Exon skipping and dystrophin protein levels in heart were lower compared with skeletal muscles. This is probably due to differences in the nature of the dystrophin-negative muscle fibres and cardiomyocytes. In the absence of dystrophin, skeletal muscle fibres become leaky. AONs can migrate through these holes into the muscle fibers.[161,218] However, the heart is built up of individual cardiomyocytes, which do not become leaky, thereby making the targeting of the heart more difficult and requiring higher doses.[200,203]

2OMePS (PRO051/GSK2402968/drisapersen) and PMO (AVI-4658/eteplirsen) targeting human exon 51 have been tested in clinical trials. Dystrophin restoration has been observed after local treatment[225,226] as well as after systemic treatment.[229,231] Drisapersen resulted in dystrophin restoration in ten out of 12 patients up to 15.6% of levels in healthy controls after five weeks of weekly subcutaneous injections. For eteplirsen dystrophin was restored in seven out of nine patients. The three highest responders showed levels up to 18% of controls. Further dose optimisation studies are ongoing for eteplirsen. Drisapersen is currently tested in a large, randomized, double-blinded, placebo-controlled phase III clinical study.

It is known that 2OMePS AONs have a plasma half-life of ~4 weeks in patients[229] and a half-life of ~2-6 weeks in *mdx* mouse muscle.[200] Thus, due to clearance and turnover of the AONs, but also of the dystrophin transcript and protein, repeated, life-long AON treatment will be required. In the 2OMePS clinical trials currently a weekly dose of 6 mg/kg/wk is used. However, little is known about which dose and which dosing schedule is optimal. Dividing

the same dose over multiple smaller injections might give better results than applying the same total amount at once, as has been shown for PMOs in *mdx* mice.[239] Furthermore, it is known that dystrophin transcripts and proteins have a relatively long half-life.[206,660] Thus, after having initiated the restoration of dystrophin protein, injection with lower amounts might be sufficient to maintain the same effect. Studies to optimise dosing and maintenance regimens are difficult in humans, where only limited material is available (often only a biopsy from a superficial muscle and a small amount of blood plasma). Therefore, (DMD) animal models are a useful tool for PK/PD modelling studies. These can be used to assess the ratio between AON levels and the amount of exon skipping in muscles which can be used to extrapolate the results to the human situation.

The aim of this study was to compare different dosing and maintenance regimens through assessment of PK and PD profiles for different AON treatment regimens in *mdx* mice.

Materials and methods

All experiments were approved by the local ethical committee for animal experiments of the LUMC (project code 07151). Mice were housed in individually ventilated cages in the animal facility of the LUMC 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 different 23AON dosing regimens*

Four-weeks old *mdx* mice (*n*=7 or 8 per group) were injected subcutaneously with 200 mg/kg body weight/week 23AON (3'-uccauucggcuccaaaccgg-5'; a 2'-*O*-methyl phosphorothioate AON described previously as M23D(+2-18)[197] (Prosensa Therapeutics), divided evenly over 1, 2, or 7 injections per week for 8 weeks (*i.e.* 1 weekly dose of 200 mg/kg versus 2 times 100 mg/kg versus 7 times 28.6 mg/kg). The RNA 23AON was produced in 3 gram-scale batches on an ÄKTA OP-100 oligonucleotide synthesizer (GE Healthcare) using standard phosphoramidite chemistry protocols. After synthesis, a two-step cleavage and deprotection procedure was applied; on-resin 23AON was first treated with diethylamine to remove phosphorothioate protecting groups and subsequently subjected to concentrated ammonia treatment for 16 hours at 55°C. Crude 23AON (DMT-off, *i.e.* without 4,4'-dimethoxytrityl group) was purified by IEX and transformed into its sodium salt by addition of NaCl and subsequent desalting by ultrafiltration. Negative mode electrospray ionization-mass spectrometers analysis confirmed the identity of 23AON (MW 6888) and purity (ultra-high performance liquid chromatography) of all batches was found to be acceptable (>84%). Mice were sacrificed by cervical dislocation 1 week after the last injection for their group and muscles (gastrocnemius, quadriceps, tibialis anterior, triceps, diaphragm, and heart) and organs (liver, kidney, and spleen) were isolated. Muscles were snap frozen in liquid nitrogen-cooled 2-methylbutane, and all tissues were stored at -80°C.

Treatment of mdx *mice with different maintenance doses of 23AON*

In the first week 4-weeks old *mdx* mice were treated subcutaneously with a loading dose of 5 times (*i.e.* daily for 5 days) 250 mg/kg body weight 23AON. This was followed by treatment with different maintenance regimens for 8 weeks: weekly 2 times 100 mg/kg (*n*=6), weekly 100 mg/kg (*n*=6), 100 mg/kg biweekly (*n*=5), 100 mg/kg monthly (*n*=3), or no further injections (*n*=4). Mice were sacrificed in week 8 by cervical dislocation, and muscles

(gastrocnemius, quadriceps, tibialis anterior, triceps, diaphragm, and heart) and organs (liver, kidney, and spleen) were isolated. Muscles were snap frozen in liquid nitrogen-cooled 2-methylbutane and all tissues were stored at -80°C.

Plasma sampling

Blood samples were taken weekly during treatment to assess creatine kinase (CK) levels. Blood samples were taken via the tail vein and were centrifuged at 18 000 *g* for 5 minutes at 4°C to generate plasma. This was diluted 10 times in Dulbecco's phosphate-buffered saline (Invitrogen) and CK levels were measured with a Reflotron system (Roche Diagnostics) with CK-strips (Roche). For the dosing experiments additional blood samples were taken at several time points after the first injection in the first (week 0) and the last (week 7) week of treatment (0, 15 minutes, 1 hour, 5 hours, and 24 hours from 4 animals per group). Finally, for all mice, a larger blood sample was taken prior to sacrifice to determine AON levels by enzyme-linked immunosorbent assay.

Functional testing

Mice were functionally tested weekly by forelimb grip strength and/or rotarod analysis. A grip strength meter (Columbus Instruments) was used to assess the forelimb grip strength according to the standardized operating procedure published on the translational research in Europe to accelerate treatments for neuromuscular disorders website.[663] Mice were tested 15 times (5 rounds of 3 consecutive measurements with 2 minutes in between). The forelimb grip strength corrected for body weight was calculated by dividing the average of the 3 highest values (the absolute strength) by the body weight in grams. For rotarod analysis mice were placed on a rotarod (Ugo Basile) accelerating from 5 to 45 rotations per minute in the first 15 seconds. The longest running time until the mouse fell off in 3 trials was notated, with a maximum of 500 seconds.

RNA extraction and analysis of exon skipping by reverse transcriptase polymerase chain reaction

Muscles were minced in TriPure isolation reagent (Roche) using MagNA Lyser green beads (Roche Diagnostics) and a MagNA Lyser (Roche) according to the manufacturer's instructions. Total RNA was extracted and 400 ng was used for reverse transcriptase polymerase chain reaction (RT-PCR) analysis, using Transcriptor reverse transcriptase polymerase (Roche) in 20 µL at 42°C for 45 minutes with random hexamer primers (20 ng/µL). Subsequently, 1.5 µL was amplified in a 50 µL PCR-reaction with 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, as previously described.[663] PCR products were visualized on 1.5% agarose gels and quantified using a DNA 1000 LabChip on the Agilent 2100 bioanalyzer (Agilent Technologies).

Dystrophin protein analysis

Muscles were homogenized in treatment buffer containing 75 mM Tris-HCl pH 6.8-15% (w/v) sodium dodecyl sulphate (SDS) using zirconium beads (1.4 mm; OPS Diagnostics) by grinding in a MagNA Lyser (Roche). Protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. Samples containing 30 µg of protein were made in treatment buffer with 20% (v/v) glycerol, 5% (w/v) β-mercaptoethanol, and 0.001% (w/v) bromophenol blue, and

heated for 5 minutes at 95°C. Samples were loaded on 1.0 mm thick native polyacrylamide gel electrophoresis Tris-acetate (polyacrylamide) gels, with a linear resolving gel gradient of 3-8% (BioRad) and run on the Trans-Blot Turbo system for 1 hour at 75 V (0.07 A) and 2 hours at 150 V (0.12 A) in running buffer (XT Tricine; Biorad) in an ice container (Hulsker *et al.*, in preparation). Proteins were blotted on a nitrocellulose membrane using the Ready to use Trans-Blot Turbo transfer packs and the Trans-Blot Turbo transfer system from BioRad at 2.5 A and ~25 V for 10 minutes. Membranes were blocked in 10 mM Tris-HCl (pH 8) and 0.15 M NaCl (Tris-buffered saline (TBS))-5% nonfat dried milk (Elk) for 1 hour, washed in TBS-0.05% (v/v) Tween20 (TBST) and incubated overnight with first antibodies 1:125 NCL-Dys1 (Dy4; NovoCastra) and, as a loading control, alpha actinin (1:5 000; AB72592) in TBS. Membranes were washed in TBST, incubated 1 h with the fluorescent secondary antibodies 1:5 000 IRDye 800CW goat α-mouse IgG (Li-Cor, Lincoln, NE, USA) and 1:10 000 IRDye 680LT donkey -α-rabbit immunoglobuline G (IgG; Li-Cor) in TBS, washed in TBST and TBS, and analysed with the Odyssey system and software (Li-Cor). Dystrophin expression in wild type control samples containing 10%, 3.3%, 1.1%, and 0.4% of protein were used as reference to determine the dystrophin percentages in the tested samples.

Hybridization-ligation assay for measuring 23AON concentration

For measuring the concentration of 23AON, an assay based on a previously published hybridization-ligation assay was used.[664] Tissue were homogenized in 100 mM Tris-HCl pH 8.5, 200 mM NaCl, 0.2% SDS, 5 mM ethylene-diaminetetraacetic acid, and 2 mg/mL protK using zirconium beads (1.4 mm; OPS Diagnostics) in a MagNA Lyser (Roche). Samples were diluted 10 times in PBS (plasma), 500 and 1 000 times (muscle), or 1 000 and 5 000 (liver and kidney) in pooled control *mdx* tissue in PBS. A signal probe (containing the peptide for antibody recognition) and a template (complementary to 23AON and the probe) were added to homogenized tissue samples. The subsequent ligation step only takes place when both 23AON and probe are bound to the template. Thereafter, unbound probe was washed away and enzyme-linked antibodies were used to detect the amount of probe-23AON. Calibration curves of the analysed 23AON prepared in 60 times pooled control mouse *mdx* tissue in PBS were included. All analyses were performed in duplicate.

Statistical analysis

Data are represented as mean ± standard deviation. 23AON levels, exon skipping levels and protein levels were compared between all different groups using one-way analysis of variance (ANOVA), followed by a Bonferroni correction for multiple testing in case of significance ($p<0.05$) in SPSS 17.0.2 (SPSS). To assess a possible treatment effect on the weight of mice over time a longitudinal analysis was perform in R,[665] using the lme4 package.[666] A baseline corrected model was used, including fixed linear and quadratic time effects; the mouse effect was considered to be random. PK plasma levels in week 0 and 7 have been compared by non-compartmental analysis on sparse sampling data, using Phoenix® WinNonlin® 6.2 (Pharsight). Data are expressed as mean ± standard error of the mean.

Results

PK/PD analysis of different dosing regimens

In this study the relation between dosing regimens, exposure, and outcome (molecular and functional) has been investigated. Therefore *mdx* mice were injected for eight weeks with the same total dose of 23AON divided over a different number of injections (1, 2, or 7 per week). None of the mice, including the mice injected daily for eight weeks, showed any sign of changes in vital parameters, including changes in weight or behaviour (data not shown). Serum CK levels were elevated compared to wild type mice, but did not differ significantly from reference values in saline-treated *mdx* mice or between different dosage groups (fig. 3.1a). In addition, both rotarod (fig. 3.1b) and forelimb grip strength (fig. 3.1c) analyses did not show differences with saline-treated *mdx* mice, wild type mice or between the various dosage groups.

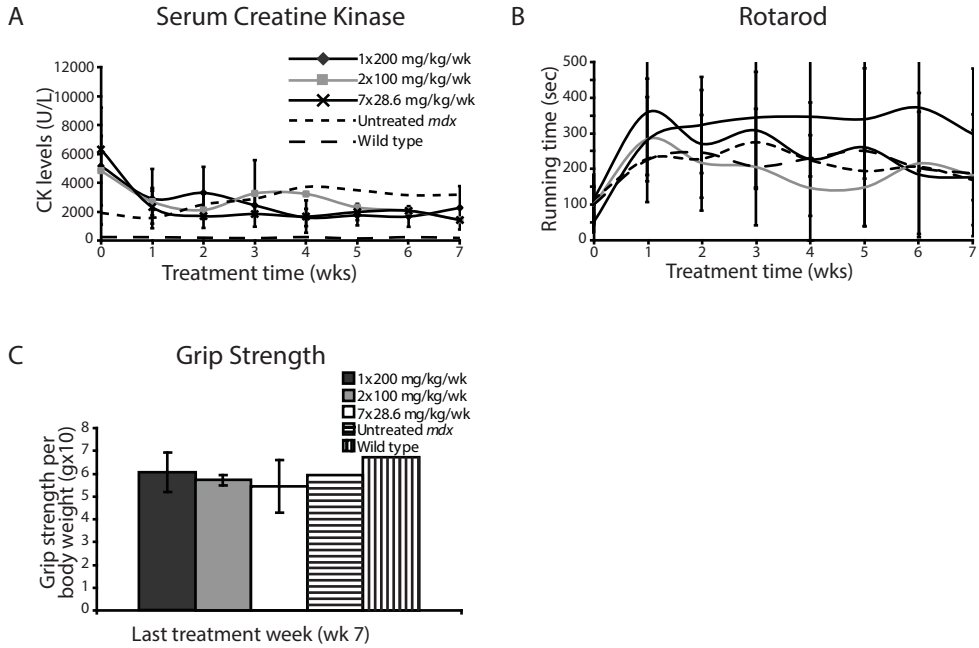

Fig. 3.1: Plasma creatine kinase (CK) and functional assessment during treatment
a) Serum CK levels are elevated in all *mdx* mice compared to wild type mice. No differences between mice of the different dosing regimens were observed. No differences between functional performance by rotarod (b) or forelimb grip strength (c) were seen between mice of various dosing regimens. Note that for rotarod analysis and forelimb grip strength the wild type mice and/or untreated *mdx* mice perform equally well.
Error bars represent the standard deviation.

The plasma profile of the AONs was assessed after the first AON injection in week 0 and week 7 (fig. 3.2a/b; table 3.1) by non-compartmental PK plasma analysis. Afterward, sacrifice AON levels in plasma, muscle, liver, kidney, and spleen were assessed as well (fig. 3.2c-e; supplementary table S3.1). Actual tissue exposures are the most relevant exposure parameters to take into account, since the process of uptake, distribution to tissue, or excretion into urine is relatively fast, while the stability of AONs in tissue is long (earlier publications suggested a tissue half-life in the range of two weeks or more in mice[200]). Plasma levels have

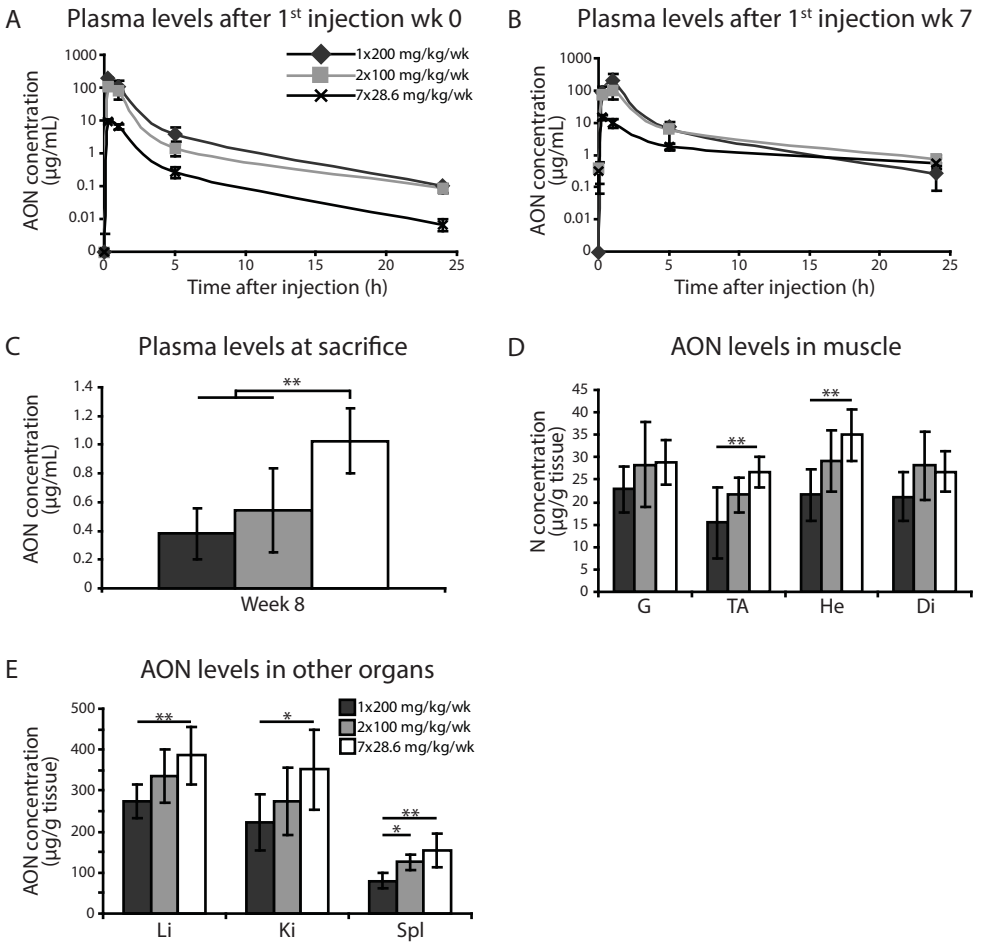

Fig. 3.2: Pharmacokinetic/pharmacodynamic profiles of antisense oligonucleotides (AONs) for the different dosing groups

a) In the first week high peak levels after 100 or 200 mg/kg were seen rapidly (± 15 minutes) after injection, which declined within hours. For injection with a lower amount of AON (28.6 mg/kg), a lower peak concentration was seen. b) The same pattern was observed after seven weeks of injection. Furthermore drug accumulation after repeated dosing was observed, indicated by an extension of the plasma profile and increase in area under the curve (AUC). c) Plasma levels at sacrifice were significantly higher for the group which had received daily injections compared to both other groups. d/e) In most muscles (the target tissue) and all other organs analysed (the non-target tissues) higher AON levels were observed in mice receiving seven injections per week compared to mice receiving the same total dose all at once.

Error bars represent the standard deviation. G=gastrocnemius; TA=tibialis anterior; He=heart; Di=diaphragm; Li=liver. Ki=kidney; Spl=spleen. *p<0.05 **p<0.01

been measured as a control to confirm dosing and detect potential non-linearities. Because blood sampling in mice is limited, only five time points have been used for the plasma PK profile using a sparse sampling approach. An overview of non-compartmental PK exposure parameters is given in table 3.1. After subcutaneous injection, AONs are taken up rapidly reaching maximum plasma levels after 15 minutes, followed by a decline to levels below 5%

	Week 0 plasma exposure		*Week 7 plasma exposure*		*Ratio week 7/week 0*	
Dosing and schedule	*Cmax (µg/µL)*	*AUC0-t (µg.h/µL)*	*Cmax (µg/µL)*	*AUC0-t (µg.h/µL)*	*Cmax*	*AUC0-t*
1 x 200 mg/kg/week	174 ± 14	381 ± 78	193 ± 70	577 ± 162	1.1	1.5
2 x 100 mg/kg/week	109 ± 11	244 ± 10	116 ± 3	407 ± 20	1.1	1.7
7 x 28.6 mg/kg/week	10 ± 1	24 ± 2	14 ± 1	56 ± 5	1.5	2.4

Table 3.1: PK analysis of plasma levels in the first and last week of treatment
Non-compartmental analysis on sparse sampling data has been performed to calculate the Cmax and AUC for the different dosing regimens, showing a more than dose-proportional increase in plasma exposure levels for both parameters at higher dosing levels.
Data are expressed as mean ± standard error of the mean.

of the maximum at 5 hours after injection (fig. 3.2a/b). Upon repeated dosing, the plasma profile gets extended and the area under the curve (AUC) increases, which is indicative of the drug accumulation in tissues (fig. 3.2b/table 3.1). The plasma exposures increase at higher dosing levels, both for the maximum plasma concentration (Cmax) and the AUC. The increase is more than dose-proportional between 28.6 and 100 mg/kg (~10 times for Cmax and ~7 to 10 times for the AUC). This indicates that mechanisms responsible for distribution to tissue can be saturated, which can explain the decreasing tissue levels at the higher dosing level of injection, while the total weekly dose remains the same (fig. 3.2c-e). The increase in exposure between 100 and 200 mg/kg appears somewhat less than dose-proportional in week 7 (fig. 3.2b). This may be due to infrequent sampling after one hour, but combined with

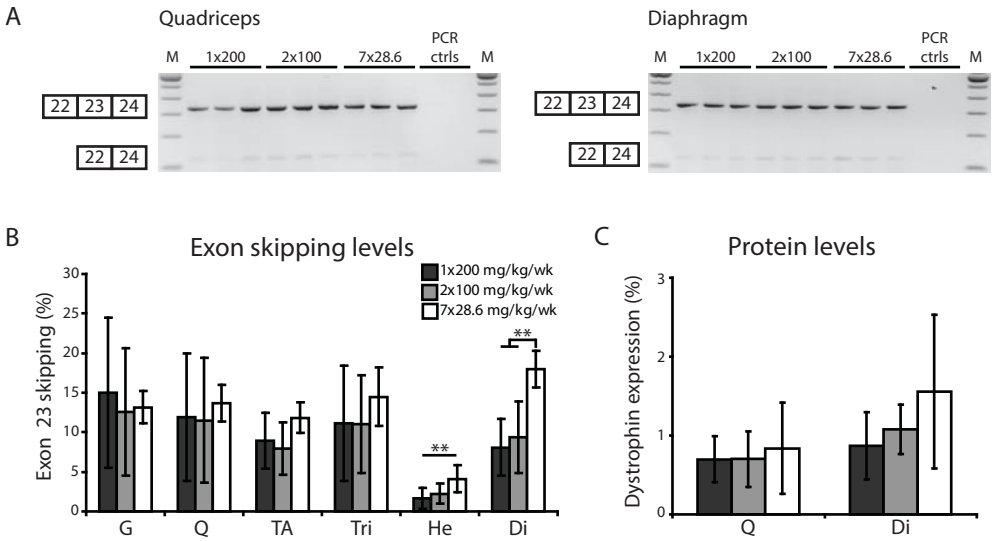

Fig. 3.3: Exon skipping levels and dystrophin protein expression
a) Examples of RT-PCR analysis of the quadriceps and diaphragm muscles showing the unskipped wild type (334 bp) and exon 23 skipped (121 bp) product. b) Quantification of exon skipping levels in different muscles. In skeletal muscle no differences between the different dosing regimens were observed. Significantly higher levels were seen in the heart and diaphragm of daily injected mice versus mice injected once and/or twice weekly. c) No differences in the expression levels of the dystrophin protein levels were observed between all dosing groups in the quadriceps. In the diaphragm a slight, but not significant, increase was found in the daily injected mice.
Error bars represent the standard deviation. M=size marker; PCR ctrls=negative PCR-controls; G=gastrocnemius; Q=quadriceps; TA=tibialis anterior; Tri=triceps; He=heart; Di=diaphragm. *p<0.05 **p<0.01

the further decrease in tissue levels, it suggests an enhanced renal excretion of free AONs in plasma when binding to plasma proteins gets saturated at higher plasma concentrations.

When comparing exon skipping levels between the different groups of mice, no differences were seen between the different groups in muscle (fig. 3.3a/b; supplementary table S3.1). Only in the heart and diaphragm an increase in exon skipping levels was found in the daily injected mice compared to the mice injected once and/or twice weekly (fig. 3.3b). A direct correlation between AON and exon skipping levels was not found, except for the heart (fig. 3.2d versus 3.3b). Furthermore the higher AON and/or exon skipping levels in daily injected mice did not result in higher protein levels (fig. 3.3c; supplementary table S3.1). These were low and comparable for all groups in the quadriceps. Slightly higher dystrophin protein levels were observed for daily injected animals in both quadriceps and diaphragm, but this was not significant due to the low levels in general and high interindividual differences (fig. 3.3c). No exon skipping or dystrophin restoration was observed in non-treated animals (data not shown).

PK/PD analysis of different maintenance regimens

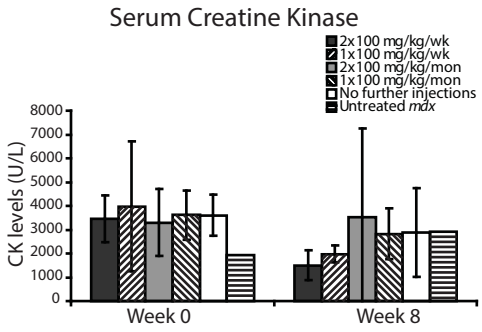

Fig. 3.4: Plasma CK before initial treatment and after eight weeks of maintenance treatment

Plasma CK is highly variable in mice, and no differences were observed between different maintenance regimens after treatment.

Error bars represent the standard deviation.

To study the effect of different maintenance regimens, *mdx* mice were subjected to different maintenance schemes for eight weeks. After an initial 'loading' treatment with five times 250 mg/kg 23AON in the first week (proven to be effective in previous experiments[200,259]), mice were injected twice weekly, once weekly, twice monthly, or once monthly with 100 mg/kg or did not receive any further injections for the subsequent eight weeks. During this period, no differences in weight or functional performance by rotarod analysis were seen between all groups (data not shown). Furthermore, CK levels did not show differences at the end of the eight weeks treatment period (fig. 3.4).

The amount of 23AON in several muscle groups and organs was assessed for the different regimens (fig. 3.5a/b; supplementary table S3.2). The 23AON was detectable in all samples analysed, even in those that did not receive any further injections for eight weeks after the initial treatment in the first week. For all muscles (fig. 3.5a) and organs (fig. 3.5b) levels were highest ($p<0.05$) in the mice injected twice weekly compared with other groups. Also, the mice injected once weekly, had significantly higher levels for most muscles and organs compared with the mice, which had received no injections and/or only once a month during the maintenance period. Only in the tibialis anterior and heart differences were observed between the group that received injections twice per month and the group that received no further injections. A clear trend with the number of injections and tissue levels was observed for all tissues.

In contrast to the dose optimisation phase, in this maintenance phase the exon skipping levels followed the pattern observed for AON levels (fig. 3.5a versus 3.5c; supplementary

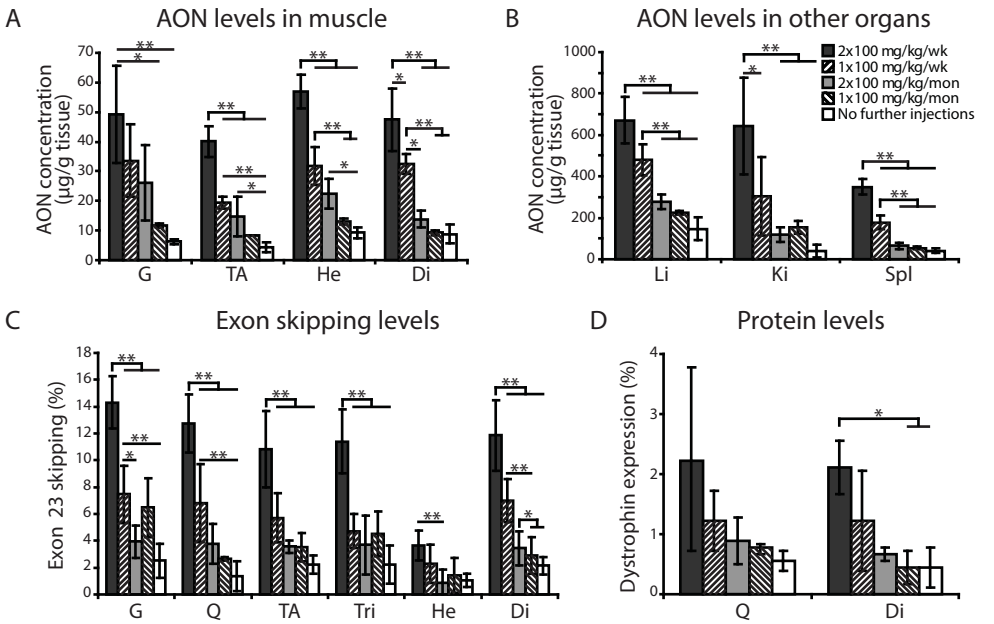

Fig. 3.5: Biodistribution, exon skipping levels and protein expression

a/b) A dose-dependent pattern was observed for the number of maintenance injections and the AON levels in all analysed muscles and other organs. For all muscle groups and other organs, levels were significantly higher in the mice which received two times 100 mg/kg/wk during the maintenance period compared to almost all other groups. Mice which had received one injection per week also showed higher levels compared to mice which had received less injection in all tissues, except for the gastrocnemius and kidney. c) The exon skipping levels also showed a dose-dependent pattern with the number of injections. However, levels were only significantly higher for mice injected twice and/or once per week, and less differences were observed between mice injected with less maintenance injections. d) Protein levels were only increased in the diaphragm for mice that were injected twice per week compared to mice injected once per month or that did not receive maintenance injections.

Error bars represent the standard deviation. G=gastrocnemius; TA=tibialis anterior; He=heart; Di=diaphragm; Li=liver; Ki=kidney; Spl=spleen; Q=quadriceps; Tri=triceps. *$p< 0.05$ **$p< 0.01$

table S3.2), that is, a decrease in exon skipping percentages with decreasing numbers of injections (and thus total dose). Only in heart, the differences were less clear, partly due to higher variations between individual mice and partly due to the fact that the exon skipping levels in heart were lower in mice injected twice a week compared with the skeletal muscles. For the mice that received AONs twice weekly, exon skipping levels were significantly increased compared with less frequently injected mice in all muscles, except for heart. Mice injected once weekly showed higher levels only in the gastrocnemius, quadriceps, and diaphragm and differences between the other groups were seen only for the diaphragm (fig. 3.5c). For dystrophin protein expression (fig. 3.5d; supplementary table S3.2) only in the diaphragm significantly higher protein levels were found in the twice-weekly injected mice versus the mice injected once monthly or not at all after the loading phase. The reason that no clear differences were observed in protein levels is the overall low expression levels and high variation between individual mice.

Detailed PK/PD analysis of dosing and maintenance regimens

The ratios between exon skipping and AON levels observed in the different muscle groups

were assessed (fig. 3.6a/b). The ratios were much higher, *i.e.* more exon skipping for a certain amount of AON, in skeletal muscles than in heart, suggesting that in heart the majority of AON is probably trapped in the interstitium and therefore ineffective. No clear pattern was observed for the different dosing regimens (fig. 3.6a). In limb muscles the ratios were higher, albeit with large interindividual variations, for the mice injected once a week, suggesting that lower AON levels were able to induce the same amount of exon skipping levels or that more AON was taken up by the muscle fibres. By contrast, in the diaphragm a higher ratio was observed for the mice injected seven times a week, suggesting that for this organ daily injections may lead to improved uptake of AONs.

For the maintenance study mice, receiving less frequent injections (and thereby also a lower total dose) showed a higher exon skipping/AON ratio for the gastrocnemius and tibialis anterior (fig. 3.6b). This may be a reflection of the fact the exon skipping is a stepwise process (AON uptake, exon skipping, dystrophin restoration) and suggests that dystrophin transcripts have a relatively long half-life.

The ratios of the average AON levels in skeletal muscles (gastrocnemius, tibialis anterior, and diaphragm) and those in non-target organs (kidney, liver, and spleen) were also calculated (fig. 3.6c/d). For the different dosing regimens, no differences were observed (fig. 3.6c). For the comparison of the different maintenance regimens the ratios, were generally similar (fig. 3.6d).

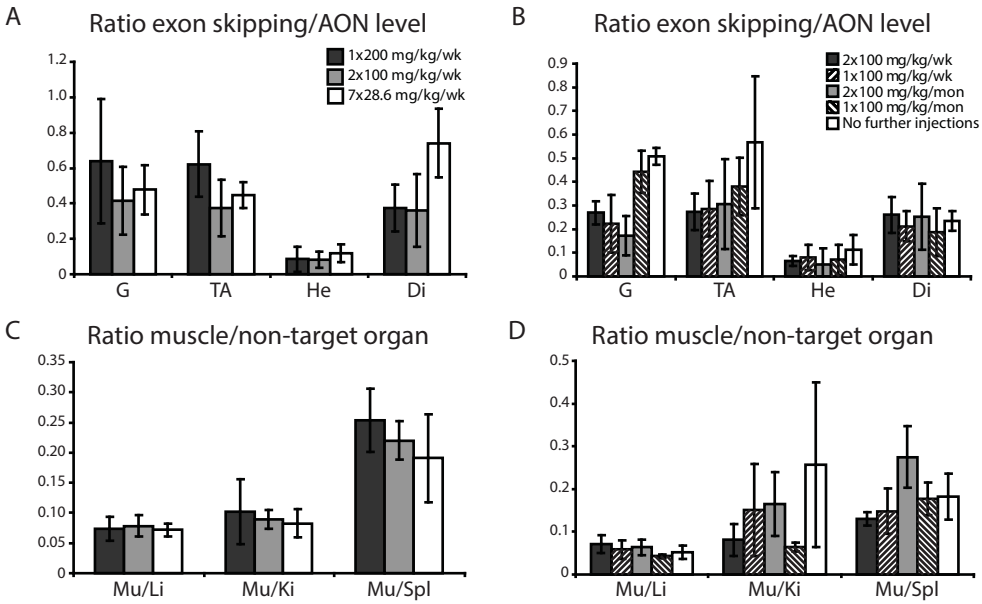

Fig. 3.6: Ratio of exon skipping versus AON levels in different muscle groups and AON levels in the muscle (target tissue) versus non-target organs

a) The exon skipping/AON ratios did not reveal large differences between the different dosage regimens. Only in the diaphragm relatively more exon skipping for a certain amount of AON was observed for the daily injected mice. The ratios were markedly lower in the heart compared to other muscles. b) In gastrocnemius and tibialis anterior exon skipping/AON ratios were a bit higher in mice receiving less frequent maintenance injections. Here, heart ratios were also markedly lower. c) Ratios between the AON levels in the target organ (muscle) versus the non-target organs were similar for the different dosage regimens. d) More variation was observed between muscle/non-target organ AON level ratios for the different maintenance regimens for the kidneys and spleen.

Error bars represent the standard deviation. G=gastrocnemius; TA=tibialis anterior; He=heart; Mu=muscle; Di=diaphragm; Li=liver; Ki=kidney; Spl=spleen.

Discussion

Antisense-mediated exon skipping is currently one of the most promising therapeutic approaches for Duchenne muscular dystrophy. This approach has shown encouraging results in preclinical experiments *in vitro* and *in vivo* in animal models and in early phase clinical trials.[667] However, to improve the therapeutic effect, optimisation of the treatment regimen is necessary. In *mdx* mice, the optimal dose is around 200 mg/kg body weight for 2OMePS.[200] This is much higher than the dosages used in clinical trials (6 mg/kg body weight). However, a correction factor must be applied when translating from mice to humans (see Guidance for Industry[605]). When applying this correction factor, 200 mg/kg in mice would correspond to 16 mg/kg in humans. This is slightly higher than the dose used in humans, but this can be explained by differences in clearance and regeneration capacity between mice and humans, interexon differences, and differences in PK/PD properties of 23AONs versus 51AONs. Although some preclinical studies into the PK/PD profiles of AONs have been done in animal models,[200,239] it is uncertain whether the currently used dosing schedule in clinical trials is the best. During optimisation there will always be a trade-off between the efficacy (exon skipping and dystrophin restoration) due to AONs in the targeted muscles and the amount that ends up in other organs, like the liver and kidneys, where it potentially can have adverse effects. Therefore, detailed PK/PD analyses were done for different 2OMePS 23AON dosing and maintenance regimens in order to model the AON and exon skipping effects.

Interestingly, dividing the same total dose of 2OMePS AONs over multiple, daily injections was more effective than giving the same dose in one or two weekly injections, especially for heart and diaphragm. This is in concordance with a study on PMO AONs in *mdx* mice, which also reported increased effectiveness with multiple, low dose injections compared to a single, high dose injection.[239] In the present study, a single injection with a high amount of AON resulted almost immediately in high plasma levels, which rapidly declined. This is partly due to uptake by, and potentially saturation of the tissues, but also largely due to clearance by the kidneys. For 2OMePS AONs, in contrast to PMOs, this is partly prevented by the serum binding properties of the PS-backbone.[668] However the binding capacity of serum proteins is limited, and at high concentrations saturation is reached, leading to urinary excretion,[669] explaining the higher concentrations of AONs in the muscles after multiple injections with low dose of AONs, which were significant in tibialis anterior and heart. Due to lower peak levels, which do not exceed the binding capacity of the serum proteins, all AON can bind serum proteins, resulting in higher availability of AON, indicated by the accumulation over time seen in this group, as reflected by the plasma levels at sacrifice. However, this effect is not only seen in the muscles, but also in the other organs (liver, kidney, and spleen). When comparing the amount of AON in the muscles and non-target organs the ratio was roughly similar between the different dosage regimens, indicating that the uptake of AONs in the different tissues increases with the same proportion with increasing numbers of smaller injections.

The exon skipping levels measured here were lower than those reported in our previous studies.[161,200] This is most likely due to the fact that previously a nested PCR was used to detect exon skipping. It has now been shown that this gives an overestimation of exon skipping.[663] Therefore, to more accurately determine skipping percentages, a single PCR was used in our current study. Indeed the exon skipping levels reported here are more comparable to another report using a single PCR.[259]

For the different dosing regimens, no clear correlation was observed between the biodistribution pattern to individual muscles and the exon skipping levels observed (fig. 3.6a). A

possible explanation could be that a treatment period of eight weeks is not sufficient to reach a steady state. This is confirmed by our earlier findings that upon longer treatment exon skipping levels increase up to 12 weeks,[219] while they do not increase further after 24 weeks.[201] In the gastrocnemius and tibialis anterior, where no increase in skipping was observed, this ratio was higher for mice who received the AONs all at once. Notably, in heart multiple, small injections did result in higher AON levels, which did correlate to higher exon skipping levels. This suggests that optimising the dosing regimen is possibly a way to improve heart targeting. This is important, since previous studies have shown that targeting heart so far has been challenging.[200,203]

No clear increase in protein levels was seen for the different dosing groups, except for a small increase in the diaphragm. This is mainly due to the high standard deviation and because levels are low in general, which is expected after such a short treatment period in mice. Notably, in clinical studies higher dystrophin levels were observed already after five weeks of treatment.[229] This could be due to differences in muscle fibre permeability between DMD patients and *mdx* mice and suggests there may be dissimilarities in biodistribution between DMD patients and *mdx* mice.

Furthermore, different regimens to maintain the effect achieved by initial treatment were compared in this study. In contrast to the dosing regimen study, here a clear dose-dependent pattern was observed, where more maintenance injections resulted in higher AON, exon skipping and dystrophin levels. This difference can be due to the initial loading with a very high dose of AON, the fact that this study was longer and/or because the total dose received by the mice was similar in the dosing study, whereas different groups received different total doses in the maintenance study. Notably, the exon skipping/AON ratio was higher in the groups receiving the lowest number of injections. This could reflect differences in turnover of AON and RNA transcripts. Animals were sacrificed one week after the last injection of the group receiving maintenance injections twice weekly. This means that the animals that did not receive maintenance injections had their last AON treatment nine weeks before sacrifice. For these animals it is anticipated that part of the AON is already turned over (as reflected by the lower AON levels in the muscles, fig. 3.5a). However, apparently the turnover of exon skipped transcript is slower, resulting in a higher exon skipping/AON ratio. Our results underline the long half-life of the 2OMePS AONs in muscle, as low levels of AON and exon skipping were still detected eight weeks after the last injection. More extensive studies are required to study the turnover of the different components (AON, transcript, and proteins) in more detail.

For the selection of an optimal dosage and maintenance regimen in humans, clinical studies are indispensable. However, preclinical animal studies provide important indications of the effects of different treatment regimens and can be used in data models to enable further optimisation. Achieving high levels of exon skipping and dystrophin restoration are of course aimed for. However, as with most drugs, one also has to balance this with how much AON accumulates in non-target tissues, mainly liver, kidney, and spleen.

Additional studies are needed for more extensive PK/PD modelling and to further elucidate the long term effects of AONs on different levels (*i.e.* RNA and protein).

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Author disclosure statement

AAR discloses being employed by LUMC, which has patent applications on exon skipping that are licensed to Prosensa Therapeutics. As a co-inventor on some of these patents, AAR is entitled to a share of royalties. IV and CTdW declare no conflict of interest. TK, IK, SdK, and JvD report being employed by Prosensa Therapeutics. JvD discloses being co-inventor on exon skipping patents and being entitle to a share of royalties.

Supplementary data

	AON levels							
	Muscle					*Other organs*		
Dosing and schedule	*Plasma*	*Gastroc-nemius*	*Tibialis anterior*	*Heart*	*Dia-phragm*	*Liver*	*Kidney*	*Spleen*
1 x 200 mg/kg/week	0.38 ± 0.18	22.9 ± 5.1	15.4 ± 8.0	21.6 ± 5.8	21.2 ± 5.5	273 ± 41	223 ± 70	80 ± 19
2 x 100 mg/kg/week	0.54 ± 0.29	28.4 ± 9.5	21.6 ± 3.9	29.3 ± 6.8	28.1 ± 7.6	336 ± 66	273 ± 82	126 ± 20
7 x 28.6 mg/kg/week	1.03 ± 0.23	28.8 ± 4.9	26.7 ± 3.3	35.0 ± 5.8	26.7 ± 4.5	386 ± 70	352 ± 97	155 ± 42

	Exon skipping levels						*Protein levels*	
	Gastrocne-mius	*Quadri-ceps*	*Tibialis anterior*	*Triceps*	*Heart*	*Dia-phragm*	*Quadri-ceps*	*Dia-phragm*
1 x 200 mg/kg/week	15.0 ± 9.5	11.9 ± 8.0	8.9 ± 3.5	11.1 ± 7.3	1.7 ± 1.3	8.1 ± 3.6	0.7 ± 0.3	0.9 ± 0.4
2 x 100 mg/kg/week	12.6 ± 8.1	11.5 ± 7.9	8.0 ± 3.3	11.0 ± 6.1	2.2 ± 1.3	9.4 ± 4.5	0.7 ± 0.3	1.1 ± 0.3
7 x 28.6 mg/kg/week	13.2 ± 2.0	13.7 ± 2.3	11.8 ± 2.0	14.5 ± 3.7	4.1 ± 1.7	18.0 ± 2.3	0.8 ± 0.6	1.6 ± 1.0

Supplementary Table S3.1: Overview of PK/PD data of different dosing regimens
Summary of the AON, exon skipping and protein levels at sacrifice in week 8 of the different dosing regimens. 23AON levels are expressed as µg/mL (for plasma) or µg/g tissue (for organs). Exon23 skipping levels are expressed as skipped transcript as percentage of total transcript. Dystrophin protein levels are expressed as percentage of wild type levels in the same muscle.
Data are expressed as mean ± standard deviation.

	AON levels						
	Muscle				*Other organs*		
Dosing and schedule	*Gastrocne-mius*	*Tibialis anterior*	*Heart*	*Diaphragm*	*Liver*	*Kidney*	*Spleen*
2 x 100 mg/kg/week	49.3 ± 16.5	40.1 ± 5.1	56.9 ± 5.8	47.4 ± 10.6	671 ± 113	645 ± 234	348 ± 38
1 x 100 mg/kg/week	33.6 ± 12.2	19.4 ± 2.0	31.9 ± 6.4	32.6 ± 3.3	481 ± 77	305 ± 190	178 ± 35
2 x 100 mg/kg/month	26.0 ± 12.7	14.8 ± 6.6	22.5 ± 5.0	13.9 ± 2.8	277 ± 35	119 ± 34	65 ± 16
1 x 100 mg/kg/month	11.7 ± 0.6	8.5 ± 0.0	13.1 ± 1.1	9.2 ± 0.7	224 ± 9	154 ± 32	55 ± 5
No further injections	6.2 ± 0.8	4.4 ± 1.7	9.2 ± 1.9	8.9 ± 3.1	146 ± 55	41 ± 30	41 ± 12

	Exon skipping levels						*Protein levels*	
	Gastroc-nemius	*Quadri-ceps*	*Tibialis anterior*	*Triceps*	*Heart*	*Dia-phragm*	*Quadri-ceps*	*Dia-phragm*
2 x 100 mg/kg/week	14.3 ± 1.9	12.7 ± 2.2	10.8 ± 2.8	11.4 ± 2.4	3.7 ± 1.1	11.9 ± 2.6	2.2 ± 1.5	2.1 ± 0.4
1 x 100 mg/kg/week	7.5 ± 2.1	6.8 ± 2.9	5.7 ± 1.8	4.7 ± 1.3	2.3 ± 1.4	7.0 ± 1.6	1.2 ± 0.5	1.2 ± 0.8
2 x 100 mg/kg/month	3.9 ± 1.2	3.8 ± 1.5	3.6 ± 0.5	3.7 ± 2.2	0.9 ± 1.0	3.4 ± 1.3	0.9 ± 0.4	0.7 ± 0.1
1 x 100 mg/kg/month	6.5 ± 2.2	2.6 ± 0.1	3.5 ± 1.0	4.5 ± 1.7	1.4 ± 1.3	2.9 ± 1.4	0.8 ± 0.1	0.4 ± 0.3
No further injections	2.5 ± 1.3	1.4 ± 1.1	2.2 ± 0.7	2.2 ± 1.4	1.0 ± 0.5	2.1 ± 0.7	0.5 ± 0.2	0.4 ± 0.3

Supplementary Table S3.2: Overview of PK/PD data of different maintenance regimens
Summary of the AON, exon skipping and protein levels at sacrifice in week 8 of the different dosing regimens. 23AON levels are expressed as µg/g tissue. Exon23 skipping levels are expressed as skipped transcript as percentage of total transcript. Protein levels are expressed as percentage of wild type levels in the same muscle.
Data are expressed as mean ± standard deviation.