Developing tissue specific antisense oligonucleotide-delivery to refine treatment for Duchenne muscular dystrophy

Jirka, S.

Citation

Jirka, S. (2017, July 4). *Developing tissue specific antisense oligonucleotide-delivery to refine treatment for Duchenne muscular dystrophy*. Retrieved from https://hdl.handle.net/1887/51132

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden]

Downloaded from: https://hdl.handle.net/1887/51132

Note: To cite this publication please use the final published version (if applicable).

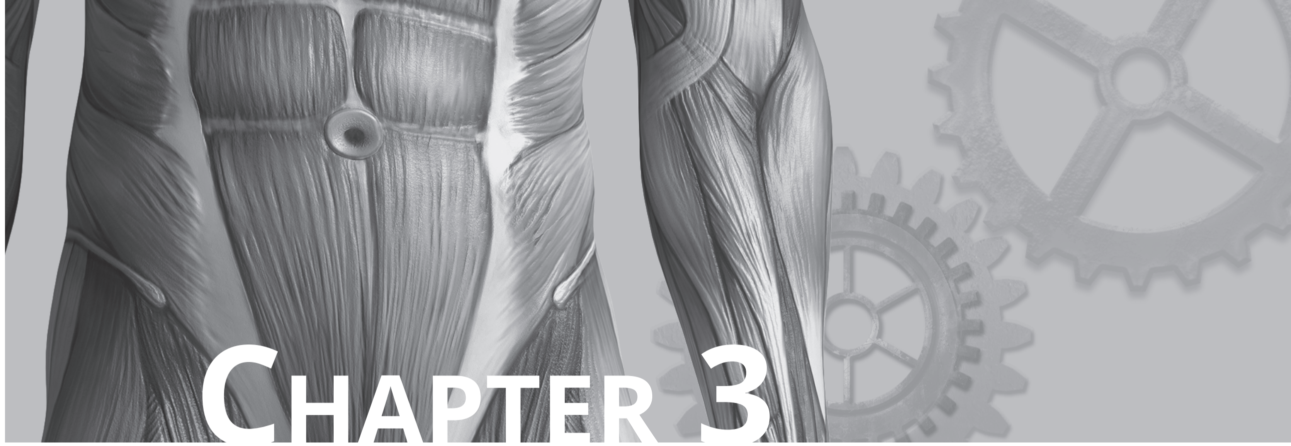
Cover Page

Universiteit Leiden

Leiden University
Repository

The handle http://hdl.handle.net/1887/51132 holds various files of this Leiden University dissertation

Author: Jirka, Silvana
Title: Developing tissue specific antisense oligonucleotide-delivery to refine treatment for Duchenne muscular dystrophy
Issue Date: 2017-07-04



CHAPTER 3

Peptide conjugation of 2'-*O*-methyl phosphorothioate antisense oligonucleotides enhances cardiac uptake and exon skipping in *mdx* mice

Silvana M.G. Jirka[a], Hans Heemskerk[a], Christa L. Tanganyika-de Winter[a], Daan Muilwijk[b], Kar Him Pang[b], Peter C. de Visser[b] , Anneke Janson[b], Tatyana G. Karnaoukh[b], Rick Vermue[b], Peter.A.C.'t Hoen[a], Judith C.T. van Deutekom[b], Begoña Aguilera[b] and Annemieke Aartsma-Rus[a]

[a]Department of Human Genetics, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

[b]Prosensa Therapeutics B.V., 2333 CH Leiden, The Netherlands

Nucleic Acid Ther. 2014 Feb;24(1):25-36

Abstract

Antisense oligonucleotide (AON)-mediated exon skipping is a promising therapeutic approach for Duchenne muscular dystrophy that is currently being tested in various clinical trials. This approach is based on restoring the open reading frame of dystrophin transcripts resulting in shorter but partially functional dystrophin proteins as found in patients with Becker muscular dystrophy. After systemic administration a large proportion of AONs ends up in the liver and kidneys. Therefore, enhancing AON uptake by skeletal and cardiac muscle would improve the AONs' therapeutic effect. For phosphorodiamidate morpholino oligomer they use nonspecific positively charged cell penetrating peptides to enhance efficacy. However, this is challenging for negatively charged 2'-*O*-methyl phosphorothioate oligomer. Therefore, we screened a 7-mer phage display peptide library to identify muscle and heart homing peptides *in vivo* in the *mdx* mouse model and found a promising candidate peptide capable of binding muscle cells *in vitro* and *in vivo*. Upon systemic administration in dystrophic *mdx* mice, conjugation of a 2'-*O*-methyl phosphorothioate AON to this peptide indeed improved uptake in skeletal and cardiac muscle, and resulted in higher exon skipping levels with a significant difference in heart and diaphragm. Based on these results, peptide conjugation represents an interesting strategy to enhance the therapeutic effect of exon skipping with 2'-*O*-methyl phosphorothioate AONs for Duchenne muscular dystrophy.

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe X-linked muscle-wasting disorder typically caused by out of frame mutations in the *DMD* gene, and affecting 1 in 5,000 newborn boys [1]. DMD patients suffer from progressive muscle weakness, are generally wheel-chair dependent before the age of 12, and die around the third decade of their life due to respiratory and cardiac failure [2]. Exon skipping is a promising therapeutic approach for DMD that is currently being tested in clinical trials [3]. This approach is based on restoration of the dystrophin transcript's open reading frame by manipulating pre-mRNA splicing using antisense oligonucleotides (AONs). This results in shorter but partially functional dystrophin proteins as found in patients with the less severe Becker muscular dystrophy (BMD) [4-7]. The exon skipping approach depends on adequate uptake by the target tissues of the AONs. Over 30% of the human body consists of muscle; therefore a body-wide effect is required to treat DMD. AONs with 2'-*O*-methyl phosphorothioate (2OMePS) and phosphorodiamidate morpholino oligomer (PMO) chemistries have been studied in most detail for DMD.

AONs are modified DNA or RNA oligomers, and the modifications render the AONs more resistant to RNases and DNases, improving stability *in vivo*. However, while PMOs are apparently fully resistant to nucleases, 2OMePS AONs still suffer from some breakdown. Upon systemic treatment there are certain barriers that an AON needs to overcome to reach the nucleus of skeletal muscle fibers. AONs are very small (~8 kDa) and as such will be filtered out by the kidney efficiently. For PMOs the vast majority is cleared through urine within an hour of treatment, leading to very short serum half-lives. By contrast, due to low affinity binding to serum proteins by 2OMePS AONs, renal filtration is largely prevented, leading to longer half-lives. In addition, to be effective, AONs also need to pass the endothelium of their target tissues, enter the tissue and (when taken up by endocytosis) have to escape from the endosomes to reach the nucleus, where splicing takes.

Based on positive results from different preclinical animal experiments and phase 1/2 clinical trials, showing dystrophin restoration after subcutaneous injections with 2OMePS AONs and intravenous injections with PMOs, one can conclude that systemic delivery is feasible [8-15]. However, a large proportion of the AON ends up in liver and kidney (2OMePS) or kidney (PMO), and is lost for targeting muscle [16]. Furthermore, while AONs appear to be taken up by dystrophic skeletal muscles, animal models have shown that the effect of AONs in heart is less (2OMePS) or minimal (PMO). Therefore, improving the uptake of AONs by skeletal but especially also by cardiac muscles is anticipated to further enhance the therapeutic effect of AON treatment.

Cell penetrating peptides (CPP) have been explored to improve uptake of PMOs by target tissues. Basic, positively charged, arginine rich peptides (PPMO) [17] indeed enhanced uptake of PMOs by most tissues, including muscle and heart, and significantly increase exon skipping levels and dystrophin restoration after systemic delivery in *mdx* mice [18-20]. However, in monkeys, 4-9 mg/kg weekly systemic injections during 4 weeks with AVI-5038, a PPMO targeting human dystrophin exon 50, resulted in tubular degeneration in the kidney [18]. The fact that apparently higher animals are more sensitive to arginine-rich peptide related toxicity than mice may limit their clinical application [17,19].

Tissue-specific homing peptides have also been investigated to improve uptake of AONs. These peptides home to the target of interest and bind or are taken up by the cells of interest, but do not necessarily penetrate the cells like CPPs. Phage display biopanning is a well-described technique to identify homing peptides for specific targets [21,22]. Several muscle or heart targeting peptides have been reported to date [23-25], of which the ASSLNIA muscle targeting peptide [24] has been further tested in conjugation to PPMOs, resulting in improved dystrophin restoration. Interestingly, this was only seen when the muscle-specific peptide was located between the arginine-rich peptide and the PMO, while linking the muscle-specific peptide to the 5' end of the PPMO reduced the efficiency [26]. For 2OMePS AONs, conjugation of highly positively charged peptides (such as arginine rich peptides) is challenging due to strong electrostatic interactions between the positive peptide and the negatively charged AON backbone, leading to aggregate formation. Therefore in a different approach we screened a 7-mer phage display peptide library for non-charged homing peptides to enhance uptake and exon skipping in muscle and heart *in vivo* in the *mdx* mouse model. We here report the identification of a 7-mer peptide which, after conjugation to a 2OMePS AON, resulted in significantly improved AON uptake and effect by heart and diaphragm upon systemic administration in the *mdx* mouse model.

2. Material and Methods

In Vivo Biopanning

Four to five weeks old *mdx* mice were injected intravenously with $2x10^{11}$ phages from the naïve or enriched libraries (Ph.D.-7™ Phage Display Peptide Library kit, New England Biolabs, Beverly, Maryland, one animal per round). Mice were perfused with PBS after one hour to remove unbound phages. The gastrocnemius and heart muscles were isolated and washed 3 times with Tris-buffered saline (TBS). Subsequently the organs were homogenized in 2 ml TBS with MagNA Lyser green beads (Roche Diagnostics, the Netherlands) in the MagNA Lyser (Roche Diagnostics, the Netherlands) according to the manufacturer's protocol. The suspension was titrated and amplified according to the protocol provided with the phage library. Up to 4 rounds of biopanning were performed for each fraction. After the third and fourth round, phages were plated and colonies were picked. DNA was isolated and prepared for Sanger sequencing with the ABI PRISM® 3730 Analyzer (Applied Biosystems).

Peptide synthesis

7-mer peptides were synthesized by standard Fmoc solid phase peptide chemistry on a PS3 or Tribute Peptide synthesizer (Protein Technologies, USA), using 5eq. HCTU (Protein Technologies, USA eq.) as activating reagent and DIPEA (10 eq.) as base. A fluorescent label was manually coupled to the resin-bound peptides by treatment with 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester and triethylamine (all from Acros, Belgium) in N,N-dimethylformamide (DMF). After cleavage and deprotection TFA trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O 95/2.5/2.5 (v/v)

or TFA/ thioanisole/TIS/H_2O 90/2.5/2.5/5 (v/v) for peptide sequences containing Met], filtration, precipitation in cold diethyl ether and centrifugation, crude 5(6)-carboxyfluorescein amide (FAM) - labeled peptides were obtained. Peptides were purified by RP-HPLC and analyzed by electrospray ionization mass spectrometry (ESI-MS) (positive mode). Fluorescently labeled peptide concentrations were determined by spectrophotometric analysis at 490 nm at pH 7.5.

In vitro uptake studies of FAM-labeled peptides

Primary human control myoblasts were grown in NutMix F-10 (HAM) medium supplemented with GlutaMax-I, 20% fetal bovine serum (FBS) and 1% Penicillin/streptomycin (P/S (all from Gibco-BRL, the Netherlands) in flasks coated with purified bovine dermal collagen for cell culture (Nutacon B.V. the Netherlands). Cells were plated on collagen coated glass slides, in 6 wells plates and grown to 90% confluence before switching to differentiation medium (Dulbecco's medium (without phenol red) with 2% FBS, 1% P/S, 2% glutamax and 1% glucose (all from Gibco-BRL, the Netherlands)). Cells were allowed to differentiate for 7-14 days. Peptides were dissolved in water and if necessary acidic acid was used to properly dissolve the peptide. Final concentrations were determined by spectrophotometric analysis at 490 nm at pH 7.5. Upon sufficient differentiation, cells were washed twice with hank's balanced salt solution (HBSS), and 1 ml of medium without serum was added with 0.45 nM or 2.25 nM of fluorescent peptide. After incubation for 3 hours at 37 °C and 5% CO_2, 2 ml of differentiation medium was added. Cells were fixed 2 days later for 5 minutes with methanol (-20 ºC) and air dried for 30 minutes. The glass slides were taken out and mounted in mounting medium (Vectashield hard set (Vector laboratories)). After drying for 30 minutes, the glass slides were analyzed with fluorescence microscopy (Leica DM RA2) using a CCD camera (Leica DC 350 FX).

In vivo evaluation of FAM labeled peptides by intramuscular injection in the *mdx* mouse model

Four to six week old *mdx* mice were injected in the gastrocnemius muscle with 2.5 nmol FAM- labeled peptides in 40 μl saline (n=1 for each peptide). After 3 days the mice were sacrificed and the muscles were snap frozen in liquid nitrogen-cooled isopentane. Subsequently, 8 μm sections were cut with a cryotome, perpendicular to the muscle fibers and collected on a positively charged glass slide. The slides were fixed with -20 ºC acetone and mounted in mounting medium (Vectashield hard set). The slides were analyzed with fluorescence microscopy (Leica DM RA2) using a charge-coupled device (CCD) camera (Leica DC 350 FX).

Oligonucleotide synthesis

The 20-mer 2'-*O*-methyl phosphorothioate RNA (5'-ggc caa acc ucg gcu uac cu-3') targeting mouse exon 23 (23AON)[29] was synthesized on an AKTA prime OligoPilot-100 (GE Healthcare, UK) synthesizer using the protocols recommended by the supplier. The oligonucleotide was cleaved and deprotected in a two-step sequence (diethylamine followed by concentrated ammonia treatment), purified by reversed phase (RP)-HPLC, dissolved in water and an excess of NaCl was added to exchange ions. After evaporation, the oligonucleotide was redissolved in water, desalted by FPLC, and lyophilized. Mass spectrometry confirmed the identity of the AON, and its purity (determined by UPLC) was 86%.

Peptide oligonucleotide conjugate synthesis

Peptide P4 was assembled on a peptide synthesizer as described above and maleimide propionic acid was subsequently coupled on-line. Deprotection, cleavage from the resin, and subsequent precipitation in cold ether, afforded crude maleimide-peptide, which was used without further purification in the conjugation step. The oligonucleotide for conjugation to the peptide was synthesized with a thiol modifier C6 S-S phosphoramidite (Link Technologies) coupled on-line to the 5’ end. Treatment of the crude resin with 40% aqueous ammonia and 0.1 M dithiotreitol led to the concomitant cleavage of the solid support, deprotection of the nucleobases and internucleoside linkages, and reduction of the disulfide bond. Crude thiol-modified oligonucleotide was purified by RP- HPLC, and directly conjugated to the maleimide-peptide (5 eq.) *via* thiol-maleimide coupling in phosphate buffer (pH 7) at ambient temperature for 16 hours. Crude product was purified by HPLC, dissolved in water, and an excess of NaCl was added to exchange ions. After evaporation of the solution, the peptide-conjugated AON (P4-23AON) was redissolved in water, desalted by FPLC and lyophilized. Mass spectrometry confirmed the identity of the compound.

Transfection of (P4-)23AON and sense staining on primary human control myotubes

Differentiated primary human control myoblasts cells were washed twice with HBSS, and 1 ml of medium without serum was added with 2.25 nmol of P4-23AON or 23AON. After incubation for 3 hours at 37 ºC and 5% CO_2, 2 ml of fusion medium was added with 2% serum. Two days later the cells were washed tree times with PBS and fixed for 30 minutes in fixation buffer. The slides were washed with 1x PBS/5 mM $MgCl_2$ for 30 minutes and pre-hybridized for 30 minutes in 125 µl pre-hybridization buffer (40% deionized formamide, 2 times SSC buffer (300mM NaCl and 30nM trisodium citrate, pH 7.0)), followed by an overnight incubation in 125µl hybridization mix (32% deionized formamide, 2 times SSC buffer, 0.2mg/ml BSA, 10mg/ml Dextran sulphate,1 mg/ml fish sperm and the 5’-Cy5 labeled sense oligonucleotide (5’□3’, ccg gtt tgg agc cga atg ga) in a water bath at 37 ºC in the dark. The slides were washed in wash buffer (40% deionized formamide 2 times SSC buffer) for 2 times 20 minutes in a water bath at 37 ºC in the dark followed by a wash step with SSC buffer for 20 minutes at room temperature in the dark and a quick incubation with 1:5,000 4’,6-diamidino-2-fenylindool (DAPI) in PBS to stain nuclei. Finally the slides were mounted in mounting medium (vectashield hard set (vector laboratories)) and after 30 minutes drying, the glass slides were analyzed with fluorescent microscopy (Leica DM RA2) using a CCD camera (Leica DC 350 FX).

AON treatment, RNA isolation and RT-PCR analysis of (P4-)23AON in differentiated mouse myogenic cells

C2C12 cells were grown in Dulbecco’s medium (without phenol red) supplemented with 10% FBS, 1% P/S, 2% Glutamax and 1% glucose (all from Gibco-BRL, the Netherlands) in collagen coated flasks. Cells were seeded in collagen coated 6 wells plate with proliferation medium and grown until confluence. The cells were washed twice with HBSS and Dulbecco’s medium (without phenol red) supplemented with 2% FBS, 1% P/S, 2% Glutamax and 1% glucose (all from Gibco-BRL, the Netherlands)) was added to induce differentiation. At day 8 of differentiation the cells were incubated with 500nM P4-23AON or 23AON with or without 3.5 µl PEI (as transfection reagent)/mg AON in differentiation medium (1 ml). After 3 hours the medium was removed and fresh differentiation medium added (3 ml). After 48 hours the cells were washed twice with HBSS and RNA was isolated by adding 500 µL TriPure (Roche diagnostics, the Netherlands) to each well to lyse the cells. This was followed by chloroform extraction in a 1/5 ratio on ice for 5 minutes. The remaining cell debris was pelleted down by centrifugation (4 ^{0}C, 15 minutes, 15,4000 rcf) and the upper aqueous phase precipitated for 30 minutes on ice with equal volume of isopropanol. The RNA was pellet down by centrifugation (4 ^{0}C, 15 minutes, 15,400 rcf) and the pellet washed with 70% ethanol. The final RNA pellet was dissolved in milli-Q water. For complementary DNA (cDNA) synthesis, 400 ng of RNA was used in a 20 µl reaction with a specific primer (ctt taa ggc ttc ctt tct ggt g) and transcriptor reverse transcriptase (Roche Diagnostics, the Netherlands) for 30 minutes at 55°C and 5 minutes at 85°C to terminate the reaction. For reverse transcriptase PCR (RT-PCR) analysis 3 µl of cDNA was incubated with 0.625 U AmpliTaq polymerase (Roche Diagnostics, the Netherlands), 10 pM of primers (reverse primer ctt taa ggc ttc ctt tct ggt g, forward primer: aga aaa ggg aca ggg gcc a) and 1 times supertaq PCR buffer (Enzyme technologies Ltd) and amplified for 20 cycles each consisting of an incubation for 40 sec at 94 °C, 40 sec at 60 °C and 80 sec at 72 °C. This PCR was followed by a nested PCR. For the nested PCR analysis 1.5 µl of the first PCR was incubated with 1.25 U AmpliTaq polymerase (Roche Diagnostics, the Netherlands), 20 pM of primers (reverse primer: cag cca tcc att tct gta agg, forward primer: atc cag cag tca gaa agc aaa) and 1 times supertaq PCR buffer (Enzyme technologies Ltd) and amplified for 32 cycles each consisting of an incubation for 40 sec at 94 °C, 40 sec at 60 °C and 60 sec at 72 °C. PCR fragments were analyzed using 1% agarose gel electrophoresis. Exon skip levels were semi-quantitatively determined as the percentages of the total (wild type and skipped) product with the Agilent 2100 bioanalyzer.

Systemic study with P4-23AON and 23AON in *mdx* mice

Four to five weeks old *mdx* mice (5 mice per group) were subcutaneous injected 4 times per week, with 50 mg/kg of AON in 100 µl of saline or the molar equivalent of peptide conjugated AON (6.8 µmol) for 6 weeks. Blood samples were obtained from the tail vein for plasma pharmacokinetics analysis (PK) 10 minutes, 30 minutes, 1 hour and 6 hours after the last injection. All mice were sacrificed 1 week after the last injection by perfusion with PBS, after taking blood from the tail vein of all mice to determine plasma parameters. Gastrocnemius, quadriceps, tibialis anterior, triceps and diaphragm muscles, heart, liver and kidney were isolated to determine exon skipping levels and AON concentrations in these tissues.

Plasma parameters

Blood was collected in microvettes (Sarstedt B.V. the Netherlands). Glutamate pyruvate transaminase (GPT), alkaline phosphates (ALP) glutamic oxaloacetic transaminase (GOT) hemoglobin (HB), urea and creatine kinease (CK) were determined using Reflotron strips (Roche Diagnostics, the Netherlands) in the Reflotron Plus machine (Roche Diagnostics, the Netherlands).

Quantification of AON levels in plasma and tissue samples

For measuring the concentration of 23AON in plasma and tissue samples a hybridization-ligation assay based on one previously published was used [35]. Tissues were homogenized in 100 mM Tris-HCl pH 8.5, 200 mM NaCl, 0.2% SDS, 5 mM EDTA and 2 mg/mL proteinase K using zirconium beads (1.4 mm; OPS Diagnostics, Lebanon, NJ) in a MagNA Lyser (Roche Diagnostics, the Netherlands). Samples were diluted 600 and 6000 times (muscle) or 6000 and 60000 (liver and kidney) in pooled control *mdx* tissue in PBS.

Calibration curves of the analyzed 23AON prepared in 60 times pooled control mouse *mdx* tissue in PBS were included. All analyses were performed in duplicate. For plasma the samples were diluted (t=0 100 times and 200 times, t=10 min, 30 min, 1h 1000 times and 10000 times and t=6h 100 times and 1000 times) in pooled control plasma of *mdx* mice in PBS. Calibration curves of the analyzed 23AON prepared in 100 times pooled control plasma of *mdx* mice in PBS were included.

Determination of exon skipping levels in *mdx* mice

Samples from muscle and heart were homogenized in RNA- Bee (Campro Scientific, Veenendaal, the Netherlands) solution using a MagNA Lyser (Roche Diagnostics, the Netherlands) and MagNA Lyser green beads (Roche Diagnostics, the Netherlands). Total RNA was isolated and purified according manufacturer's instructions. For complementary DNA (cDNA) synthesis, 400 ng of RNA was used in a 20 µl reaction with random hexamers and transcriptor reverse transcriptase (Roche Diagnostics, the Netherlands) for 45 minutes at 42°C. For PCR analysis 1.5 µl of cDNA was incubated with 1.25 U taq polymerase (Roche Diagnostics, the Netherlands), 20 pM of primers (reverse primer: cag cca tcc att tct gta agg, forward primer: atc cag cag tca gaa agc aaa) and 1 times supertaq PCR buffer (Enzyme technologies Ltd) and amplified for 30 cycles each consisting of an incubation for 30 sec at 94 °C, 30 sec at 60 °C and 30 sec at 72 °C. PCR fragments were analyzed by 1% agarose gel electrophoresis. Exon skipping levels were semi-quantitatively determined as the percentages of the total (wild type and skipped) product with the Agilent 2100 bioanalyzer.

Table 1. Overview of selected peptide sequences

Peptide (P)	Sequence	Detected	Tissue	Comments
1	LYQDYSL	twice	heart 3rd round	
2	LPWKPLG	twice	heart 3rd and 4th round	
3	TPAHPNY	twice	heart 3rd and 4th round	
4	LGAQSNF	once	heart 4th round	low prevalence
5	PGAQSNF	once	heart 3rd round	
6	VNSPTHS	once	heart 3rd round	
7	VNSATHS	once	heart 4th round	
8	YQDSAKT	once	heart 3rd round	negative control
9	TALPPSY	twice	muscle 3rd and 4th round	
10	AMISAIH	once	muscle 3rd round	low prevalence
11	HVIANAG	once	muscle 3rd round	low prevalence
12	EPLQLKM	twice	heart and muscle 4th round	reported as nuclei targeting
13	GNTPSRA	once	muscle 4th round	negative control

3. Results

To identify peptides homing to muscle and heart, an *in vivo* biopanning was performed in the *mdx* mouse model with a 7-mer phage display peptide library. 2x10^{11} phages from the PhD-7 library were injected intravenously and mice were perfused after 1 hour, after which gastrocnemius and heart muscles were isolated. Tissues were homogenized and recovered phages were amplified for the next screening round. After the third and fourth round of screening, a total of 52 colonies were selected and sequenced. For each peptide sequence we consulted Sarotup [27] and Pepbank [28] databases to identify false positives. This led to the exclusion of 8 peptides for further experiments, as these peptides had been identified before and were therefore not likely to be target specific binders. A series of peptide sequences occurring more than once or with recurrent stretches of 6 amino acids was identified (Table 1). Because not all amino acids occur in the library with the same frequency, some peptide sequences are less common than others. After calculating the prevalence of each of the found peptide sequences, two peptide sequences (P10 and P11) that were found only once were also included based on the fact that they had a very low prevalence in the library. As negative controls we included three peptide sequences from the selection, which showed up in the Sarotup database (P8, P12 and P13).

To evaluate uptake and intracellular distribution, selected peptides were synthesized with a FAM label and incubated with primary human control myotube cultures for 48 hours (2.25 and 0.8 nM respectively) and analyzed with fluorescence microscopy (figure 1A). Clear fluorescence was observed for 7 of the 12 peptides, also at a lower concentration of 0.8 nM (figure 1B). Based on the highest fluorescence intensity, P1, P2, P4 and P11 were chosen for *in vivo* evaluation. Four to six weeks old *mdx* mice were intramuscularly injected with 2.5 nmol of labeled peptide in the gastrocnemius muscle. Muscles were isolated 3 days after injection, and cross-sections were analyzed with fluorescence microscopy (figure 2). Clear fluorescence was observed for P4 at the membrane. In contrast, P11 was found negative, and P2 showed only some weak staining close to the injection site. P1 resulted in strong fluorescence, but as acetic acid was needed to dissolve this peptide, it is likely that this resulted in damaged muscle fibers leading to the observed uptake of the labelled peptide or to autofluorescence of necrotic cells.

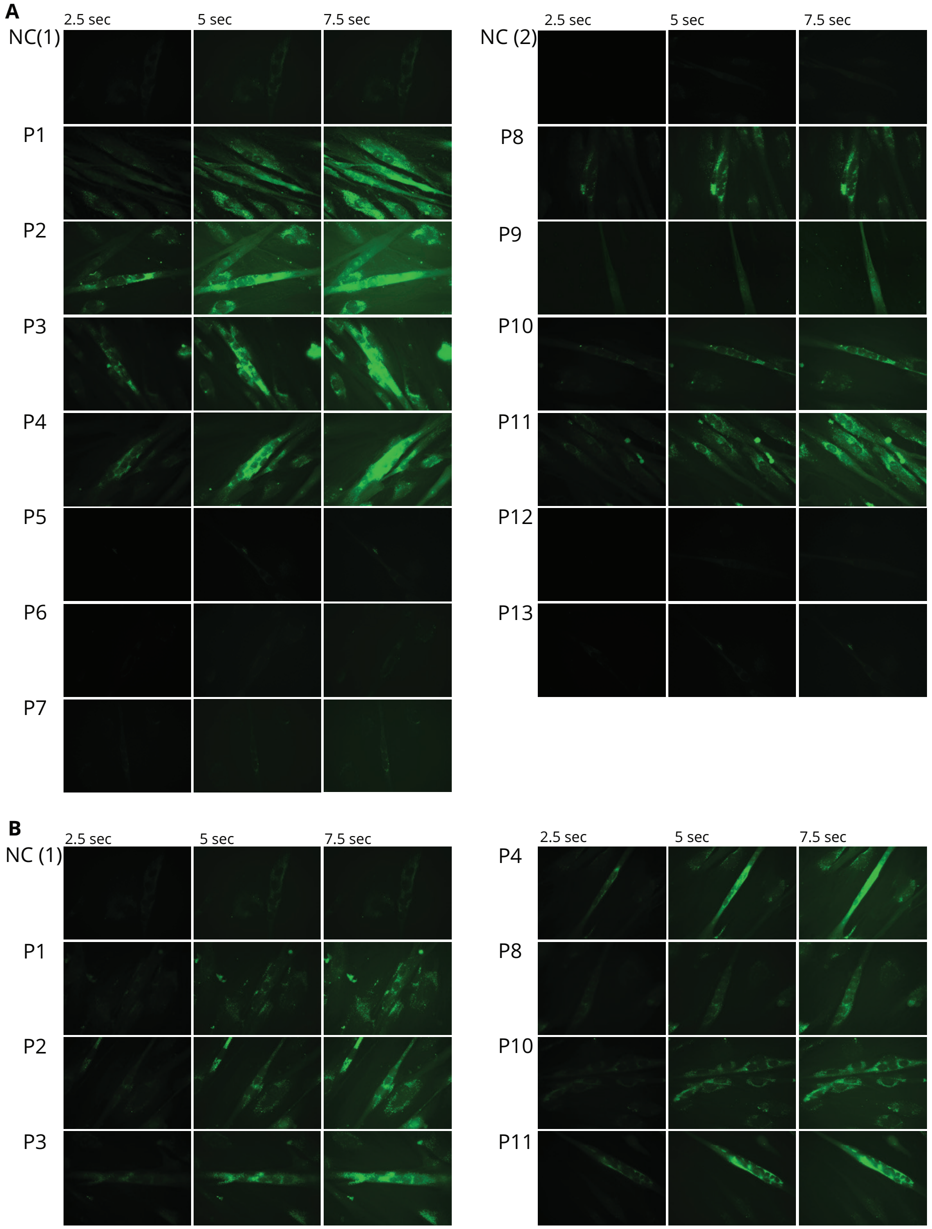


Figure 1. Uptake of FAM-labeled peptides by primary human control myotubes. Primary human control myotubes were incubated with (A) 2.25 or (B) 0.45 nmol of FAM-labeled peptide. After 48 hours the cells were washed and analysed at three different exposure times (2.5, 5 and 7.5 seconds) with fluorescence microscopy. Representative pictures are shown. NC is un-transfected control, P1-13 are the peptides.

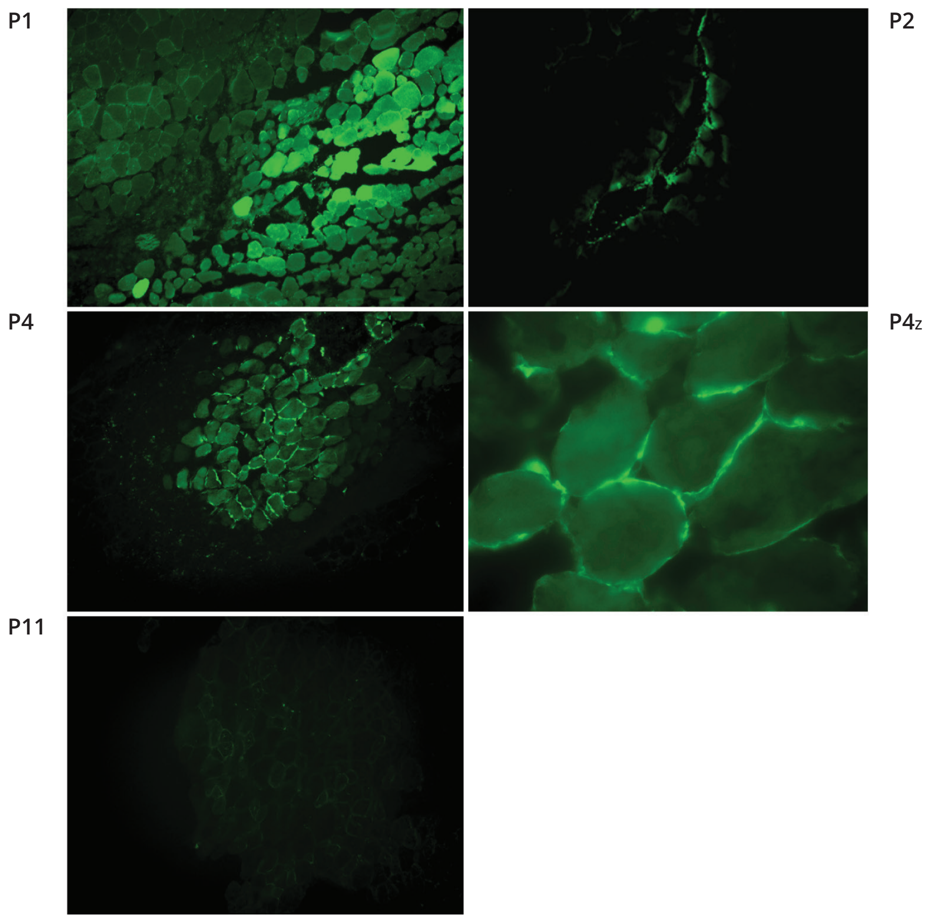


Figure 2. Uptake of FAM-labeled peptides in vivo after intramuscular injection in the mdx mouse model. Four to six weeks old mdx mice were intramuscularly injected with 2.5 nmol FAM-peptide in the gastrocnemius muscle. After 3 days the muscle was isolated and muscle sections were analysed using fluorescence microscopy. Representative pictures are shown. P1, 2, 4 and 11 are candidate peptides. P4z is a 5 times enlargement of P4.

Showing the best results, P4 was conjugated to a 2OMePS AON specific for mouse dystrophin exon 23 (23AON), which can induce exon skipping and restore dystrophin in the *mdx* mouse [29]. 23AON and P4-conjugated 23AON (P4-23AON) were incubated with primary human control myotube cultures without a transfection reagent. To detect the location of the (P4-)23AON, we used a sense DNA oligonucleotide with a Cy5 label and observed more a punctuate fluorescence pattern for P4-23AON compared to 23AON (figure 3).

To confirm that the efficacy of the 23AON is not influenced by the conjugation of the peptide, differentiated C2C12 mouse myogenic cells were incubated with 500 nM of P4-23AON or 23AON with PEI as transfection reagent and without a transfection reagent. RNA was isolated 48 hours after incubation, and exon skipping percentages were determined by RT-PCR (figure 4). No exon skipping was seen when the cells were incubated with P4-23AON or 23AON without the use of a transfection reagent; a possible explanation for this is endosomal entrapment [30] of the AONs, as is suggested by the punctuate staining observed in figure 3. PEI is known to facilitate endosomal escape of compounds after transfection and indeed, in cells incubated with PEI as a transfection reagent and AONs, ~50% exon skipping was observed for both 23AON and P4-23AON. This indicates that the conjugation of P4 to the 23AON does not interfere with the exon skipping ability of the 23AON.

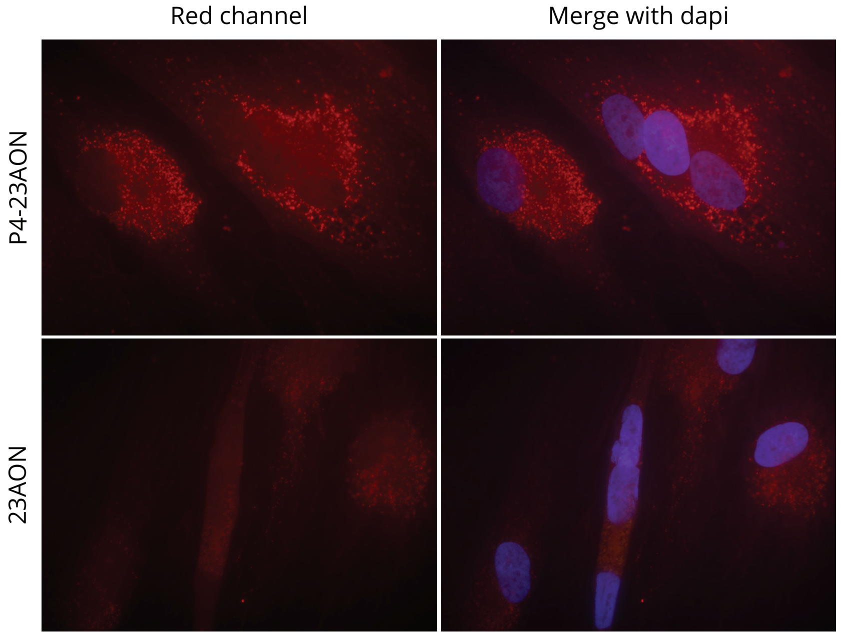


Figure 3. Sense staining of 23AON and P4-23AON transfected primary human control myotube cells. Differentiated primary humane control myotubes were incubated with 2.25 nmol of P4-23AON or 23AON respectively in serum free differentiation medium. After 3 hours 2 ml of differentiation medium with 2% serum was added and 48 hours later the cells were hybridized with a Cy5-labeled sense DNA oligonucleotide and analyzed with fluorescence microscopy. Representative pictures are shown.

Finally, we tested whether this peptide could enhance 23AON uptake by heart and muscle after systemic treatment of an animal model. For 2OMePS AONs the optimal dose and route for systemic AON treatment in *mdx* mice appears to be 200 mg/kg/week by subcutaneous administration[12,31], which would correspond to a dose of 16 mg/kg in humans after correcting for differences in body surface area between small and large animals http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf). In this study *mdx* mice (5 per group) were subcutaneously injected with 4 weekly doses of 50 mg/kg of 23AON or the molar equivalent of P4-23AON for 6 weeks. Plasma samples were collected at different time points after the last injection, mice were sacrificed one week later and several tissues were harvested for bioanalysis. AON levels in plasma, organs and tissues were determined. Compared to 23AON, P4-23AON showed higher AON levels in plasma for all time points (figure 5a). This could relate to a higher availability of the conjugate due to lower immediate clearance rate by liver and kidney. In most isolated tissues, P4-23AON levels were slightly higher than 23AON levels, with a significant difference in heart (p=0.0017, figure 5b). Since we also observed slightly higher levels in liver and kidney for P4-23AON than 23AON, we calculated the muscle/liver, muscle/kidney, heart/liver, and heart/kidney ratios. This revealed improved relative uptake of the P4-23AON conjugate in muscle and especially heart compared to 23AON for both liver and kidney (figures 5c,d). Accordingly, exon skipping levels were higher in all tissues for P4-23AON with a significant difference for heart and diaphragm (P=0.02 and P=0.001, respectively, figure 5e)

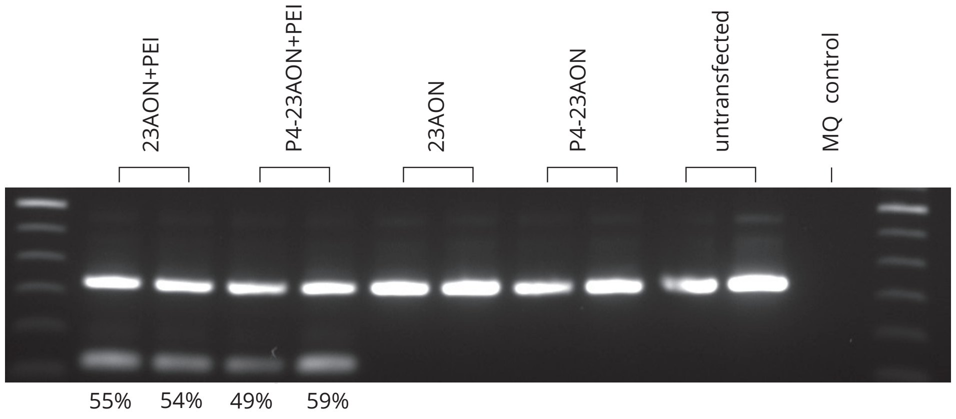


Figure 4. Exon skipping efficacy of 23AON and P4-23AON in differentiated mouse myogenic cells. Mouse myogenic cells were differentiated and transfected in duplo with 500 nM of 23AON or P4-23AON with (1-4) and without (5-8) PEI. The cells were incubated for 48 hours after which RNA was isolated and exon skipping levels analyzed by RT- PCR. Full length and exon skipping fragments were visualized on agarose gel and semi-quantitatively determined by lab-on a chip as the percentages of the total (skip and wild type) product.

To evaluate safety of P4-23AON, body weight was measured over time and plasma parameters for liver and kidney function and damage and muscle integrity were assessed at time of sacrifice (figure 6). Alkaline phosphatase (ALP) a marker for hepatobiliary function, glutamate pyruvic transaminase (GPT) and glutamic oxaloacetic pyruvate transaminase (GOT), which are enzymes that leak in to the bloodstream upon liver and muscle damage, showed no significant differences for P4-23AON and 23AON treated animals and were in the normal ranges for *mdx* mice (figure 6a,b). Also urea (a marker for kidney function) and hemoglobin showed no significant differences between the groups and were within normal levels (figure 6c). We finally measured creatine kinase levels (marker for muscle damage) and observed no difference between P4-23AON and 23AON treated animal (figure 6d). No differences were found when measuring body weight over time (figure 6e).

4. Discussion

For DMD AON-mediated exon skipping has reached phase 3 clinical trials for drisapersen (2OMePS) and eteplirsen (PMO) and seems a promising therapeutic approach. Since over 30% of the human body consists of muscle, systemic treatment is necessary, and seems feasible. However, most of the injected AONs end up in liver and kidney, which decreases the amount that can be taken up by muscle.

Preclinical animal studies suggest that upon systemic AON delivery, body wide dystrophin restoration in skeletal muscles is feasible, while dystrophin restoration in heart may be less effective. There is a possibility that improved muscle function due to dystrophin restoration leads to a higher demand on the heart [32,33]. Therefore, enhancing uptake of AONs by skeletal but especially cardiac muscle would further improve their therapeutic effect.

Several papers describe arginine rich cell-penetrating peptides to enhance the uptake of PMOs by all tissues [17]. The resulting peptide-PMO conjugates (PPMOs) showed increased exon skipping and dystrophin levels in skeletal and cardiac muscle when systemically tested in *mdx* mice. However these peptides are not tissue specific and peptide-related toxicity problems have been observed [18,19]. In a different approach to improve specific AON uptake by heart and skeletal muscle without using a cell penetrating peptide, we used a phage display peptide library screen to specifically identify muscle and heart homing peptides. Sequencing single colonies after the pre-final and final rounds of selection gave insight in library enrichment. Phage display screenings are known to result in false positive peptides caused by binding to non-target related materials or propagation advantages. Therefore we used Pepbank and Sarotup databases to filter for these false positive peptides [27,28].

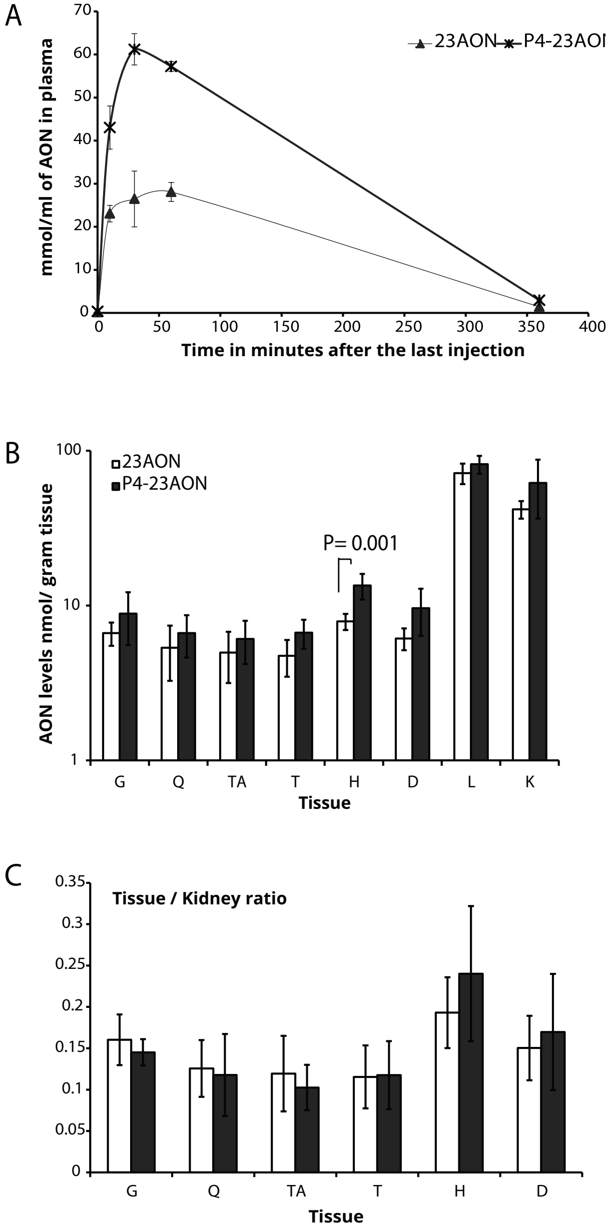
Samoylova et al describe finding the muscle targeting peptide ASSLNIA [24]. We did not find this peptide in our biopanning. There are several possible explanations for this. First, we had a different experimental setup (only *in vivo* compared to first *in vitro* than *in vivo*). Second, since one selects peptides from a library containing theoretically $2x10^8$ different peptide sequences and analyses only 10-100 clones from the enriched library, it is possible that different peptides are found in different biopanning experiments (including experiments that are performed in parallel). The identification of enriched peptides by DNA sequencing of inserts of 10-100 clones is laborious, while it gives only a small insight in possible candidates and phages that are prone to preferential amplification will constitute a significant subset of sequenced clones. As an alternative approach one can use deep sequencing to analyse millions of insert sequences after a more limiting biopanning [34]. Third, according to the sarotup target unrelated peptide scanner[27], ASSLNIA has a high probability of binding avidin-family proteins, implying that it is possible that this peptide is not entirely target specific.

Ten candidate peptides and 3 control peptides were tested *in vitro*. Of the 3 control peptides, only P8 revealed positive fluorescence *in vitro*, but with lower intensity than some other candidates. Since this peptide was already identified in other biopanning experiments for different targets it is most likely not muscle and/or heart specific [27]. Control peptide P12 was suggested to target the nuclei of mammary epithelial cells, but was negative for targeting cultured muscle cells; P13 was negative as expected. An interesting finding is the high fluorescence for P4 and the lack of fluorescence for P5, since these peptides only differ in their N-terminal amino acid. This finding highlights the sequence specificity of homing peptides.

Of the four candidate peptides (P1, P2, P4 and P11) selected as promising based on *in vitro* and *in vivo* results, P4 conjugation to 23AON resulted in significantly increased exon skipping in heart and diaphragm. For heart, this is most likely due to the higher levels of P4-23AON compared to 23AON. However, for diaphragm, we did not find significantly improved levels of P4-23AON. Note that it is not possible to distinguish between AONs taken up by cells and AONs sequestered in interstitium with the ELISA used to measure AON levels in tissue homogenates. Therefore there may not always be a clear correlation between the increase in exon skipping and increase of AON levels in the tissues as seen here for the diaphragm. This probably also underlies the fact that exon skipping levels in heart are lower than in skeletal muscles, while AON levels in these tissues are comparable. No significant increase was seen in other skeletal muscle, which is as expected since P4 was selected for uptake by heart (see table 1). The generally higher tissue P4-23AON levels are possibly the result of a lower clearance rate by liver and kidney or faster uptake by muscle. This is underlined by the higher P4-23AON plasma levels after injection. While we also observed increased P4-23AON levels in liver and kidney at time of sacrifice, the relative levels of P4-23AON in skeletal and cardiac muscle compared to liver and kidney were favourable.

We investigated the safety profile of our P4 conjugate. Based on the fact that all markers for liver and kidney function and damage were unchanged after short term systemic treatment with P4-23AON, we conclude that P4 conjugation does not alter the safety profile of the 23AON in short term treatment of *mdx* mice; however extended studies are required for longer term safety evaluation.

In summary, peptide P4 conjugation slightly enhances skeletal and cardiac muscle uptake of 23AON, resulting in higher exon skipping levels, significant in heart and diaphragm, and is well-tolerated by *mdx* mice after short term treatment. The application of P4 for clinical application will depend on more extensive efficacy and safety studies and mechanism of action, but the approach may be very interesting to further improve the efficacy of both 2OMePS and potentially also PMOs currently in clinical development for DMD.



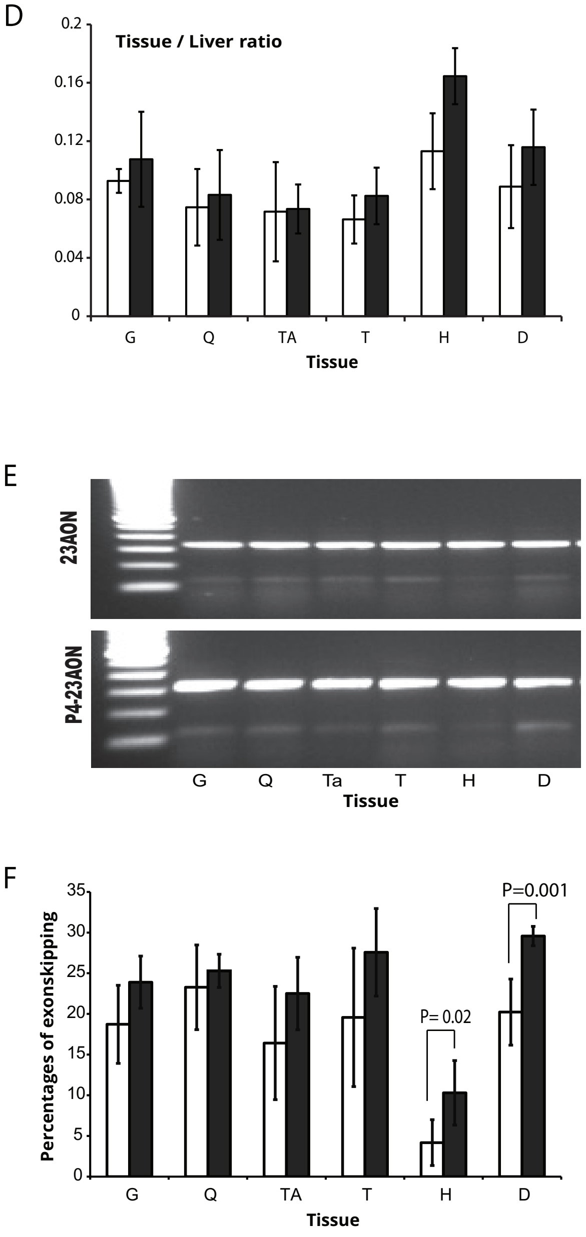


Figure 5. AON levels and exon skipping efficacy after systemic treatment of (P4-)23AON in mdx mice. Mdx mice (5 mice per group) were subcutaneosly injected 4 times per week with 50 mg/kg of 23AON or equimolar P4-23AON for 6 weeks. Tissues were harvested for bioanalysis one week after the last injection. A) A hybridization ligation assay was used to measure plasma AON levels before, and 10 minutes, 30 minutes, 1 hour and 6 hours after the last injection. B) The same assay is used to determine tissue AON levels. C) Ratios of AON levels in muscle and heart compared to kidney. D) Ratios of AON levels in muscle and heart compared to liver. E) Exon skipping assessed by RT-PCR visualized on agarose gel (two representative pictures, one mouse from each group) and semi-quantitatively determined by lab-on a chip as the percentages of the total (skip and wild type) product Bars represent means ± SD.(T-test for significant P<0.05 G= Gastrocnemius, Q= Quadriceps, TA= Tibialis Anterior, T= Triceps, H= Heart, D= Diaphragm, L = Liver K=Kidney.

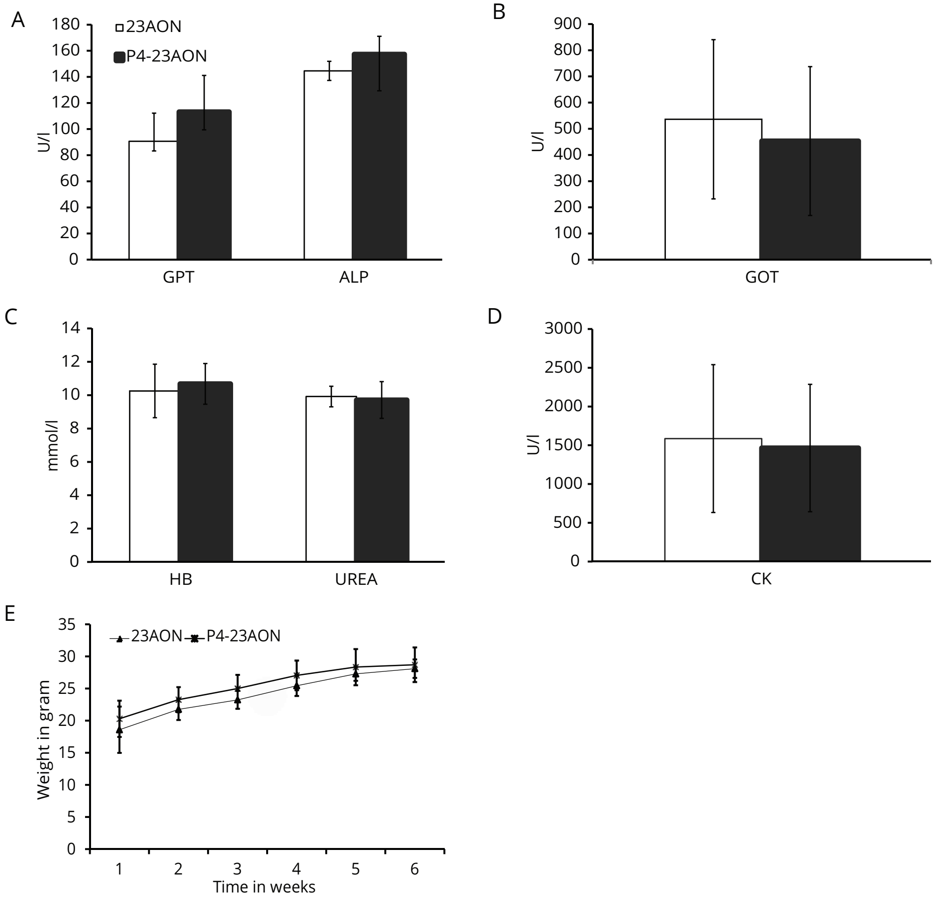


Figure 6. Assessment of safety parameters. *Mdx* mice were subcutaneously injected 4 times per week with 50 mg/kg of 23AON or P4-23AON for 6 weeks. Upon sacrifice (one week after the last injection) blood samples were taken from all mice and plasma was analyzed for levels of A) glutamate pyruvate transaminase (GPT), alkaline phosphates (ALP) B) glutamic oxaloacetic transaminase (GOT) C) hemoglobin (HB), urea and D) creatine kinease (CK). E) Weight over time. Bars represent means ± SD.

Acknowledgements

This work was funded by a grant from the Dutch Duchenne Parent Project and ZonMw (the Netherlands). All peptides and peptide-AON conjugates were synthesised by Prosensa Therapeutics. All *in vitro* and *in vivo* experiments were performed at the LUMC.

Author disclosure statement

Hans Heemskerk ”a”., Annemieke Aartsma –Rus “a”. and Judith van Deutenkom “b”. report being coinventors on patents of the LUMC on exon skipping, licensed by LUMC to Prosensa Therapeutics, and being entitled to a share of royalties. Judith van Deutenkom., Begona Aguilera., Peter de Visser., Anneke Janson., Daan Muilwijk, Kar Him Pang, Rick Vermue and Tatyana G. Karnaoukh report being employed by Prosensa Therapeutics.

Reference

1. Moat, SJ, Bradley, DM, Salmon, R, Clarke, A, Hartley, L (2013) Newborn bloodspot screening for Duchenne muscular dystrophy: 21 years experience in Wales (UK). Eur J Hum Genet;

2. Emery, AE (2002) The muscular dystrophies. Lancet; 359: 687-695.

3. van Putten M., Aartsma-Rus, A (2011) Opportunities and challenges for the development of antisense treatment in neuromuscular disorders. Expert Opin Biol Ther; 11: 1025-1037.

4. Monaco, AP, Bertelson, CJ, Liechti-Gallati, S, Moser, H, Kunkel, LM (1988) An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics; 2: 90-95.

5. Muntoni, F, Torelli, S, Ferlini, A (2003) Dystrophin and mutations: one gene, several proteins, multiple phenotypes. Lancet Neurol; 2: 731-740.

6. Aartsma-Rus, A, Janson, AA, Kaman, WE, Bremmer-Bout, M, den Dunnen, JT, Baas, F et al. (2003) Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. Hum Mol Genet; 12: 907-914.

7. Aartsma-Rus, A (2010) Antisense-mediated modulation of splicing: therapeutic implications for Duchenne muscular dystrophy. RNA Biol; 7: 453-461.

8. McClorey, G, Moulton, HM, Iversen, PL, Fletcher, S, Wilton, SD (2006) Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. Gene Ther; 13: 1373-1381.

9. Yokota, T, Lu, QL, Partridge, T, Kobayashi, M, Nakamura, A, Takeda, S et al. (2009) Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. Ann Neurol; 65: 667-676.

10. Bremmer-Bout, M, Aartsma-Rus, A, de Meijer, EJ, Kaman, WE, Janson, AA, Vossen, RH et al. (2004) Targeted exon skipping in transgenic hDMD mice: A model for direct preclinical screening of human-specific antisense oligonucleotides. Mol Ther; 10: 232-240.

11. Fletcher, S, Honeyman, K, Fall, AM, Harding, PL, Johnsen, RD, Wilton, SD (2006) Dystrophin expression in the mdx mouse after localised and systemic administration of a morpholino antisense oligonucleotide. J Gene Med; 8: 207-216.

12. Heemskerk, H, de, WC, van, KP, Heuvelmans, N, Sabatelli, P, Rimessi, P et al. (2010) Preclinical PK and PD studies on 2’-O-methyl-phosphorothioate RNA antisense oligonucleotides in the mdx mouse model. Mol Ther; 18: 1210-1217.

13. Alter, J, Lou, F, Rabinowitz, A, Yin, H, Rosenfeld, J, Wilton, SD et al. (2006) Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat Med; 12: 175-177.

14. Goemans, NM, Tulinius, M, van den Akker, JT, Burm, BE, Ekhart, PF, Heuvelmans, N et al. (2011) Systemic administration of PRO051 in Duchenne’s muscular dystrophy. N Engl J Med; 364: 1513-1522.

15. Cirak, S, Arechavala-Gomeza, V, Guglieri, M, Feng, L, Torelli, S, Anthony, K et al. (2011) Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. Lancet; 378: 595-605.

16. Heemskerk, HA, de Winter, CL, de Kimpe, SJ, van Kuik-Romeijn, P, Heuvelmans, N, Platenburg, GJ et al. (2009) In vivo comparison of 2’-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. J Gene Med; 11: 257-266.

17. Moulton, HM, Wu, B, Jearawiriyapaisarn, N, Sazani, P, Lu, QL, Kole, R (2009) Peptide-morpholino conjugate: a promising therapeutic for Duchenne muscular dystrophy. Ann N Y Acad Sci; 1175: 55-60.

18. Moulton, HM, Moulton, JD (2010) Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy. Biochim Biophys Acta; 1798: 2296-2303.

19. Wu, B, Lu, P, Cloer, C, Shaban, M, Grewal, S, Milazi, S et al. (2012) Long-term rescue of dystrophin expression and improvement in muscle pathology and function in dystrophic mdx mice by peptide-conjugated morpholino. Am J Pathol; 181: 392-400.

20. Yin, H, Saleh, AF, Betts, C, Camelliti, P, Seow, Y, Ashraf, S et al. (2011) Pip5 transduction peptides direct high efficiency oligonucleotide-mediated dystrophin exon skipping in heart and phenotypic correction in mdx mice. Mol Ther; 19: 1295-1303.

21. Scott, JK, Smith, GP (1990) Searching for peptide ligands with an epitope library. Science; 249: 386-390.

22. Kehoe, JW, Kay, BK (2005) Filamentous phage display in the new

millennium. Chem Rev; 105: 4056-4072.

23. Zahid, M, Phillips, BE, Albers, SM, Giannoukakis, N, Watkins, SC, Robbins, PD (2010) Identification of a cardiac specific protein transduction domain by in vivo biopanning using a M13 phage peptide display library in mice. PLoS One; 5: e12252.

24. Samoylova, TI, Smith, BF (1999) Elucidation of muscle-binding peptides by phage display screening. Muscle Nerve; 22: 460-466.

25. Seow, Y, Yin, H, Wood, MJ (2010) Identification of a novel muscle targeting peptide in mdx mice. Peptides; 31: 1873-1877.

26. Yin, H, Moulton, HM, Betts, C, Seow, Y, Boutilier, J, Iverson, PL et al. (2009) A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice. Hum Mol Genet; 18: 4405-4414.

27. Huang, J, Ru, B, Li, S, Lin, H, Guo, FB (2010) SAROTUP: scanner and reporter of target-unrelated peptides. J Biomed Biotechnol; 2010: 101932.

28. Shtatland, T, Guettler, D, Kossodo, M, Pivovarov, M, Weissleder, R (2007) PepBank--a database of peptides based on sequence text mining and public peptide data sources. BMC Bioinformatics; 8: 280.

29. Mann, CJ, Honeyman, K, McClorey, G, Fletcher, S, Wilton, SD (2002) Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. J Gene Med; 4: 644-654.

30. Juliano, RL, Ming, X, Nakagawa, O (2012) Cellular uptake and intracellular trafficking of antisense and siRNA oligonucleotides. Bioconjug Chem; 23: 147-157.

31. Verhaart, IE, Tanganyika-de Winter, CL, Karnaoukh, TG, Kolfschoten, IG, de Kimpe, SJ, van Deutekom, JC et al. (2013) Dose-dependent pharmacokinetic profiles of 2'-O-methyl phosphorothioate antisense oligonucleotidesin mdx mice. Nucleic Acid Ther; 23: 228-237.

32. Malerba, A, Boldrin, L, Dickson, G (2011) Long-term systemic administration of unconjugated morpholino oligomers for therapeutic expression of dystrophin by exon skipping in skeletal muscle: implications for cardiac muscle integrity. Nucleic Acid Ther; 21: 293-298.

33. Townsend, D, Yasuda, S, Chamberlain, J, Metzger, JM (2009) Cardiac consequences to skeletal muscle-centric therapeutics for Duchenne muscular dystrophy. Trends Cardiovasc Med; 19: 50-55.

34. 't Hoen, PA, Jirka, SM, Ten Broeke, BR, Schultes, EA, Aguilera, B, Pang, KH et al. (2012) Phage display screening without repetitious selection rounds. Anal Biochem; 421: 622-631.

35. Yu, RZ, Baker, B, Chappell, A, Geary, RS, Cheung, E, Levin, AA (2002) Development of an ultrasensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay for the determination of phosphorothioate oligodeoxynucleotide in plasma. Anal Biochem; 304: 19-25.