

Title: In Vivo Delivery of Morpholino Oligos by Cell-Penetrating Peptides

Running title: In Vivo Delivery of PPMO

Hong M. Moulton

Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331.
hong.moulton@oregonstate.edu. 541-737-2764

Keywords: Morpholino oligos, Cell-penetrating peptide, PPMO, Duchenne muscular dystrophy, Antisense

ABSTRACT

Morpholino oligos (Morpholinos) are widely used tools for knocking down gene expression and are currently in a clinical trial for treatment of Duchene muscular dystrophy. A Morpholino analog has been in a clinical trial as a potential anti-bioterrorism agent for inhibiting replication of deadly Marburg viral infection. The cellular uptake of Morpholinos can be greatly increased by conjugation with cell-penetrating peptides (CPP). The use of the CPP-Morpholino conjugates (PPMOs) *in vivo* has been broadly demonstrated in viral, bacterial, genetic and other diseases. The following aspects of PPMOs will be discussed in this paper including chemistry, stability, antisense specificity, mechanism of cellular uptake, *in vivo* efficacy, tissue distribution, pharmacokinetics, toxicity and the human clinical trials. PPMOs are powerful research tools for studying gene function in animals and their properties are being improved as potential human therapeutic agents.

1. INTRODUCTION

Phosphorodiamidate morpholino oligomers (also known as Morpholinos or PMOs) are a type of antisense oligos that are widely used in developmental biology to knockdown gene expression. The efficacy, sequence specificity, stability and low toxicity of Morpholinos have been demonstrated in about 5000 publications (pubs.gene-tools.com), mostly describing work in embryos. The therapeutic development of Morpholinos is underway, currently in on-going clinical trials for treatment of Duchenne muscular dystrophy (DMD) and against infection and spread of the hemorrhagic filovirus Marburg (www.sarepta.com). In this paper, the chemistry and mechanism of action of Morpholinos will be briefly introduced. The main part of the paper will be devoted to the delivery of Morpholinos by cell-penetrating peptides (CPPs) including discussion of the chemistry, sequence specificity, stability and cellular uptake mechanism of CPP-Morpholino conjugates (PPMO). In addition, *in vivo* efficacy, pharmacokinetics, tissue distribution and toxicity of PPMO will be discussed in the context of systemic delivery for DMD research. Lastly, I will highlight the two human clinical trials of PPMO for cardiovascular diseases.

2. PHOSPHORODIAMIDATE MORPHOLINO OLIGOMERS

2.1. Nomenclature, chemical structure and stability.

Phosphorodiamidate morpholino oligomers are referred to by a number of different names and acronyms. In this paper, two commonly used terms, PMO and Morpholino, will be used interchangeably to describe this class of antisense compounds. In the literature, phosphorodiamidate morpholino oligomers are also referred to as MO or MORF.

The structure of a Morpholino is superficially similar to a nucleic acid oligo; however, upon closer inspection there are significant differences. Nucleic acids consist of a backbone of sugar rings (ribose or deoxyribose) bearing pendant nucleic acid bases and are coupled via phosphates. In contrast, Morpholino oligos replace the ribose rings of RNA with morpholine rings which bear the nucleic acid bases (usually A, C, G or T), and the morpholino-base moieties are linked through methylene phosphordiamidates (Figure 1). The phosphordiamidate group contains two nitrogen-phosphorous bonds, one with the morpholine nitrogen and one with a dimethylamine. The phosphorodiamidate linkage is non-ionic, in contrast to the anionic phosphate linkage of natural nucleic acids [1]. Morpholinos are usually 18-31 subunits in length with each subunit bearing an A,T,C or G base; a typical 25-base Morpholino oligo has a mass of ~8000 Daltons, with the exact mass dependent on the specific base sequence. Morpholinos are stable in basic conditions and sensitive to acidic conditions at pH < 3. Morpholinos are not degraded by enzyme action [2] and they are biochemically stable in human serum and in cells [3] and tissue lysates [4].

2.2. Mechanism and Biological Functions.

Binding of a Morpholino to its target RNA sequence prevents other processes from occurring at that target by steric blocking, simply getting in the way of macromolecules [1, 5]. Morpholinos bind to sequences of RNA in antiparallel orientation by complementary base pairing. They do not require enzymes for their activity [1, 5, 6].

Depending on their target sequence, Morpholinos have been shown to have the following functions: blocking translation of mRNA [7], modifying of splicing of pre-mRNA [8], blocking miRNA maturation and activity [9], and blocking other RNA sites – splice regulation sequences [10], ribozymes [11], translocation signals [12] and slippery sequences [13] .

2.3. Toxicity.

On-target Morpholino exhibits very low toxicity in living systems unless knockdown of the targeted gene is itself toxic. Morpholinos are broadly-used tools for gene knockdown in studies of the development of zebrafish [14], frogs [15], chicken eggs [15], urchins [16] and tunicates [17]. Their successful and widespread use for gene knockdown in those exquisitely sensitive embryonic systems indicates their low toxicity and low off-target effects [14, 18, 19]. This success is probably due to their charge-neutrality, good aqueous solubility, reasonable RNA binding affinity and low protein binding affinity. Morpholinos don't activate Toll-like receptors or otherwise trigger innate immune responses [20]. The safety of Morpholinos has been demonstrated in mice, in monkey [21-24] and in human clinical trials [25, 26].

2.4. Delivery.

For most applications, delivery assistance is required to get Morpholinos into cells in order to perform their functions. Various methods have been used for *in vivo* delivery of Morpholinos, including microinjections into the cytoplasm of early embryos, injection then electroporation for local delivery in later embryos, conjugation of cell-penetrating peptides to form PPMO [3, 27-35] or conjugation to an octa-guanidinium dendrimers [36]. Below I will focus on studies of PPMOs and *in vivo* systemic delivery of PPMOs in DMD animal models.

3. CELL-PENETRATING PEPTIDE MORPHOLINO CONJUGATES

3.1. Chemistry.

An uncharged Morpholino does not form a complex with a CPP so simply mixing a Morpholino and a CPP and putting the mixture onto cells failed to produce antisense effects [37]. Therefore, covalent conjugation of a CPP to a Morpholino is usually required for the delivery except with the peptide Endoport. Endoport, developed by Jim Summerton, has been shown to successfully deliver Morpholinos in cell cultures [38, 39] and in organ explants [40] without covalently conjugating the peptide to Morpholino; however, its efficacy for systemic delivery of Morpholinos *in vivo* has not been demonstrated.

A CPP can be conjugated at either end (5' or 3') of a Morpholino and the resulting PPMO conjugates have similar *in vivo* activity (unpublished data). Several linker types including amide, thioether, disulfide and ester have been tested to determine the linkage's influence on activity [30, 41] and stability [3] of PPMOs. In cell culture, the nuclear antisense activities of the PPMO were similar for the three linkage types based on a splicing assay [30]. An amide linkage is the most common way to conjugate a CPP to Morpholino because a Morpholino can easily be conjugated to the carboxyl group of a CPP via an amine at an end of the Morpholino (Figure 1B). For this conjugation, only one

reaction step is required as compared to a two-step strategy for other linkages. In addition, an amide bond is chemically more stable than the other linkages.

3.2. Cellular Uptake Mechanism.

Study of the mechanism of uptake of PPMOs revealed that cell-surface proteoglycans were involved in binding the conjugates, which were subsequently taken up by endocytosis. When the CPPs R_9F_2 , Tat and $(RXR)_4$ (R = arginine, F = phenylalanine, X = 6-aminohexanoic acid) were studied as components of PPMOs, the internalization of the PPMOs was found to be energy- and temperature-dependant, as expected for endocytotic uptake. The majority of the conjugates co-localized in cells with a marker for endocytotic/lysosomal vesicles. A small amount of the conjugates escaped the vesicles to enter the cytosol and nucleus by an unknown mechanism. The degree of endosomal escaping seems related to structural features of the CPPs, with details of this structure activity relationship yet to be defined. A significant portion of the conjugates were found to be trapped within vesicles and could be released by chloroquine treatment [41].

A hypothesis is that proteoglycans tightly binding to a PPMO hinders its endosomal release. Two peptides, $(RXR)_4$ and R_8 , were compared for their ability to deliver Morpholino into cells to the target RNA and for their binding kinetics with a model proteoglycan, heparin sulfate. Those two peptides contain the same number of positive charges but differ in charge density. As demonstrated by an antisense splice correction assay done in cell culture and in mice, $(RXR)_4$ PPMO had significantly higher antisense activity than the R_8 PPMO [35, 42]. A binding study by surface plasmon resonance reveals that both PPMOs bind to heparin sulfate with similar stoichiometric ratios but have different heparin sulfate binding kinetics (Figure 2). The $(RXR)_4$ PPMO exhibits about a 10-fold greater dissociation constant for heparin sulfate than the R_8 PPMO; this is reflected in the $(RXR)_4$ PPMO's 2 x slower on-rate and 5 x faster off-rate compared to the R_8 PPMO. Taken together, the studies support the idea that while binding to proteoglycans aids PPMO internalization, tight binding to proteoglycans may not be beneficial for endosomal release of PPMO.

3.3. Stability.

Twelve PPMOs each with a different CPP were investigated to determine the effects of peptide sequence, linker type and stereochemistry of the amino acids on the stability of a PPMO in cells and in human serum. The stability of the peptide moiety in serum ranked in the order D-CPPs = $(RX)_8 = (RB)_8 > (RXR)_4 > R_9F_2 = \text{Tat}$. Stabilities of peptide moieties within cells was found to be D-CPPs $> (RB)_8 > (RX)_8 = (RXR)_4 = R_9F_2 = \text{Tat}$ (X = 6-aminohexanoic acid, B=beta-alanine). Stabilities of linkers in serum or in cells were found to be amide = maleimide $>$ disulfide [3]. In addition, the stability of an $(RXR)_4$ PPMO was determined in rat serum and organ lysates. The CPP portion of the conjugate exhibited time- and tissue-dependent degradation, with biological stability ranked in the order of liver $>$ heart = kidney $>$ plasma. Up to 6 hr, the CPP portion of PPMO was partially degraded in the plasma but had no apparent degradation in tissues [4]. In all cases, the Morpholino portions of PPMOs were completely stable in cells, human or rat serum and in rat tissue lysates.

3.4. Specificity.

Off-target effects of PPMOs depend on the intracellular stability of the CPPs and the concentrations of PPMO in the cytosol and nuclei of cells. Effects of CPPs (R_9F_2 , $R_4F_2R_4$, $(RXR)_4$) on the specificity of PPMO have been investigated in various models from cell-free translation assays to cell cultures and *in vivo* models. Cell-free translation studies showed that these PPMOs had minimum off-target effects at $< 1 \mu\text{M}$ but off-target effects were observed at higher concentrations [43-46]. The non-specificity was caused by the cationic charged nature of PPMO and the degree of non-specificity of PPMO appears to depend on the Morpholino sequences [45]. However, the non-specificity was not observed either in cell culture up to $20 \mu\text{M}$ treatment concentrations [43-51] or in mouse models [43, 47, 51, 52].

There are two major reasons giving rise to the discrepancy between cell-free and cell/animal models. First, the intracellular instability of CPP reduces off-target effects. In cells most of the CPP was degraded from the MORPHOLINOs and intact conjugates were not detected [3]. Non-specificity in cells was observed for the non-degradable D-isomer of R_9F_2 PPMO [45]. Second, low PPMO concentration in cytosol and nuclei reduce off-target effects. Although the treatment concentrations were $> 1 \mu\text{M}$ in most applications, the concentration of PPMO available in the cytosol and nucleus is only a small fraction of the treatment concentration because only a small amount of PPMO entered cells and the majority of the PPMOs were trapped within endosomal/lysosomal vesicles [41].

4. IN VIVO DELIVERY OF PPMO

PPMOs have been used for numerous applications in animal models, mostly in the areas of genetic, bacterial and viral diseases including various strains of influenza virus [49, 53, 54], respiratory syncytial virus [55, 56], herpes simplex viruses [57, 58], severe acute respiratory syndrome (SARS) [31, 59], murine hepatitis virus [52] and Ebola virus [33, 43], etc. There is a good review paper for the *in vivo* applications of PPMOs against viral diseases [60]. PPMO's antibacterial *in vivo* applications include *Burkholderia cepacia* complex [61] and *Escherichia coli* [62]. Here I will focus on systemic delivery of PPMOs for the genetic disease Duchenne muscular dystrophy (DMD) in animal models and highlight the two clinical trials with a PPMO.

4.1. Systemic Delivery of PPMO in DMD Mouse Models

PPMO delivery has been extensively studied in animal models of DMD. DMD is an X-linked disease usually caused by frameshift or nonsense mutation of the human dystrophin transcript, leading to muscle degeneration and decreased span and quality of life in affected humans. Using splice-switching oligos (such as Morpholinos) to modify splicing can result in targeted exon exclusion and restoration of the correct reading frame which has been shown to restore much dystrophin function, offering hope of changing the pathology of Duchenne to resemble the milder Becker muscular dystrophy.

A dystrophin-deficient mouse model of muscular dystrophy (*mdx* mouse) carries a nonsense mutation in exon 23. Excision of exon 23 in muscle cells of *mdx* mice by a Morpholino can restore the expression of a partially-functional dystrophin protein [8]. Skipping exon 23 maintains the reading frame of downstream sequence, allowing expression of an in-frame protein product when the stop codon in exon 23 has been deleted from the mature mRNA along with the rest of the exon. The 25-base Morpholino sequence complementary to the splice donor site, the e23i23 junction (PMO_{E23}), has been used by several groups and showed various degrees of efficacy with different dose regimens [8, 63, 64]. Several CPPs with different sequences have been tested for their ability to deliver PMO_{E23} to the muscles of the *mdx* mice [29, 65]. I will review the studies with a CPP named “B peptide” Morpholino conjugates as the peptide is one of the most extensively studied CPPs for the DMD application and has been tested in two DMD mouse models, wild type mice and in non-human primates [66]. The B peptide has the sequence of (RXRRBR)₂XB where R = arginine, X = 6-aminohexanoic acid, and B = beta-alanine. The discussion includes 1) PPMO improved exon-skipping efficiency and reduced disease pathology, 2) PPMO improved cardiac function, 3) PPMO caused sustained dystrophin production and reduced disease pathology, 4) plasma pharmacokinetics, 4) tissue distribution and residence time, and 6) toxicity.

4.1.1. Improved Exon-Skipping Efficiency and Reduced Disease Pathology.

The dystrophin band of muscle lysate from *mdx* mice is totally absent on a Western blot. Lack of dystrophin causes many downstream effects including 1) leaking of muscle cell membranes, evaluated by elevated serum creatine kinase (CK) and uptake of normally cell-impermeable dyes and antibodies, 2) changes in cellular architecture including absence of proteins in the dystrophin-associated protein complex, 3) regeneration and degeneration of muscle fibers, evaluated by the fiber sizes and the location of nuclei, and 4) decreased muscle functions, evaluated by grip strength, treadmill running, etc.

Because an unmodified Morpholino has rapid renal clearance and poor cellular uptake, the efficacy of delivery to various muscles varies greatly and delivery across skeletal muscle tissue is patchy [8, 67]. Dystrophin expression was found low and uneven and only in a fraction of some muscle types. In addition, MORPHOLINOs do not enter cardiomyocytes sufficiently to relieve heart pathology [8, 67]. Delivery of a Morpholino by CPPs significantly improves intracellular concentration of the Morpholino and results in much greater levels of dystrophin expression in all muscles including heart [34, 42, 68]. A single IV injection of the PPMO_{E23} through a retro-orbital (RO) route at 30 mg/Kg resulted in production of exon-skipped transcript accounting for >80% of dystrophin RT-PCR product in all skeletal muscles and accounting for 50% of the transcript in the cardiac muscle. The amount of dystrophin protein restored in the skeletal muscles was near the normal level in the TA of a wild-type mouse. Every skeletal muscle fiber and 94% of cardiac muscle fibers stained positive for dystrophin [34]. Six biweekly treatments over three-months led to even higher amounts of exon-skipped dystrophin mRNA and protein. In addition, the dystrophin protein was detected in smooth muscles of large blood vessels, the pulmonary artery, and the small intestine. Leaky cell membranes were repaired as demonstrated by lack of uptake of Evan's blue dye and mouse IgG [34].

A one-year long term study of the PPMO_{E23} in *mdx* mice was carried out to determine the effect on dystrophin restoration and disease pathology at lower or less frequent dosing regimen[69]. Monthly injections of 30 mg/Kg PPMO_{E23} restored dystrophin expression to > 50% normal levels in all skeletal muscles and 15% in cardiac muscle. This was associated with greatly reduced serum CK levels, near normal histology, and functional improvement of skeletal muscles. Biweekly injection of 6 mg/Kg PPMO_{E23} for one year produced > 20% dystrophin expression in all skeletal muscles and up to 5% in cardiac muscles, with improvement in muscle function and pathology and reduction in the levels of serum creatine kinase (CK). However, biweekly injection of the PPMO at 1.5 mg/Kg for one year resulted in < 5% dystrophin expression in skeletal muscles and nothing in the heart. The low expression correlates with no improvement of muscle function and disease pathology.

Efficacy of a human-targeted a B peptide PPMO to cause targeted exon 50 skipping was tested in healthy cynomolgus monkeys. With once-weekly IV injection for four weeks at 9 mg/Kg, the PPMO induced an average of 40%, 25% and 2% exon-skipped RT-PCR product in diaphragm, quadriceps and heart (respectively) of four monkeys [70]. The level of exon-skipping is probably an underestimate because the exon-skipped product is out-of-frame and undergoes nonsense-mediated decay in a healthy monkey. With the same dose schedule at a lower dose of 3 mg/Kg little exon-skipped product was detected, similar to the saline-treated monkeys.

4.1.2. Improved Cardiac Function

The first study showing the B peptide PPMO_{E23} improved heart function of *mdx* mice employed a hemodynamic model [34]. *Mdx* mice were treated twice with the PPMO_{E23} at 30 mg/Kg with a 2 week interval. Three weeks after the 2nd treatment, the PPMO_{E23}-treated *mdx* mice, untreated *mdx* mice and the wild type mice were challenged with dobutamine, a β -adrenergic stimulant that can increase cardiac workload and magnify the dysfunctions of the heart of *mdx* mice. All ten wild-type mice and all eight PPMO_{E23}-treated *mdx* mice survived the challenge, while only three of nine untreated *mdx* mice survived the challenge. In addition, the cardiac parameters, including end-diastolic volume, end-systolic pressure, dp/dt_{max}, and cardiac pressure/volume loops of PPMO-treated *mdx* mice were similar to the wild-type mice but different from the untreated *mdx* mice.

The long term benefit of the PPMO_{E23} treatment prior to the development of cardiomyopathy was shown in another study[68]. The effect of the PPMO_{E23} was investigated by treating 16 week old *mdx* mice, the age just before the development of cardiomyopathy. The PPMO_{E23} was injected IV at 12 mg/Kg in two cycles of four once-daily injections with a 2-week interval. Dystrophin restoration was found throughout the heart of treated *mdx* mice and was about 30% of wild type level. The cardiac marker creatine kinase-MB, a hallmark for cardiomyopathy, was significantly reduced, reaching wild type concentration 5 weeks after the first injection. A sham PPMO was used as the specificity control. No detectable dystrophin expression was observed in sham-treated *mdx* mice. Restoration of dystrophin prevented cardiac hypertrophy compared to the sham-treated *mdx* mice as measured by significant reduction in the thickness of anterior and posterior walls in diastole and systole, total heart mass and left ventricle mass to body mass ratio[68]. In addition, the PPMO_{E23} treatment prevented the typical *mdx* age-associated

development of diastolic dysfunction. The seven-month study also showed that earlier PPMO intervention can potentially reduce cardiomyopathy.

4.1.3. Sustained Dystrophin Production and Effect On Disease Pathology

Sustained dystrophin production was first demonstrated in a study when the “peptide B” PPMO_{E23} was IV injected into *mdx* mice at 12 mg/Kg daily for four days. Exon-skipping efficacy, evaluated by detection of exon-skipped RNA transcript, and dystrophin restoration, evaluated by immune blot, was determined from day 1 up to 17 weeks after the last injection [42]. Nine weeks after the last injection, the exon 23-skipped RNA transcript was the major band detected in diaphragm and quadriceps; the band was less intense but still detectable in the heart. Up to 17 weeks after the last injection, dystrophin protein was clearly detected in quadriceps and to a lesser degree in the diaphragm. In the heart, dystrophin protein was detected up to 9 weeks but not 17 weeks after the injection. No tests of disease pathology and muscle function were done in this study.

In a recent study, the half-life of dystrophin induced by a single IV injection of the same PPMO_{E23} was determined[69]. The half-life of dystrophin induced in the skeletal muscles of *mdx* mice was found to be about 2 months. Single IV injection of the PPMO_{E23} at 30 mg/Kg into *mdx* mice resulted in production of dystrophin in skeletal muscles that was still detectable at 30% and 10% of wild-type level 4 and 5 months after the injection, respectively. The dystrophin level in skeletal muscle was very high at >80% of wild type level and this level was sustained from 2 weeks to 1.5 months and then decreased to 50% at 2.5 months after the injection. Dystrophin expression induced in the cardiac muscle was at a lower level and had a shorter half-life than that in skeletal muscles. The half-life of dystrophin in cardiac muscle was about 1 month. Cardiac dystrophin induced by the single 30mg/Kg PPMO treatment peaked at 1 month after the injection at about 70% of wild type levels, and then decreased to less than 50% and 25% at 1.5 and 2.5 months after the injection respectively. Dystrophin was not detected at 4 or 5 months in cardiac muscles either by immunohistochemistry or by Western blot.

Dystrophin restoration improved disease pathology of the *mdx* mice. Unlike in the saline-treated *mdx* mice, areas of muscle degeneration and mononucleocyte infiltration were absent in the examined skeletal muscles including TA, quadriceps, biceps and gastrocnemius between 2 weeks and 5 months after the single dose PPMO treatment at 30 mg/Kg. PPMO treatment increased the % muscle fibers that didn't have centralized nuclei from 20% to 40% from 1 month to 4 months post injection. Percentage of fibers with uncentralized nuclei went down to 30% by 5 months in those skeletal muscles after the PPMO_{E23} injection.

A single dose of PPMO treatment provides a shorter period of protection to diaphragm than to other skeletal muscles. In diaphragm, the percentage of fibers with uncentralized nuclei increased to the highest level by 2.5 months, but then decreased again by 4 months, which was earlier than for other skeletal muscles. This was associated with the temporary amelioration then return of areas with degenerating fibers and foci of mononucleocyte infiltration. CK levels were reduced significantly and remained at similar low levels from 1 month to 5 months after the injection.

4.1.4. Plasma Pharmacokinetics

The pharmacokinetics (PK) profile and parameters of the B peptide PPMO_{E23} in *mdx* mice is yet to be published. However, it is expected to be similar to a published study with a similar peptide (RXR)₄XB (P7) conjugated to a Morpholino targeted to human *c-myc* (PMO_{cmyc}) [4]. P7 peptide and B peptide only differ in two amino acid residues, with two of the four 6-aminohexanoic residues in P7 replaced with two beta-alanine residues in the B peptide. When the P7 PPMO_{cmyc} was IV injected to rats at 15 mg/Kg, its plasma PK was characterized by rapid distribution from the vascular space to tissues with an initial distribution half-life ($T_{1/2\alpha}$) of 0.40 h. The PPMO_{cmyc} showed a prolonged elimination half-life ($T_{1/2\beta}$) of 5 hr, representing a 2-fold increase compared to the corresponding unmodified PMO_{cmyc} at the same dose. The volume of distribution of the P7 PPMO_{cmyc} was increased by 4-fold over PMO_{cmyc}. P7 PPMO_{cmyc} had a small increase in area under the curve over PMO_{cmyc}.

4.1.5. Tissue Distribution and Residence Time

B, P7 and other arginine-rich CPP conjugated Morpholinos are found to exhibit a broad tissue distribution as shown by measuring Morpholino tissue concentrations [4] or by an *in vivo* antisense functional assay [42]. Kidney and liver are the primary sites of accumulation of the PPMOs [4, 71]. Relatively lower concentrations were detected in spleen, lungs, heart, brain and muscles. At equivalent dose, the tissue recovery data showed that conjugation of the P7 peptide improved tissue uptake of the MORPHOLINO in all of these tissues except brain [4]. In addition, tissue concentrations of the Morpholino in the P7 PPMO-treated rats were greater than in the tissues of the corresponding Morpholino-treated rats 5 days post injection, indicating that CPP conjugation prolongs the duration of tissue retention of the Morpholino. Functional biodistribution of 14 CPP PPMOs varying in amino acid sequences of the CPPs were determined in a splice correction reporter transgenic mouse model, with EGFP as the reporter for functional readout [72]. Antisense activity of the PPMOs was found in all of the tested organs and tissues including kidney, liver, lungs, heart, skeletal muscles, diaphragm, and intestine [42].

4.1.6. Toxicity

PPMO exhibits dose-dependent toxicity [4]; there seems to be a dose threshold for the toxicity. Below the threshold, toxicity was not observed. Above it, the severity of toxicity is dose-dependent. Toxicity includes lethargy, rapid breathing, tubular degeneration in kidney and weight loss. The severity of toxicity is also dependent on the dose frequency. Animals can tolerate doses and recover better if infrequent dose schedules are followed. The B peptide PPMO_{E23} was found not toxic when it was IV injected to *mdx* mice at 6 mg/Kg biweekly or at 30 mg/Kg monthly for one year [69]. At higher doses, PPMO-related toxicity was observed with the LD₅₀ of the “B-peptide” PPMO_{E23} determined to be 85 mg/Kg in *mdx* mice.

The threshold level of PPMO toxicity varies among animal species.. The B peptide PPMO_{E23} exhibited no toxic effect in kidneys at either 20 mg/Kg weekly injection to the wild type mice for 6 weeks (data not shown) or 30 mg/Kg biweekly injection to *mdx* mice for three months [34]. However, the same peptide conjugated to another Morpholino targeted to human dystrophin exon 50 (PPMO_{E50}) was found to cause mild tubular degeneration in the kidneys of monkeys at 9 mg/Kg weekly injections for 4 weeks [70]. However, one needs to be cautious in drawing any conclusion from one limited study because other factors could also contribute to the higher level of toxicity in monkeys. For example, the antisense component may also contribute to the toxicity in several possible ways. If there is an off-target RNA interaction related to antisense sequence, that may contribute to the observed toxicity. In addition, unlike PPMO_{E23} which skips exon 23 in mice and does not frameshift downstream dystrophin, the PPMO_{E50}-induced exon 50 skipping frameshifts the dystrophin sequence downstream of exon 50 in monkeys, which might contribute to toxicity.

No specific humoral immune response against the B peptide PPMO_{E23} was found after a single systemic injection or multiple injections over several months [34]. Another study examined both humoral and T cell responses to a B peptide derivative, B-MSP, conjugated to PMO_{E23}. After this PPMO was injected into *mdx* mice at 6 mg/Kg biweekly for 12 weeks, anti-PPMO antibody was not detected in the mouse serum. In addition, CD3⁺ and CD8⁺ T lymphocytes in muscle tissue sections did not differ significantly between age-matched PPMO-treated and untreated *mdx* mice [73].

4.2. PPMO Clinical Trials

AVI-5126 is a PPMO containing the P7 peptide conjugated PMO_{cmyc}. It was the first PPMO used in a safety and efficacy clinical trial. The PPMO_{cmyc} was intended to prevent eventual blockage of a transplanted vein after cardiovascular by-pass surgery by inhibiting cell proliferation. The vein was excised and immersed in a solution containing 10 μM AVI-5126 and then re-implanted as a bypass graft. The study was titled “Clinical Study to Assess the Safety and Efficacy of ex-Vivo Vein Graft Exposure to AVI-5126 in Coronary Artery By-Pass Grafting to Reduce Clinical Graft Failure” (<http://clinicaltrials.gov/ct2/results?term=AVI+5126>). Unfortunately, the trial was terminated based on the results from limited number of patients; the company felt that “the probability of successfully attaining the study’s clinical endpoint, even at this early stage, was deemed to be too low to warrant continuing the trial”. However, termination decision was not based on clinical safety concerns since there was no significant difference between the AVI-5126 or placebo groups with respect to Major Cardiac Adverse Events (MACE) (http://www.clinicaspace.com/news_print.aspx?NewsEntityId=99791). The same PPMO_{cmyc} renamed as GTX 5126 was tested in another clinical trial in Germany for prevention of restenosis of coronary artery after balloon angioplasty, sponsored by COOK (<http://clinicaltrials.gov/ct2/show/NCT00777842>). PPMO_{cmyc}-loaded GTXTM Drug Eluting Coronary Stent System is intended for the treatment of patients with a lesion in the coronary artery. Thirty patients were enrolled in the trial and the trial was terminated for an unknown reason. No efficacy and safety data have been published.

5. CONCLUDING REMARKS

CPP conjugation is one of the most effective techniques for systemic delivery of Morpholinos *in vivo*. They have been valuable research tools for studying Morpholino-mediated gene knockdown in cell culture and in animals. The therapeutic potential of PPMO is promising. Improvement of endosomal release and lowering the toxicity of PPMOs through structure-activity-relationship studies will result in optimized CPP, able to deliver Morpholinos with improved therapeutic windows.

Acknowledgments

The author wishes to thank Dr. Jon Moulton for critical reading of the manuscript.

References

- [1] J. Summerton, D. Weller, Morpholino antisense oligomers: design, preparation, and properties, *Antisense Nucleic Acid Drug Dev* 7 (1997) 187-195.
- [2] R.M. Hudziak, E. Barofsky, D.F. Barofsky, D.L. Weller, S.B. Huang, D.D. Weller, Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation, *Antisense Nucleic Acid Drug Dev* 6 (1996) 267-272.
- [3] D.S. Youngblood, S.A. Hatlevig, J.N. Hassinger, P.L. Iversen, H.M. Moulton, Stability of cell-penetrating peptide-morpholino oligomer conjugates in human serum and in cells, *Bioconjug Chem* 18 (2007) 50-60.
- [4] A. Amantana, H.M. Moulton, M.L. Cate, M.T. Reddy, T. Whitehead, J.N. Hassinger, D.S. Youngblood, P.L. Iversen, Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate, *Bioconjug Chem* 18 (2007) 1325-1331.
- [5] J. Summerton, Morpholino antisense oligomers: the case for an RNase H-independent structural type, *Biochim Biophys Acta* 1489 (1999) 141-158.
- [6] J.E. Summerton, Morpholino, siRNA, and S-DNA compared: impact of structure and mechanism of action on off-target effects and sequence specificity, *Curr Top Med Chem* 7 (2007) 651-660.
- [7] R.M. Hudziak, J. Summerton, D.D. Weller, P.L. Iversen, Antiproliferative effects of steric blocking phosphorodiamidate morpholino antisense agents directed against c-myc, *Antisense Nucleic Acid Drug Dev* 10 (2000) 163-176.
- [8] J. Alter, F. Lou, A. Rabinowitz, H. Yin, J. Rosenfeld, S.D. Wilton, T.A. Partridge, Q.L. Lu, Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology, *Nat Med* 12 (2006) 175-177.
- [9] W.P. Kloosterman, A.K. Lagendijk, R.F. Ketting, J.D. Moulton, R.H. Plasterk, Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development, *PLoS Biol* 5 (2007) e203.
- [10] I.G. Bruno, W. Jin, G.J. Cote, Correction of aberrant FGFR1 alternative RNA splicing through targeting of intronic regulatory elements, *Hum Mol Genet* 13 (2004) 2409-2420.
- [11] L. Yen, J. Svendsen, J.S. Lee, J.T. Gray, M. Magnier, T. Baba, R.J. D'Amato, R.C. Mulligan, Exogenous control of mammalian gene expression through modulation of RNA self-cleavage, *Nature* 431 (2004) 471-476.
- [12] P.K. Arthur, M. Claussen, S. Koch, K. Tarbashevich, O. Jahn, T. Pieler, Participation of *Xenopus* Elr-type proteins in vegetal mRNA localization during oogenesis, *J Biol Chem* 284 (2009) 19982-19992.
- [13] M.T. Howard, R.F. Gesteland, J.F. Atkins, Efficient stimulation of site-specific ribosome frameshifting by antisense oligonucleotides, *RNA* 10 (2004) 1653-1661.
- [14] B.R. Bill, A.M. Petzold, K.J. Clark, L.A. Schimmenti, S.C. Ekker, A primer for morpholino use in zebrafish, *Zebrafish* 6 (2009) 69-77.
- [15] M.S. Mimoto, J.L. Christian, Manipulation of gene function in *Xenopus laevis*, *Methods Mol Biol* 770 55-75.
- [16] Z. Wei, R.C. Angerer, L.M. Angerer, Direct development of neurons within foregut endoderm of sea urchin embryos, *Proc Natl Acad Sci U S A* 108 (2011) 9143-9147.
- [17] L. Christiaen, E. Wagner, W. Shi, M. Levine, Microinjection of morpholino oligos and RNAs in sea squirt (*Ciona*) embryos, *Cold Spring Harb Protoc* 2009 (2009) pdb prot5347.
- [18] H. Yin, H.M. Moulton, Y. Seow, C. Boyd, J. Boutilier, P. Iverson, M.J. Wood, Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function, *Hum Mol Genet* 17 (2008) 3909-3918.
- [19] J.S. Eisen, J.C. Smith, Controlling morpholino experiments: don't stop making antisense, *Development* 135 (2008) 1735-1743.
- [20] J.D. Moulton, Y.L. Yan, Using Morpholinos to control gene expression, *Curr Protoc Mol Biol* Chapter 26 (2008) Unit 26 28.
- [21] P. Sazani, K.P. Ness, D.L. Weller, D. Poage, K. Nelson, A.S. Shrewsbury, Chemical and mechanistic toxicology evaluation of exon skipping phosphorodiamidate morpholino oligomers in mdx mice, *Int J Toxicol* 30 (2011) 322-333.

- [22] P. Sazani, K.P. Ness, D.L. Weller, D.W. Poage, K. Palyada, S.B. Shrewsbury, Repeat-dose toxicology evaluation in cynomolgus monkeys of AVI-4658, a phosphorodiamidate morpholino oligomer (PMO) drug for the treatment of duchenne muscular dystrophy, *Int J Toxicol* 30 (2011) 313-321.
- [23] P. Sazani, D.L. Weller, S.B. Shrewsbury, Safety pharmacology and genotoxicity evaluation of AVI-4658, *Int J Toxicol* 29 (2010) 143-156.
- [24] B. Wu, B. Xiao, C. Cloer, M. Shaban, A. Sali, P. Lu, J. Li, K. Nagaraju, X. Xiao, Q.L. Lu, One-year treatment of morpholino antisense oligomer improves skeletal and cardiac muscle functions in dystrophic mdx mice, *Mol Ther* 19 (2010) 576-583.
- [25] S. Cirak, L. Feng, K. Anthony, V. Arechavala-Gomez, S. Torelli, C. Sewry, J.E. Morgan, F. Muntoni, Restoration of the dystrophin-associated glycoprotein complex after exon skipping therapy in Duchenne muscular dystrophy, *Mol Ther* 20 (2011) 462-467.
- [26] M. Kinali, V. Arechavala-Gomez, L. Feng, S. Cirak, D. Hunt, C. Adkin, M. Guglieri, E. Ashton, S. Abbs, P. Nihoyannopoulos, M.E. Garralda, M. Rutherford, C. McCulley, L. Popplewell, I.R. Graham, G. Dickson, M.J. Wood, D.J. Wells, S.D. Wilton, R. Kole, V. Straub, K. Bushby, C. Sewry, J.E. Morgan, F. Muntoni, Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study, *Lancet Neurol* 8 (2009) 918-928.
- [27] H.M. Moulton, J.D. Moulton, Peptide-assisted delivery of steric-blocking antisense oligomers, *Curr Opin Mol Ther* 5 (2003) 123-132.
- [28] H.M. Moulton, J.D. Moulton, Arginine-rich cell-penetrating peptides with uncharged antisense oligomers, *Drug Discov Today* 9 (2004) 870.
- [29] H.M. Moulton, J.D. Moulton, Morpholinos and their peptide conjugates: Therapeutic promise and challenge for Duchenne muscular dystrophy, *Biochim Biophys Acta* (2010).
- [30] H.M. Moulton, M.H. Nelson, S.A. Hatlevig, M.T. Reddy, P.L. Iversen, Cellular uptake of antisense morpholino oligomers conjugated to arginine-rich peptides, *Bioconjug Chem* 15 (2004) 290-299.
- [31] B.W. Neuman, D.A. Stein, A.D. Kroeker, H.M. Moulton, R.K. Bestwick, P.L. Iversen, M.J. Buchmeier, Inhibition and escape of SARS-CoV treated with antisense morpholino oligomers, *Adv Exp Med Biol* 581 (2006) 567-571.
- [32] S. Svasti, T. Suwanmanee, S. Fucharoen, H.M. Moulton, M.H. Nelson, N. Maeda, O. Smithies, R. Kole, RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice, *Proc Natl Acad Sci U S A* 106 (2009) 1205-1210.
- [33] D.L. Swenson, K.L. Warfield, T.K. Warren, C. Lovejoy, J.N. Hassinger, G. Ruthel, R.E. Blouch, H.M. Moulton, D.D. Weller, P.L. Iversen, S. Bavari, Chemical modifications of antisense morpholino oligomers enhance their efficacy against Ebola virus infection, *Antimicrob Agents Chemother* 53 (2009) 2089-2099.
- [34] B. Wu, H.M. Moulton, P.L. Iversen, J. Jiang, J. Li, C.F. Spurney, A. Sali, A.D. Guerron, K. Nagaraju, T. Doran, P. Lu, X. Xiao, Q.L. Lu, Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer, *Proc Natl Acad Sci U S A* 105 (2008) 14814-14819.
- [35] R.P. Wu, D.S. Youngblood, J.N. Hassinger, C.E. Lovejoy, M.H. Nelson, P.L. Iversen, H.M. Moulton, Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity, *Nucleic Acids Res* 35 (2007) 5182-5191.
- [36] Y.F. Li, P.A. Morcos, Design and synthesis of dendritic molecular transporter that achieves efficient in vivo delivery of morpholino antisense oligo, *Bioconjug Chem* 19 (2008) 1464-1470.
- [37] H.M. Moulton, M.C. Hase, K.M. Smith, P.L. Iversen, HIV Tat peptide enhances cellular delivery of antisense morpholino oligomers, *Antisense Nucleic Acid Drug Dev* 13 (2003) 31-43.
- [38] V.C. Pillai, R. Yesudas, I.H. Shaik, T.J. Thekkumkara, U. Bickel, K.S. Srivenugopal, R. Mehvar, Delivery of NADPH-cytochrome P450 reductase antisense oligos using avidin-biotin approach, *Bioconjug Chem* 21 (2010) 203-207.
- [39] J.E. Summerton, Endo-Porter: a novel reagent for safe, effective delivery of substances into cells, *Ann N Y Acad Sci* 1058 (2005) 62-75.
- [40] G.N. Nikopoulos, T.L. Adams, D. Adams, L. Oxburgh, I. Prudovsky, J.M. Verdi, The use of Endo-Porter to deliver morpholinos in kidney organ culture, *Biotechniques* 44 (2008) 547-549.

- [41] S. Abes, H.M. Moulton, P. Clair, P. Prevot, D.S. Youngblood, R.P. Wu, P.L. Iversen, B. Lebleu, Vectorization of morpholino oligomers by the (R-Ahx-R)₄ peptide allows efficient splicing correction in the absence of endosomolytic agents, *J Control Release* 116 (2006) 304-313.
- [42] N. Jearawiriyapaisarn, H.M. Moulton, B. Buckley, J. Roberts, P. Sazani, S. Fucharoen, P.L. Iversen, R. Kole, Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice, *Mol Ther* 16 (2008) 1624-1629.
- [43] S. Enterlein, K.L. Warfield, D.L. Swenson, D.A. Stein, J.L. Smith, C.S. Gamble, A.D. Kroeker, P.L. Iversen, S. Bavari, E. Muhlberger, VP35 knockdown inhibits Ebola virus amplification and protects against lethal infection in mice, *Antimicrob Agents Chemother* 50 (2006) 984-993.
- [44] K.L. Holden, D.A. Stein, T.C. Pierson, A.A. Ahmed, K. Clyde, P.L. Iversen, E. Harris, Inhibition of dengue virus translation and RNA synthesis by a morpholino oligomer targeted to the top of the terminal 3' stem-loop structure, *Virology* 344 (2006) 439-452.
- [45] M.H. Nelson, D.A. Stein, A.D. Kroeker, S.A. Hatlevig, P.L. Iversen, H.M. Moulton, Arginine-rich peptide conjugation to morpholino oligomers: effects on antisense activity and specificity, *Bioconjug Chem* 16 (2005) 959-966.
- [46] Y.J. Zhang, K.Y. Wang, D.A. Stein, D. Patel, R. Watkins, H.M. Moulton, P.L. Iversen, D.O. Matson, Inhibition of replication and transcription activator and latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus by morpholino oligomers, *Antiviral Res* 73 (2007) 12-23.
- [47] T.S. Deas, C.J. Bennett, S.A. Jones, M. Tilgner, P. Ren, M.J. Behr, D.A. Stein, P.L. Iversen, L.D. Kramer, K.A. Bernard, P.Y. Shi, In vitro resistance selection and in vivo efficacy of morpholino oligomers against West Nile virus, *Antimicrob Agents Chemother* 51 (2007) 2470-2482.
- [48] T.S. Deas, I. Binduga-Gajewska, M. Tilgner, P. Ren, D.A. Stein, H.M. Moulton, P.L. Iversen, E.B. Kauffman, L.D. Kramer, P.Y. Shi, Inhibition of flavivirus infections by antisense oligomers specifically suppressing viral translation and RNA replication, *J Virol* 79 (2005) 4599-4609.
- [49] Q. Ge, M. Pastey, D. Kobasa, P. Puthavathana, C. Lupfer, R.K. Bestwick, P.L. Iversen, J. Chen, D.A. Stein, Inhibition of multiple subtypes of influenza A virus in cell cultures with morpholino oligomers, *Antimicrob Agents Chemother* 50 (2006) 3724-3733.
- [50] R.M. Kinney, C.Y. Huang, B.C. Rose, A.D. Kroeker, T.W. Dreher, P.L. Iversen, D.A. Stein, Inhibition of dengue virus serotypes 1 to 4 in vero cell cultures with morpholino oligomers, *J Virol* 79 (2005) 5116-5128.
- [51] J. Yuan, D.A. Stein, T. Lim, D. Qiu, S. Coughlin, Z. Liu, Y. Wang, R. Blouch, H.M. Moulton, P.L. Iversen, D. Yang, Inhibition of coxsackievirus B3 in cell cultures and in mice by peptide-conjugated morpholino oligomers targeting the internal ribosome entry site, *J Virol* 80 (2006) 11510-11519.
- [52] R. Burrer, B.W. Neuman, J.P. Ting, D.A. Stein, H.M. Moulton, P.L. Iversen, P. Kuhn, M.J. Buchmeier, Antiviral effects of antisense morpholino oligomers in murine coronavirus infection models, *J Virol* 81 (2007) 5637-5648.
- [53] G. Gabriel, A. Nordmann, D.A. Stein, P.L. Iversen, H.D. Klenk, Morpholino oligomers targeting the PB1 and NP genes enhance the survival of mice infected with highly pathogenic influenza A H7N7 virus, *J Gen Virol* 89 (2008) 939-948.
- [54] C. Lupfer, D.A. Stein, D.V. Mourich, S.E. Tepper, P.L. Iversen, M. Pastey, Inhibition of influenza A H3N8 virus infections in mice by morpholino oligomers, *Arch Virol* 153 (2008) 929-937.
- [55] S.H. Lai, D.A. Stein, A. Guerrero-Plata, S.L. Liao, T. Ivanciuc, C. Hong, P.L. Iversen, A. Casola, R.P. Garofalo, Inhibition of respiratory syncytial virus infections with morpholino oligomers in cell cultures and in mice, *Mol Ther* 16 (2008) 1120-1128.
- [56] T. Opriessnig, D. Patel, R. Wang, P.G. Halbur, X.J. Meng, D.A. Stein, Y.J. Zhang, Inhibition of porcine reproductive and respiratory syndrome virus infection in piglets by a peptide-conjugated morpholino oligomer, *Antiviral Res* 91 (2011) 36-42.
- [57] K. Eide, M. Moerdyk-Schauwecker, D.A. Stein, R. Bildfell, D.M. Koelle, L. Jin, Reduction of herpes simplex virus type-2 replication in cell cultures and in rodent models with peptide-conjugated morpholino oligomers, *Antivir Ther* 15 (2010) 1141-1149.
- [58] M. Moerdyk-Schauwecker, D.A. Stein, K. Eide, R.E. Blouch, R. Bildfell, P. Iversen, L. Jin, Inhibition of HSV-1 ocular infection with morpholino oligomers targeting ICP0 and ICP27, *Antiviral Res* 84 (2009) 131-141.

- [59] B.W. Neuman, D.A. Stein, A.D. Kroeker, M.J. Churchill, A.M. Kim, P. Kuhn, P. Dawson, H.M. Moulton, R.K. Bestwick, P.L. Iversen, M.J. Buchmeier, Inhibition, escape, and attenuated growth of severe acute respiratory syndrome coronavirus treated with antisense morpholino oligomers, *J Virol* 79 (2005) 9665-9676.
- [60] D.A. Stein, Inhibition of RNA virus infections with peptide-conjugated morpholino oligomers, *Curr Pharm Des* 14 (2008) 2619-2634.
- [61] D.E. Greenberg, K.R. Marshall-Batty, L.R. Brinster, K.A. Zarembler, P.A. Shaw, B.L. Mellbye, P.L. Iversen, S.M. Holland, B.L. Geller, Antisense phosphorodiamidate morpholino oligomers targeted to an essential gene inhibit Burkholderia cepacia complex, *J Infect Dis* 201 (2010) 1822-1830.
- [62] B.L. Mellbye, S.E. Puckett, L.D. Tilley, P.L. Iversen, B.L. Geller, Variations in amino acid composition of antisense peptide-phosphorodiamidate morpholino oligomer affect potency against Escherichia coli in vitro and in vivo, *Antimicrob Agents Chemother* 53 (2009) 525-530.
- [63] S. Fletcher, K. Honeyman, A.M. Fall, P.L. Harding, R.D. Johnsen, S.D. Wilton, Dystrophin expression in the mdx mouse after localised and systemic administration of a morpholino antisense oligonucleotide, *J Gene Med* 8 (2006) 207-216.
- [64] A. Malerba, F.C. Thorogood, G. Dickson, I.R. Graham, Dosing regimen has a significant impact on the efficiency of morpholino oligomer-induced exon skipping in mdx mice, *Hum Gene Ther* 20 (2009) 955-965.
- [65] H. Yin, A.F. Saleh, C. Betts, P. Camelliti, Y. Seow, S. Ashraf, A. Arzumanov, S. Hammond, T. Merritt, M.J. Gait, M.J. Wood, Pip5 transduction peptides direct high efficiency oligonucleotide-mediated dystrophin exon skipping in heart and phenotypic correction in mdx mice, *Mol Ther* 19 (2011) 1295-1303.
- [66] H.M. Moulton, J.D. Moulton, Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy, *Biochim Biophys Acta* 1798 (2010) 2296-2303.
- [67] T. Yokota, Q.L. Lu, T. Partridge, M. Kobayashi, A. Nakamura, S. Takeda, E. Hoffman, Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs, *Ann Neurol* 65 (2009) 667-676.
- [68] N. Jearawiriyapaisarn, H.M. Moulton, P. Sazani, R. Kole, M.S. Willis, Long-term improvement in mdx cardiomyopathy after therapy with peptide-conjugated morpholino oligomers, *Cardiovasc Res* 85 (2010) 444-453.
- [69] B. Wu, P. Lu, C. Cloer, M. Shaban, S. Grewal, S. Milazi, S.N. Shah, H.M. Moulton, Q.L. Lu, 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 (2012) 392-400.
- [70] P. Sazani, R. Blouch, D. Weller, S. Leow, R. Kole, S. Shrewsbury, AVI 5038: Initail Efficacy and Safety Evaluation in Cynomolgus Monkeys, Treat-NMD/NIH Conference 2009, Brussels, Belgium, 2009.
- [71] H. Yin, H.M. Moulton, C. Betts, Y. Seow, J. Boutilier, P.L. Iversen, M.J. Wood, A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice, *Hum Mol Genet* 18 (2009) 4405-4414.
- [72] P. Sazani, F. Gemignani, S.H. Kang, M.A. Maier, M. Manoharan, M. Persmark, D. Bortner, R. Kole, Systemically delivered antisense oligomers upregulate gene expression in mouse tissues, *Nat Biotechnol* 20 (2002) 1228-1233.
- [73] H. Yin, H.M. Moulton, C. Betts, T. Merritt, Y. Seow, S. Ashraf, Q. Wang, J. Boutilier, M.J. Wood, Functional rescue of dystrophin-deficient mdx mice by a chimeric peptide-PMO, *Mol Ther* 18 (2010) 1822-1829.

FIGURE LEGENDS

Figure 1. Chemical structures of (A) Morpholino and (B) (RXR)₄XB CPP Morpholino conjugate (PPMO).

Figure 2. Heparin sulfate binding SPR sensorgrams (top graph) and parameters (bottom table) of (RXR)₄ PPMO (red) or R₈ PPMO (blue). Biotin conjugated heparin sulfate was immobilized onto a streptavidin sensor chip and the binding parameters of PPMO to the heparin sulfate were determined using BioCore T-100 instrument and its software (GE Healthcare).

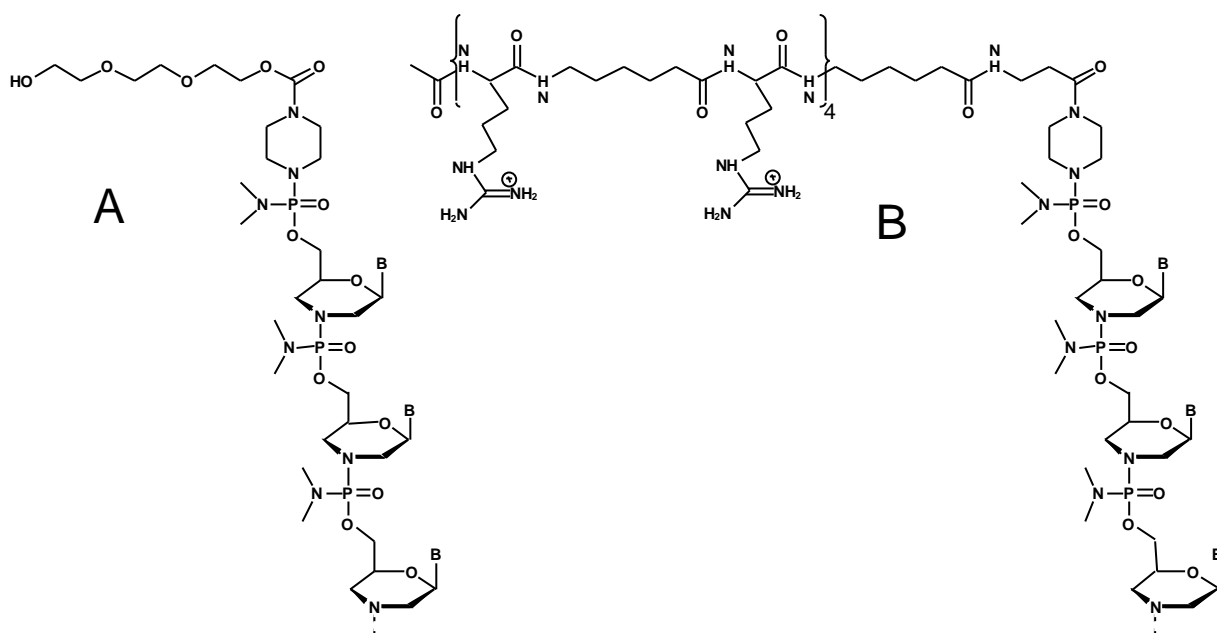
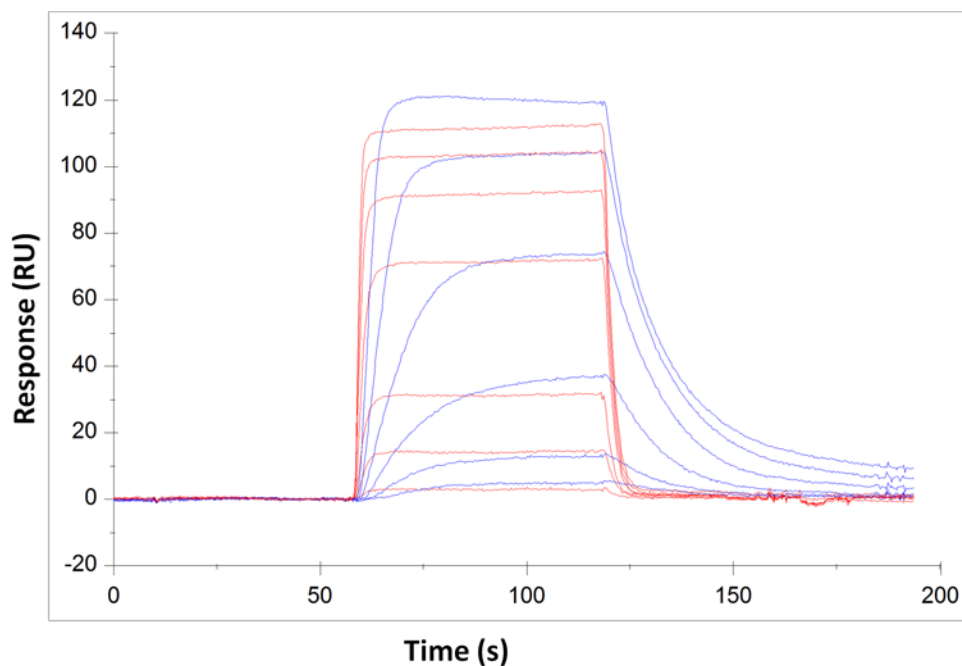


Figure 1. Chemical structures of (A) Morpholino and (B) (RXR)₄XB CPP Morpholino conjugate (PPMO).



Heparin Sulfate Binding	(RXR) ₄ PPMO	R ₈ PPMO
Stoichiometry	175	171
K _{on} (M ⁻¹ S ⁻¹)	4.4 x 10 ⁵	8.1 x 10 ⁵
K _{off} (S ⁻¹)	0.34	0.06
KD (nM)	762	77

Figure 2. Heparin sulfate binding SPR sensorgrams (top graph) and parameters (bottom table) of (RXR)₄ PPMO (red) or R₈ PPMO (blue). Biotin conjugated heparin sulfate was immobilized onto a streptavidin sensor chip and the binding parameters of PPMO to the heparin sulfate were determined using BioCore T-100 instrument and its software (GE Healthcare).