Review

Morpholinos and their peptide conjugates: Therapeutic promise and challenge for Duchenne muscular dystrophy

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Abstract

Exon-skipping efficacies of phosphodiamidate morpholino oligomers (PMOs) or the conjugates of PMOs with cell-penetrating peptides (PPMOS) have been tested in various animal models of Duchenne muscular dystrophy (DMD), including mdx mice, utrophin-dystrophin double-knockout mice, and CXMD dogs, as well as in DMD patients in clinical trials. The studies have shown that PMOs can diffuse into leaky muscle cells, modify splicing of DMD transcripts, induce expression of partially functional dystrophin, and improve function of some skeletal muscles. PMOs are non-toxic, with a report of mdx mice tolerating a 3 g/kg dose, and no drug-related safety issue in human has been reported. However, because of their poor cell uptake and rapid renal clearance, large and frequently repeated doses of PMOs are likely required for functional benefit in some skeletal muscles of DMD patients. In addition, PMOs do not enter cardiomyocytes sufficiently to relieve heart pathology, the efficacy of delivery to various muscles varies greatly, and delivery across the tissue of each skeletal muscle tissue is patchy. PPMOs enter cells at far lower doses, enter cardiomyocytes in useful quantities, and deliver more evenly to myocytes both when different muscles are compared and when assessed at the level of single muscle tissue sections. Compared to PMOs, far lower doses of PPMOs can restore dystrophin sufficiently to reduce disease pathology, increase skeletal and cardiac muscle functions, and prolong survival of animals. The biggest challenge for PPMO is determining safe and effective doses. The toxicity of PPMOs will require caution when moving into the clinic. The first PPMO-based DMD drug is currently in preclinical development for DMD patients who can benefit from skipping exon 50.

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Abbreviations: PMO, phosphodiamidate morpholino oligomer; PPMO, cell-penetrating peptide conjugated PMO; DMD, Duchenne muscular dystrophy; RO, retro-orbital; IV, intravenous; IP, intraperitoneal; IM, intramuscular; CK, creatine kinase

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1. Introduction

1.1. Phosphodiamidate morpholino oligomers (PMOs)

Antisense oligos offer a promising therapeutic strategy for treatment of the human genetic disease Duchenne muscular dystrophy (DMD). Antisense oligos are used to bind with RNA and modify gene expression by base-pairing to complementary base sequences. Phosphodiamidate morpholino oligomers (PMOs) are uncharged antisense molecules, with structures analogous to nucleic acids but with the bases linked to an unnatural backbone [1]. Each base is bound at the 2 position of a 6-methylene morpholine ring, and the methylene is also bound to a dimethylamino phosphorodiamidate group linked to the nitrogen of the next morpholine ring (Fig. 1). PMOs are typically 18–30 bases in length. They are stable in cells, and no enzyme is known to degrade them [2,3]. PMOs have been broadly adopted by developmental biology researchers to knockdown gene expression in embryos of model organisms such as zebrafish and Xenopus. Specificity and lack of immune response are critical characteristics for oligos to be used in humans; the successful and widespread use of PMOs for knockdowns in exquisitely sensitive embryonic systems shows that they are a good choice for further testing as human therapeutics [4–6].

PMOs regulate gene expression through a steric blocking mechanism. Bound to complementary sequences of RNA, the PMOs get in the way of processes that would normally take place at those sequences. By selecting the appropriate target sequence, PMOs can get in the way of proteins or snRNPs involved in splicing of pre-mRNA and this can be exploited to redirect splicing. Most commonly, splice modification results in exclusion of an exon from the mature mRNA. A forefront clinical application of PMOs is as a splicing modulator for treatment of Duchenne muscular dystrophy (DMD).

Fig. 1. Chemical structures of PMO (left) and the (RXRRBR)2-PMO (right). X = 6-aminohexanoic acid and B = beta-alanine.

1.2. Duchenne muscular dystrophy

DMD is a disabling, progressive, and deadly X-linked disease that affects 1 in 3500–6000 male births [7]. Symptoms usually appear in boys aged 1 to 6, and most boys are confined to a wheelchair at 12 years old and die as early as teenage. Respiratory complications and heart failure are common causes of death. There is no effective treatment today. DMD is caused by deficiency of dystrophin, a vital part of a protein complex that connects the cytoskeleton of a muscle fiber to the surrounding extracellular protein matrix through the cell membrane. The dystrophin deficiency is caused by mutations in the human dystrophin gene (DMD) [8], a very large and mutation-prone gene, comprised of 79 exons. Common mutations causing DMD are deletions, insertions, and nonsense mutations. Deletions or insertions of numbers of protein-coding bases that are not evenly divisible by three cause downstream sequence to be frameshifted during translation.

Sometimes, deletion of an exon can stabilize a DMD transcript by removing a mutant stop codon with the exon or by restoring the reading frame of the downstream protein-coding sequence. The resulting transcript contains an internal deletion but might still encode a functional form of dystrophin. Internal in-frame deletions in DMD occur naturally, giving rise to populations with milder allelic forms of muscular dystrophy including Becker muscular dystrophy [7]. Becker muscular dystrophy (BMD) affects 1 in 20,000 men, causing muscle wasting. However, these patients experience a less severe phenotype than the DMD patients, ranging from mild to moderately severe [9], with life spans ranging from nearly normal to deaths in their forties. Unlike in DMD patients who have undetectable or very low amount of dystrophin (<5% of wild type), protein is more readily detectable in BMD patients by Western blot although the size and amount vary among BMD patients. The amount of dystrophin has been correlated to the severity of phenotype, with >20% of wild-type concentration occurring with mild phenotypes and 10–20% occurring with moderate phenotypes [9].

Molecular analysis of nucleic acids revealed that the majority of the BMD patients have in-frame mutations; the resulting dystrophin proteins are semi-functional [10,11].

A strategy for the treatment of DMD is to turn the severe DMD phenotype into mild to moderate BMD phenotype by removing the exon(s) that disrupt the reading frame, producing internally truncated but semi-functional dystrophin protein [12,13]. A PMO can be targeted to an RNA sequence involved in splicing in order to force deletion of an exon. Splice-modifying PMOs have been shown to induce expression of modified forms of dystrophin in mice [14], dogs [15] and humans [16]. The conjugates of PMOs with cell-penetrating peptides (CPPs) have been shown to effectively restore dystrophin in dystrophic mice [17–19] and cause targeted exon skipping in monkeys [20]. One of the peptide–PMO conjugates (PPMOs, Fig. 1) is in preclinical development for treatment of DMD patients who can benefit from skipping exon 50 [20].

In this article, we will first review the DMD studies with PMO and PPMO chemistries, focusing on systemic use of these molecules. We will then discuss the promises and challenges of PMO and PPMO chemistries as DMD therapeutics.

2. PMO for DMD

2.1. Systemic delivery of PMOE23 in the mdx mouse model

mdx Mice carry a mutation in dystrophin exon 23 introducing a stop codon. The mice have undetectable levels of dystrophin besides a
few revertant fibers that can be detected by immunofluorescence staining. The dystrophin band is totally absent on an immune 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. Skipping exon 23 maintains the reading frame of downstream sequence, allowing expression of an in-frame protein product with the polypeptide moity encoded by exon 23 deleted. A 25-base PMO sequence complementary to the splice donor site, the e23i23 junction (PMOE23), has been used by several groups and showed various degrees of efficacy with different dose regimens [14,21,22].

Lu and colleagues were first to demonstrate the systemic utility of PMOE23. PMOE23 was administered at 100 mg/kg to 6-week-old mdx mice by tail vein injection [14]. Single doses, three doses at weekly intervals, and seven doses at weekly intervals were tested, and mice were sacrificed 2 weeks after their final doses. All dosing schedules resulted in increased indicators for targeted exon skipping and new dystrophin expression in skeletal muscles: increased exon-23-skipped RNA (assayed by RT-PCR), increased dystrophin (assayed by Western blots), and an increase in the number of fibers visibly stained for dystrophin (assayed by immunohistochemistry). These indicators increased with the number of injections. Among the nine muscle types evaluated, the exon-skipping effect of the PMOE23 is more prominent in quadriceps, abdominals, intercostals, and gastrocnemii; less prominent in diaphragm, biceps, triceps, and tibialis anterior (TA); and absent in heart. Compared to wild-type concentration of dystrophin transcript, exon-23-skipped RNA transcript at about 50% concentration was detected in gastrocnemii, quadriceps, and abdominal and intercostal muscles after seven injections.

Western blots revealed up to 5% of normal dystrophin concentration in intercostals and quadriceps after a single injection; about 20% of normal levels in quadriceps, intercostals, and abdominal muscles after three injections; and up to 50% of normal levels in gastrocnemii and quadriceps after seven injections. A dystrophin protein band was barely detected from the diaphragm and totally absent from the heart. Immunofluorescent staining of muscle sections revealed that the fraction of fibers staining positive for dystrophin increased with dose and the intensity of the dystrophin signal became more evenly distributed as the dose increased; however, even after seven injections, the muscle sections appeared mottled with areas of high and low staining, obvious when compared with the even staining of healthy control sections. The 7 weekly PMO treatments resulted in reduction of the serum CK levels and increased tetanic force of the tibialis anterior muscle.

Comparison of single injections versus repeated injections at lower doses shows that while the repeated lower doses restored dystrophin expression more evenly across the muscle sections, the number of centrally located nuclei was reduced more effectively by single larger doses. Apparently, while the repeated low doses reached more cells, the higher dose is needed to deliver sufficient oligo to rearrange the cell morphology [15,22]. This study showed that expression of sufficient dystrophin to enable detection by immunohistochemistry does not prove that there is enough dystrophin to ameliorate cellular pathology, and while increases in the count of fibers staining for dystrophin may present information about evenness of uptake into cells, it is not sufficient information to predict therapeutically useful doses of oligo.

2.2. Systemic delivery of PMOs in the CXMD dog model

Dogs carrying a mutation that is a model of Duchenne muscular dystrophy, canine X-linked muscular dystrophy (CXMD) beagles, were treated systemically by IV infusion with PMOs. The CXMD mutation causes exon 7 to be skipped, causing frameshift of downstream sequence, which brings a premature stop codon in-frame. The reading frame can be restored by skipping exons 6 and 8. A cocktail of three oligos was found effective in vivo; these oligos targeted an exonic site in exon 6, the splice donor site of exon 6, and an exonic site in exon 8. The cocktail also caused some excision of exon 9. Whether only exons 6 and 8 or all of exons 6, 8 and 9 were excised from the CXMD transcripts, the RNA was translated to an in-frame protein product with an internal peptide sequence deleted [15].

Systemic exon-skipping PMO treatment was tested on three CXMD dogs, ranging from 2 to 5 months old. PMO cocktail solution was injected through a 22-gauge catheter into the saphenous vein. Various dose regimens were tested, with equimolar doses of all three sequences in injections ranging from 26 to 62 ml. The 5-month-old dog was injected weekly five times at 120 mg/kg (40 mg/kg each sequence). One of the 2-month-old dogs was injected biweekly seven times at 120 mg/kg, and the other 2-month-old dog was injected weekly seven times at 200 mg/kg (66 mg/kg each sequence). Dogs were sacrificed 2 weeks after the final injection.

New dystrophin expression was found by immunofluorescence in all skeletal muscles assessed. However, the amount of dystrophin expression varied, with immunoblots showing dystrophin ranging from trace to 50% of normal concentrations. In skeletal muscles, amount of dystrophin expression was not correlated with fiber type and contralateral muscles often differed in dystrophin level. Similar to the mouse study, the exon-skipping efficiency of the PMOs varied by muscle types. The dog given seven weekly treatments of 200 mg/kg had the highest dystrophin levels, with dystrophin restored to 26% of the wild-type level in three out of the eight skeletal muscles analyzed. Increased dystrophin was detected in small patchy areas of the heart by immunofluorescence but was not detected by Western blot.

Some improvement in muscle function was observed for these dogs. The treated dogs ran faster than their littermates. Muscle inflammation and CK levels were reduced, indicating reduction in disease pathology.

2.3. Intramuscular injection of AVI-4658 in humans

AVI-4658 is a PMO targeted to skip exon 51 of the human dystrophin gene and is currently in a clinical trial for systemic treatment of DMD (NCT00844597). Results of a previous trial (NCT00159250), involving local injections of AVI-4658, have been published [16]. In that trial, seven DMD boys were treated. Each patient had a deletion somewhere upstream of exon 50 and extending into or across exon 50; in each case skipping exon 51 was predicted to restore the reading frame. The patients received intramuscular (IM) injections in the extensor digitorum brevis (EDB) of their feet, with AVI-4658 into one foot and the saline vehicle control into another foot. Two patients received AVI-4658 at 0.09 mg, and five patients received 0.9 mg, in each case dissolved in 900 μl saline. The first five patients (two at low dose, three at high dose) were treated once each with nine injections of 100 μl per injection, administered simultaneously in a 1-cm² grid using a 22-gauge multiple needle array. The remaining two patients were treated once with four 225 μl injections simultaneously in a 1-cm² grid using a similar needle array. The muscles were biopsied between 3 and 4 weeks post-injection, with most of the EDB removed.

Exon skipping was qualitatively detected by RT-PCR and Western blot. RT-PCR products at the mass expected for successful skipping of exon 51 were detected in treated muscles of all seven patients. Western blots showed some new dystrophin protein expression. Some fibers in the biopsy section appeared dystrophin-positive, and others appeared dystrophin-negative, so it was decided that quantitation should rely on a fiber-by-fiber count of dystrophin signal instead of the “averaged” quantity that would result from Western blot or qRT-PCR of batches of cells.
The quantitative assessment of the restoration of dystrophin expression was based on immunohistochemical staining of muscle fibers and assessment of the fluorescence of the stained fibers. Distribution of the dystrophin within the muscle fibers was also observed, with the dystrophin signal in high-dose treated muscles appearing localized at the sarcolemma as it is in normal (wild-type) muscle. Muscles treated with AVI-4658 were compared with saline-treated control muscles and normal muscle to determine the percent restoration of dystrophin in the treated muscle. Fluorescent signals were normalized to signals from a β-spectrin antibody.

The signal from normal (wild-type) fibers was taken as 100% signal intensity. In the sections from the patient whose muscles produced the brightest staining, the bright dystrophin-positive fibers averaged 42% of the dystrophin signal intensity of wild-type muscle fibers while dim fibers averaged 11% of wild type. Across the population of high-dose patients, the average dystrophin intensity was 26.4%. The dystrophin signal of the treated muscle sections was fairly uneven, with unstained or poorly stained fibers visible in many samples. Among the five high-dose patients, between 44% and 79% of the fibers were counted as dystrophin-positive. Though these patients underwent local injections, most of the published immunostained images of PMO-treated human DMD muscle sections show patchiness of muscle fiber staining, similar to the images from the muscles of the mouse and dog DMD models after systemic PMO treatment.

While immunofluorescent signals can indicate concentration trends, the dystrophin intensities from immunohistochemistry do not necessarily correlate with absolute dystrophin concentrations, as factors such as variations in thickness of tissue slice, fluorescence quenching at high fluorochrome concentrations, and nonlinearity of detector sensitivity can cause estimates of concentration by immunofluorescence vs. measurements by Western blot to yield differing results.

3. CPPs-mediated systemic delivery of PMOs for DMD

Published to date, five different peptides each conjugated to PMOE23 have been tested for their systemic utility for treatment of DMD in mouse models and one of them, (RXRBR)2, conjugated to a PMO targeted to exon 50, has been tested in healthy monkeys.

3.1. (RXR)4 Peptide

The first delivery moiety tested in mdx mouse model was the (RXR)4 peptide where X = 6-aminohexanoic acid. Its PMO conjugate, PMOE23, was first administrated through the intraperitoneal (IP) route [23]. Effects of the PMMO dose levels and the age of animals on exon skipping were determined. Animals started treatment with the same dose at 1 day, 4 weeks, or 1 year of age. While splice-modified dystrophin expression increased for all ages treated, treatment at younger age was clearly more beneficial as the older animals had more established pathology. A single-dose study in neonatal mice was done to determine the dose and activity relationship with the PMPO at 1, 2, 5, 10, and 25 mg/kg. Immunofluorescent detection of dystrophin clearly showed a dose-dependent relationship, with higher levels of dystrophin restoration observed at higher doses. Four doses of the PMMO at a lower dose, 5 mg/kg per dose, administrated once a week for 4 weeks led to near normal levels of dystrophin in diaphragms at 6 weeks of age. The PMMO effect was more modest in the tibialis anterior (TA), gluteus maximus, and triceps brachialis: weak in the colon and stomach; and absent in the heart. At 22 weeks after the fourth injection, dystrophin was still detected in the diaphragm but was discontinuous with some disruption of muscle architecture. Peptide-related toxicity was not observed in the 5 mg/kg dose regimen.

In another study, the same PMMO was IV injected to mdx mice [19]. Effect of dose regimen on dystrophin restoration was determined in 6- to 8-week-old mdx mice. PMMO was injected once at 25 mg/kg into the tail vein. Widespread, uniform expression of dystrophin protein over multiple tissue sections within seven muscle types was detected in hind limb, fore limb, abdominal wall, and diaphragm muscles and, importantly, in cardiac muscle. RT-PCR analysis showed nearly total exon skipping of the mutated RNA transcript in the skeletal muscles and 50% skipping in heart. Analysis of the muscles by immunoblotting indicated that dystrophin was restored at 25–100% of wild-type levels in skeletal muscles and at 10–20% of wild-type level in cardiac muscle. The muscle pathology was decreased and function improved as indicated by reduction of CK, detection of several proteins associated with the dystrophin-associated protein complex (DAPC), and enhanced grip strength. Three weekly IV injections of the PMMO at 6 mg/kg also induced exon skipping in these muscles, but this dose regimen was less effective, especially in heart, than the single 25 mg/kg dose.

The PMMO was further tested in the double-knockout (dKO) mouse model that lacks both dystrophin and utrophin. The mice carry the same mutation at exon 23 as the mdx mice in addition to another mutation disabling utrophin. Unlike mdx mice that have near normal life span with an average survival of 18 months, the dKO mice have an average survival of 8.2 weeks with much more severe and progressive dystrophic phenotypes including abnormal waddling gait, contracted and stiff limbs, very pronounced kyphosis, and limited mobility. dKO mice may be a more appropriate model to determine the effectiveness of the potential of DMD therapeutics. Two dose levels of the PMMO were tested by IP route of administration. The higher dose at 25 mg/kg/week for 6 weeks started at 10 days of age. Treated mice appeared healthy and survived until they were sacrificed for analysis at 13 weeks of age, 6 weeks after the last injection [24]. This survival duration was never seen in untreated dKO mice. Dystrophic phenotypes were significantly improved. Both RT-PCR and Western analysis showed nearly complete exon skipping and wild-type level of dystrophin in diaphragm and high levels in other skeletal muscles but much less in the cardiac muscles. Dystrophin distribution was uniformly detected in the fibers of all analyzed muscles except for cardiac muscles. However, the lower dose at 5 mg/kg was not sufficient to rescue the severe pathology of the dKO mice; treated mice exhibited no improvement in dystrophic phenotype and survival.

3.2. (RXRBR)2 Peptide

This is an analog of the (RXR)4 peptide with two of the 6-aminohexanoic acid residues replaced with β-alanine. This peptide was found to be less toxic to cells than the parent (RXR)4 peptide [25]. The utility of this peptide to deliver PMOs has been tested in mdx mice, in wild-type mice, and in non-human primates. Batteries of tests for exon-skipping efficiency were performed as was done for the (RXR)4 PMMO. The sequence specificity of (RXRBR)2–PMOE23 was investigated in mdx mice as well as its utility to improve cardiac function.

A single IV injection through a retro-orbital (RO) route of the PMMO 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% 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. Six biweekly treatments over 3 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. The same peptide conjugated to a PMO with scrambled sequence was used as a control; when tested in mdx mice, it did not cause exon skipping. Toxicity and immune response were assayed for but not observed [17].
The first study showing a PPMO-improved heart function of mdx mice employed a hemodynamic model [17]. Eight mdx mice were treated twice with the PPMO at 30 mg/kg with a 2-week interval. Three weeks after the second treatment, the PPMO-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 10 wild-type mice and all eight PPMO-treated mdx mice survived the challenge, while only 3 of 9 untreated mdx mice survived the challenge. In addition, the cardiac parameters, including end-diastolic volume, end-systolic pressure, dp/dtmax, and cardiac pressure/volume loops of PPMO-treated mdx mice were similar to the wild-type mice. The untreated mdx mice had significantly different values, indicating cardiac dysfunction.

Improvement of heart function by PPMO treatment was later demonstrated in another study without using the hemodynamic model [26]. This study demonstrated the long-term benefit of PPMO treatment prior to the development of cardiomyopathy. The effect of the PPMO was investigated by treating 16-week-old mdx mice, the age just before the development of cardiomyopathy in mdx mice. The PPMO conjugate was injected IV at 12 mg/kg in two cycles of four once-daily injections with a 2-week interval. A sham PPMO targeted to an intron of the unrelated human β-globin gene was used as the control. Cardiac CK-MB, a hallmark for cardiomyopathy, was significantly reduced, reaching wild-type concentration 5 weeks after the first injection. Dystrophin restoration was found throughout the heart of treated mdx mice. Dystrophin correctly localized at the sarcolemma of cardiac muscles and its concentration as measured by Western blot was about 30% of wild-type level. 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. In addition, the PPMO treatment prevented the mdx age-associated development of diastolic dysfunction as shown by improvement in the E and A waves from Doppler echocardiography compared to the sham-treated mice. The same PPMO treatment was applied to a group of 8-week-old mdx mice with saline-treated mdx mice as the control. Seven months after the treatment, the effect of PPMO relieving cardiac hypertrophy was still detectable as indicated by lower left ventricle mass to body mass ratio and thinner anterior and posterior walls compared to the sham-treated mice. Although dystrophin level was not determined for this time point, it is expected that dystrophin is not detectable 7 months after the treatment. An earlier study with one cycle of four once daily injections showed that dystrophin persisted in the hearts of mdx mice for about 11 weeks after dosing [27]. The 7-month study showed that even after dystrophin no longer persists in the heart, earlier PPMO intervention can potentially reduce cardiomyopathy.

AVI-5038 is a (RXRRBR)2 peptide-conjugated PMO targeted to skip human exon 50. This PPMO is currently in preclinical development for DMD patients who can benefit from the removal of exon 50. Initial efficacy of the PPMO has been demonstrated in healthy cynomolgus monkey. With once-weekly IV injection for 4 weeks at 9 mg/kg, the PPMO induced an average of 40%, 25%, and 2% exon-skipped product in diaphragm, quadriceps, and heart (respectively) of four monkeys [20]. The level of exon skipping is probably an underestimate because in a healthy monkey, the exon-skipped product is out of frame and undergoes nonsense-mediated decay. 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.

3.3. **ASSLNIAX and (RXRRBR)2-ASSLNIAX chimeric peptides**

Despite PPMOs' efficacy in the muscles as demonstrated above, the majority of (RXR)2 PMO in normal mice [28] or (RXRRBR)2 PMO in mdx mice (manuscript in preparation) were detected in livers and kidneys, with relative small amounts taken up by muscles. Using a muscle-targeting ligand may increase muscle uptake of a PMO. ASSLNIAX was identified as a muscle-specific peptide (MSP) by phage display screening of a peptide library [29]. Phage carrying this peptide had a higher selectivity for muscles than for the liver, kidney, or brain. The MSP–PMO conjugate, tested in mdx mice, was found to have activity slightly higher than PMO, but much lower than the (RXRRBR)2 PPMO [18]. These data are not unexpected as the MSP may direct more PMO to muscles but it lacks the “cell-penetrating” moiety of the (RXRRBR)2 peptide so very little PMO was available for the RNA target in the cytosol/nuclei of cells. However, tissue distribution analysis showed that the kidney and liver of mice are still two major organs targeted by the MSP–PMO conjugate (data not shown), suggesting that the selectivity of the MSP peptide is significantly reduced when it carries a PMO cargo.

Two chimeric peptides with MSP and (RXRRBR)2 combinations were designed to assess the position and effect of MSP on the exon-skipping efficacy of the PPMO. MSP–(RXRRBR)2–PMO construct had the cell-penetrating peptide positioned between the MSP and the PMO. This construct was less effective than the parent PPMO, (RXRRBR)2–PMO, as demonstrated by immunofluorescence, Western blot, and RT–PCR assays. The second construct, (RXRRBR)2–MSP–PMO, had the MSP positioned between the CPP and the PMO. This construct gave 2– to 5-fold (depending on the tissue type) higher exon-skipping activity in the skeletal muscles with no improvement in cardiac muscles compared to (RXRRBR)2–PMO. Tissue distribution analysis showed that there was little improvement of muscle selectivity with either of the chimeric peptides and the majority of the materials were still found in kidneys followed by livers (data not shown). Reduced activity of the first construct is likely caused by decreased internalization of the conjugate, probably because the position of the MSP inhibited the interaction of the CPP with cell membranes. Despite lack of evidence for its intended muscle selectivity, the (RXRRBR)2–MSP–PMO improved exon-skipping efficacy over the parent PPMO, which may reduce the therapeutic dose and dose frequency for treatment of DMD [18].

4. Discussion and conclusion

4.1. **PMO: promise and challenge for DMD**

The systemic utility of PMO chemistry for the treatment of DMD has been demonstrated in mice and dogs without safety issues. The AVI-4658 intramuscular trial provided a proof-of-principle efficacy study in human. A systemic trial of AVI-4658 PMO in humans is currently ongoing, and the data should be available in 2010. At the time of this article, PMO targeted to exon 51 has been dosed in humans up to 20 mg/kg once a week for 12 weeks and so far there are no drug-related safety issues. Mouse dosed up to 3 g/kg of the PMO at 2300 H.M. Moulton, J.D. Moulton / Biochimica et Biophysica Acta 1798 (2010) 2296–2303.
from mouse to dog as both required doses $\geq 100$ mg/kg, despite the difference in body surface area between mouse and dog [14,15]. Dose scaling across species by mass instead of by body surface area has been shown for other antisense oligos [33]. In addition, the target tissues for a DMD drug are the muscles throughout the whole body, which requires more drug than a single tissue type. We expect that large and repeated doses of PMO, as used in mouse and dogs, will be required in order to attain therapeutic effects for DMD patients. If so, the cost of a PMO drug for life-long treatment will probably be prohibitive for many patients.

The second problem with PMOs is the large variability in exon-skipping effect among muscle types and within a specific muscle. Some skeletal muscles responded to PMO better than the others, as shown in both the mouse and dog studies. Even within these responsive muscles, dystrophin was not uniformly expressed, varying from muscle section to section and within a section, with detection of patches of dystrophin-positive fibers and patches of dystrophin-negative fibers [14,15,17]. This may be because only leaky muscle cells can take in PMO. Therefore, PMO’s efficacy in humans will depend on the number of leaky muscle fibers and how well these leaky fibers can carry out the necessary biological processes to restore the dystrophin and its associated protein complex.

The last problem but not the least important is that PMO has shown very little efficacy in the hearts of the mouse and dog models unless a huge dose is used. The PMOE23 restored about 5% and 30% of wild-type level of cardiac dystrophin at 300 and 3000 mg/kg dose, respectively [30]; at a dose of 3000 mg/kg, 0.3% of the organism is PMO by mass and such an extreme treatment would be unsustainable long term. Increased skeletal muscle mobility with the PMO treatment may increase workload for the hearts of the patients [34,35]. Clinically relevant cardiomyopathy is observed in 95% of DMD patients, and 20–30% of the deaths are primarily attributable to heart disease [36]. Therefore, a safe and effective delivery strategy is needed that will reduce dose level and dose frequency, will deliver the PMO to non-leaky muscle fibers, and will deliver the PMO to the heart.

4.2. PPMO: promise and challenge for DMD

PPMOS are much more effective than PMOs because of the ability of CPPs to increase the volume of distribution of PMOs [28] to whole-body muscles and to facilitate the internalization of PMO through an active process, unlike the passive diffusion process for PMO. PPMOs are internalized by nearly all muscle cells; their delivery is not limited to leaky muscle cells as are PMOs. With 2 µg of PMO or PPMO injected intramuscularly to the TA muscles of mdx mice, 14% of the fibers were dystrophin-positive after PMO treatment versus the 85% of fibers that were positive after PPMO treatment [17]. This finding is verified by a study using systemic tail vein injection [37,38]. A single 12 mg/kg injection of the (RXRRBR)$_2$-PMOE23 resulted in restoration of dystrophin in nearly 100% of the fibers of the quadriceps muscles, while only a small percentage of dystrophin-positive fibers were detected after administration of the PMOE23 at 400 mg/kg (Fig. 2A). Uptake of PPMO does not depend on the leakiness of fibers, as demonstrated by the healthy monkey study of AVI 5038 [20] as well as by the study shown in Fig. 2B [37,38], comparing the efficacy of PMO and PPMO in wild-type C57 mice. With 6 weekly IV injections of 20 mg/kg each, >95% exon-skipped RNA transcript was detected in the skeletal muscles after PPMO treatment while very little exon-skipped transcript was detected for the PMO treatment even at a 40-fold higher dose (6 x 800 mg/kg) (Fig. 2B).

**Fig. 2.** Comparison of exon-skipping efficacy of PMOE23 and PPMOE23 in mdx mice and in wild-type C57 mice. (A) Seven- to eight-week-old mdx mice were injected with PMOE23 at 400 mg/kg (right) or the (RXRRBR)$_2$-PMOE23 conjugate at 12 mg/kg (left) through tail veins and the mice were sacrificed 1 week after the injection. The quadriceps were sectioned and stained for dystrophin according to an immunofluorescent method described elsewhere [14] (B). Twelve-week-old wild-type C57 mice were injected with PMOE23 at 800 mg/kg or the (RXRRBR)$_2$-PMOE23 conjugate at 20 mg/kg once a week for 6 weeks through tail veins and the mice were sacrificed 1 week after the injection. Muscles were taken for RT-PCR analysis according to a method described elsewhere [27]. Three mice were used for each treatment. Each lane represents RNA sample isolated from a muscle of individual mouse. The top and bottom bands in the lanes represent the unskipped and skipped (removal of exon 23) transcripts, respectively.
PMMO-treated mdx mice exhibited widespread and uniform dystrophin expression in the hearts of mdx mice, with nearly 100% of the mdx muscle fibers immunostaining positive for dystrophin with the treatment at 30 mg/kg [17]. A single tail vein injection at 15 mg/kg of the (RXRRBR)2-PMOE23 resulted in cardiac dystrophin restoration at 11% of wild-type level as measured by Western blot and nearly 100% of the cardiac myocytes of the mdx mice immunostained positive for dystrophin [37–39]. Improvement in heart function with the PMMO treatment has been demonstrated [17,26]. These studies indicate that PMMOs can be used at a much lower dose than PMOs and can achieve more widespread exon skipping throughout the whole body's muscles including the heart.

The toxicity of current PMMO chemistry poses a challenge for determination of an effective and safe dose regimen in human. The nature of the toxicity is not well understood. Likely it is due to the cationic nature of the peptide. There seems to be a dose threshold for the toxicity, and the threshold level depends on the amino acid composition of a peptide. Below the threshold, toxicity was not observed. Above it, the severity of toxicity is dose-dependent. Toxicity includes lethargy, weight loss [28] and tubular degeneration in kidney [20]. The severity of toxicity is also dependent on the dose frequency. Animals can tolerate doses and recover better if infrequent dose schedules are followed. For a given peptide, the threshold level varies among the animal species, with monkeys seeming more sensitive to the PMMO-related toxicity than mice. (RXRRBR)2-PMOE23 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 3 months [17]. However, the same peptide conjugated to the PMO targeted to human exon 50, AVI-5038, was found to cause mild tubular degeneration in the kidneys of monkeys at 9 mg/kg weekly injections for 4 weeks [20]. This indicates that PMMO toxicity is observed at lower dose level in monkeys than in mice, although the change in the PMO sequence means the antisense component might contribute to toxicity in the monkey. For instance, skipping exon 23 does not frameshift downstream dystrophin, while skipping exon 50 in healthy animals does; however, 4 weeks is not enough time to deplete all the preexisting dystrophin, and loss of dystrophin would not be expected to severely damage kidney. In addition, if there is a sequence related off-target effect, it will contribute to the observed toxicity [40].

The efficacious dose of a PMMO is also likely scaled by body weight from mouse to monkey to human. The PMOE23 at 20 mg/kg induced nearly 100% exon-skipped transcripts in the quadriceps and diaphragms of wild-type mice. The monkey equivalent dose scaled by surface area at 3 mg/kg of AVI-5038 had little exon-skipping effect in those muscles and a higher dose at 9 mg/kg caused 25–40% exon-skipped transcript in those muscles of monkeys [20]. Of course, the observation here may not be determined solely by dose scaling but also influenced by the exon-skipping efficiency of the PMO; this in turn be affected by the mechanism and regulation of pre-mRNA processing of the individual targeted exon, which may vary with the species.

In conclusion, the PMMOs have potential as DMD therapeutics. One advantage for PMMO DMD therapeutics is the long half-life of dystrophin and long tissue residence time of PMMOs [37–39]. Restoration of dystrophin in the majority of muscle fibers at the intensity of 20% of wild type will significantly reduce the DMD disease pathology [9]. These factors permit an infrequent dosing schedule to minimize toxicity in patients. Success will depend on well-designed preclinical studies to address the relationship between dose regimens, route, and rate of drug administration, to predict exon-skipping efficacy/toxicity across species and to plan efficiently yet safe dose escalation in patients.

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