Exon skipping is capable of correcting frameshift and nonsense mutations in Duchenne muscular dystrophy. Phase 2 clinical trials in the United Kingdom and the Netherlands have reported induction of dystrophin expression in muscle of Duchenne muscular dystrophy patients by systemic administration of both phosphorodiamidate morpholino oligomers (PMO) and 2′-O-methyl phosphorothioate. Peptide-conjugated phosphorodiamidate morpholino offers significantly higher efficiency than phosphorodiamidate morpholino, with the ability to induce near-normal levels of dystrophin, and restores function in both skeletal and cardiac muscle. We examined 1-year systemic efficacy of peptide-conjugated phosphorodiamidate morpholino targeting exon 23 in dystrophic mdx mice. The LD50 of peptide-conjugated phosphorodiamidate morpholino was determined to be approximately 85 mg/kg. The half-life of dystrophin expression was approximately 2 months in skeletal muscle, but shorter in cardiac muscle. Biweekly injection of 6 mg/kg peptide-conjugated phosphorodiamidate morpholino produced >20% dystrophin expression in all skeletal muscles and ≤5% in cardiac muscle, with improvement in muscle function and pathology and reduction in levels of serum creatine kinase. Monthly injections of 30 mg/kg peptide-conjugated phosphorodiamidate morpholino restored dystrophin to >50% normal levels in skeletal muscle, and 15% in cardiac muscle. This was associated with greatly reduced serum creatine kinase levels, near-normal histology, and functional improvement of skeletal muscle. Our results demonstrate for the first time that regular 1-year administration of peptide-conjugated phosphorodiamidate morpholino can be safely applied to achieve significant therapeutic effects in an animal model. (Am J Pathol 2012, 181:392–400; http://dx.doi.org/10.1016/j.ajpath.2012.04.006)

Duchenne muscular dystrophy (DMD) is the most common and lethal muscle disorder with onset in early childhood, and no effective treatment is available. DMD is caused mainly by nonsense and frameshift mutations of the dystrophin gene (DMD), leading to the lack of functional dystrophin protein in muscle. Becker muscular dystrophy (BMD), an allelic form of DMD, is caused by mutations that typically create shortened but in-frame transcripts with production of partially functional dystrophin; in BMD, patients have variable or no symptoms.1–3 Antisense oligomer-mediated exon skipping has been demonstrated to have high potential for open reading frame restoration of the dystrophin gene and for induction of BMD-like functional dystrophin; significant progress in exon skipping for DMD has been made.4–28 Therapeutic potential of exon skipping was first demonstrated in dys-

Supported by the Carolinas Muscular Dystrophy Research Endowment at the Carolinas HealthCare Foundation and Carolinas Medical Center (Charlotte, NC) and US Army Medical Research, Department of Defense (W81XWH-05-1-0616, W81XWH-09-1-0599).

Accepted for publication April 5, 2012.

Disclosure: AVI BioPharma supplied the peptide phosphorodiamidate morpholino oligomer AVI-5038.

Supplemental material for this article can be found at http://ajp.amjpathol.org or at http://dx.doi.org/10.1016/j.ajpath.2012.04.006.

Address reprint requests to Qi Long Lu, M.D., Ph.D., or Bo Wu, Ph.D., McColl-Lockwood Laboratory for Muscular Dystrophy Research, Neuromuscular/ALS Center, Department of Neurology, Carolinas Medical Center, 1000 Blythe Blvd., Charlotte, NC. E-mail: qi.lu@carolinashealthcare.org or bo.wu@carolinashealthcare.org.
trophic mdx mice, a model of DMD, by intramuscular injection.\textsuperscript{12} Since then, systemic efficacy with improvement in muscle function has been demonstrated in dystrophic mouse and dog with phosphorodiamidate morpholino oligomers (PMOs).\textsuperscript{14,16} Phase 1 clinical trials targeting human dystrophin exon 51 demonstrated dystrophin expression in muscle injected with specific antisense oligonucleotide (AO) of PMO and 2′-O-methyl phosphorothiate (2OMePS).\textsuperscript{19,20} In a phase 2a trial, systemic administration of the antisense drug PRO051 (2OMePS) was assessed in DMD patients, with a weekly subcutaneous injection regimen for 17 weeks; exon 51 skipping and increased dystrophin expression were reported in a dose-related manner in all cohorts at doses of 0.5, 2, 4, and 6 mg/kg.\textsuperscript{21} More recently, a phase 2 clinical trial with repeated weekly intravenous injections of PMO targeting the same human dystrophin exon 51 reported encouraging preliminary results. Up to 20 mg/kg per injection of AVI-4658 showed a dose-dependent restoration of dystrophin expression.\textsuperscript{22} At the doses tested, both compounds appeared to be well tolerated, although functional consequences and cumulative effects of long-term treatment remain to be demonstrated.

Use of the compounds PMO and 2OMePS as currently in clinical trials is limited by their low efficiency and high variability in exon skipping and dystrophin induction in all muscles, as revealed from studies in both animal models and the clinical trials. One special concern is the very low efficiency of exon skipping in cardiac muscle, which in boys with DMD is severely affected by the lack of dystrophin expression. Results from animal model studies suggest that a detectable dystrophin induction in cardiac muscle will require biweekly injections of PMO at >60 mg/kg.\textsuperscript{23} This has led to the use of cationic peptides and other polymers to improve the efficiency of PMO delivery.\textsuperscript{15,16,24–28} PMOs conjugated with an arginine-rich peptide (PPMO) were able to restore dystrophin expression to near-normal levels in bodywide skeletal muscle and to approximately 50% in cardiac muscle at a dose of 30 mg/kg by single injection. Such high levels of dystrophin expression significantly improved function of both skeletal and cardiac dystrophic muscle.\textsuperscript{15} However, it is well documented that the use of positively charged peptides and polymers increases toxicity considerably.\textsuperscript{15,16,26–28} This together with the requirement of lifelong AO drug administration for treating DMD necessitates investigation of these modified PMOs for their long-term applicability and efficacy in relevant animal models \textit{in vivo}.

In the present study, we investigated the acute toxicity and dose-related 1-year efficacy of PPMO treatment targeting mouse dystrophin exon 23 systemically in dystrophic mdx mice. Our results show that PPMO has a high level of acute toxicity (the LD$_{50}$ is approximately 85 mg/kg). However, the effective dose for inducing >20% dystrophin in skeletal muscle and 5% dystrophin in cardiac muscle requires only 6 mg/kg biweekly injections with no obvious acute or chronic adverse effects were detected. PPMO could therefore be an effective candidate compound as AO drugs for long-term treatment of DMD.

### Materials and Methods

#### Animals, Oligonucleotides, and in Vivo Delivery Methods

In each treatment group, 10 mdx or C57BL/6 mice aged 4 to 5 weeks were used. Experiments were approved by the Institutional Animal Care and Use Committee of the Carolinas Medical Center. The phosphorodiamidate morpholino oligomer (+07-18) (5′-GGGCAAACTCGGC- TTACCTGAAAT-3′) was used against the boundary sequences of exon and intron 23 of the dystrophin gene and was conjugated to the peptide (RXRRBR),XB (where R is arginine, X is 6-amino-hexanoic acid, and B is β-alanine) through a noncleavage amide linker to form a peptide-PMO conjugate (PPMO) (AVI BioPharma, Bothell, WA). For intravenous administration by retro-orbital injection, the PPMO was used in 100 μL saline; control mdx mice were injected with 100 μL saline only. Mice were sacrificed at various time points, and muscles were snap-frozen in liquid nitrogen–cooled isopentane and stored at −80°C.

#### Antibodies and Immunohistochemistry

Sections (6 μm thick) were cut from at least two thirds of the muscle length of tibialis anterior (TA), quadriceps, biceps, and gastrocnemius muscles at 100-μm spacing and from at least six levels from all other muscles (including cardiac, diaphragm, intercostal, and abdominal muscle) at 100-μm spacing. The intervening muscle sections were collected for Western blot and RT-PCR analysis. The serial sections were stained with rabbit polyclonal antibody P7 against dystrophin. The primary antibody was detected by goat-anti-rabbit IgG Alexa Fluor 594 (Invitrogen-Life Technologies, Carlsbad, CA). Sections were also stained with H&E for histological assessment.

#### Protein Extraction and Western Blot

The collected sections were ground into powder and lysed with 200 μL protein extraction buffer, as described previously.\textsuperscript{15,16} The protein concentration was quantified by a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins were loaded onto a 4% to 15% Tris-HCl gradient gel. Samples were electrophoresed overnight at 10 mA at 4°C and blotted onto nitrocellulose membrane overnight at 50 V. The membrane was then washed and blocked with 5% nonfat milk and probed overnight with monoclonal antibody against the dystrophin rod domain (NCL-DYS1; Vector Laboratories, Burlingame, CA). Antibody against kidney injury molecule-1 (KIM-1; also known as hepatitis A virus cellular receptor 1 homolog, or HAVcr-1) (R&D Systems, Minneapolis, MN) was used for detecting expression of KIM-1 in the kidney. The bound primary antibody was detected by horseradish peroxidase-conjugated goat anti-mouse IgG for dystrophin (Santa Cruz Biotechnology, Santa Cruz, CA) or horseradish peroxidase-conjugated donkey anti-goat IgG for KIM-1 (Millipore, Billerica, MA) and a Western blotting enzymatic chemiluminescence analysis system (Perkin
Elmer, Waltham, MA). The intensity of the bands obtained from the AO-treated muscle was measured and compared with that from normal muscle of C57BL/6 mice (ImageJ software version 1.42; NIH, Bethesda, MD).

RNA Extraction and RT-PCR

The collected sections were homogenized in TRIzol (Invitrogen) in an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany). Total RNA was then extracted, and 100 ng of RNA template was used for a 50-μL RT-PCR with a master mix from USB (Cleveland, OH). The primer sequences for the RT-PCR were Ex20Fo 5’-AGAATTCTGCCAATTGCTGAG-3’ and Ex26Ro 5’-TCTT-CAGCTTGTGTCATCC-3’ for amplification of mRNA from exons 20 to 26. In total, 40 cycles were performed for the RT-PCR. Bands with the expected size for the transcript with deletion of exon 23 were extracted and sequenced. The intensity of the bands was measured with ImageJ software; the percentage of exon skipping was calculation from 6 mg/kg, to determine the LD50. All surviv-

Grip Strength Test

Grip strength was assessed using a grip strength meter consisting of horizontal forelimb mesh and an angled hindlimb mesh (Columbus Instruments, Columbus OH). Five successful hindlimb and forelimb strength measurements within 2 minutes were recorded, and data were normalized to body weight and expressed as kilogram force.

Measurement of Serum Creatine Kinase and Other Components

Mouse blood was taken immediately after cervical dislocation and centrifuged at 200 × g for 3 minutes. Serum was separated and stored at −80°C. The level of serum components was determined by Charles Riverside Laboratories International (Wilmington, MA).

Results

LD50 of PPMO in mdx Mice

Our research group previously showed that 30 mg/kg PPMO was able to induce almost normal levels of dystrophin expression in bodywide muscle of mdx mice without clearly observable acute or chronic toxicity, even after six rounds of biweekly intravenous injection. Nonetheless, acute toxicity remains a serious concern, especially because repeated, lifelong administration is required for treating DMD. We therefore first tested the same PPMO targeting mdx mouse exon 23 by dose escalation from 6 mg/kg, to determine the LD50. All surviving mice were sacrificed 2 weeks after injection. In accord with previous report, all mice injected with 6 mg/kg PPMO survived without any observable difference from saline-injected control mice. All mice receiving 30 mg/kg PPMO also survived without loss of body weight; the only observable change was reduced activity during the first 2 hours after injection in 3 of the 10 mice in this group. When the dose of PPMO was increased to 60 mg/kg, however, all mice showed reduced activity after recovery from anesthesia. Of the 10 treated mdx mice, 2 died within 5 minutes after the intravenous injection, and 1 died within 24 hours. Further increase in dose to 90 mg/kg PPMO resulted in the death of 5 out of 10 mice within 2 days after injection, and with increase to 120 mg/kg, 8 out of 10 mice died within 2 days (Figure 1). The LD50 of PPMO in mdx mice was thus determined to be approximately 85 mg/kg.

Half-Life of Dystrophin Expression after Single Intravenous Administration of 30 mg/kg PPMO

Our previous study demonstrated that a single intravenous injection of 30 mg/kg PPMO induced 100% and 50% of normal levels of dystrophin expression in bodywide skeletal muscle and cardiac muscle, respectively. To assess the possible half-life of dystrophin expression, we applied the same dose of 30 mg/kg of the PPMO targeting exon 23 by single intravenous injection into mdx mice and examined the levels of dystrophin expression at a series of time points (2 days, 2 weeks, and 1, 1.5, 2.5, 4, and 5 months after the injection) (Figure 2A). At 2 days after intravenous injection, nearly 100% efficiency in exon 23 skipping was detected by RT-PCR (Figure 2B). Dystrophin protein was detected by Western blot at approximately 20% of normal levels (Figure 2, C and D), and ≤50% dystrophin-positive fibers were detected by immunohistochemistry in all skeletal muscles examined (Figure 2A). In cardiac muscle, ≤40% efficiency in exon 23 skipping was detected; however, dystrophin protein was not convincingly detected by Western blot (Figure 2). By 2 weeks, dystrophin expression in skeletal muscle was detected in nearly 100% of muscle fibers, with the protein reaching ≥80% of normal levels. Similar levels of dystrophin protein and percentage of dystrophin-positive fibers were maintained up to 2.5 months after the single injection; however, exon-skipping efficiency was significantly reduced, down to approximately 50% at 2.5 months after the injection. The levels of dystrophin decreased to 30% of normal levels at 4 months after the injection and to 10% at 5 months. In cardiac muscle,
dystrophin expression induced by a single dose of PPMO peaked at 1 month after the injection, reaching approximately 70% of normal levels, and then decreased to <50% at 1.5 months and to <25% at 2.5 months after the injection. No dystrophin was detected at 4 and 5 months in cardiac muscle by either immunohistochemistry or Western blot (Figure 2, A, C, and D). The data thus indicate that PPMO induces dystrophin expression approximately two times more efficiently in skeletal muscle than in cardiac muscle.

The half-life of dystrophin expression was also longer in skeletal muscle than in cardiac muscle. Consistent with our previous results, expression of high levels of dystrophin after a single 30 mg/kg PPMO treatment improved muscle pathology of the \textit{mdx} mice (Figure 3A). The percentage of muscle fibers without central nucleation increased time-dependently from 20% at 1 month after the single-dose treatment to 40% at 4 months, but then decreased to 30% by 5 months in most skeletal muscles, except diaphragm (Figure 3B). No areas of muscle de-
generation or foci of mononucleocyte infiltration were observed in any of the skeletal muscles examined (including TA, quadriceps, biceps, and gastrocnemius) between 2 weeks and 5 months after treatment. In diaphragm, the percentage of fibers without central nucleation also increased at first, reaching their highest level by 2.5 months, but then decreased again by 4 months (Figure 3B). The increase of fibers with central nucleation was associated with an increase in areas with degenerating fibers and foci of mononucleocyte infiltration. Thus, a single dose of PPMO treatment provides a shorter period of protection from degeneration for diaphragm than for other skeletal muscles. Serum creatine kinase (CK) levels were reduced significantly and remained at similar low levels from 1 month to 5 months after the injection (Figure 3C).

Biweekly Administration of 1.5 mg/kg PPMO for 1 Year Produces Limited Dystrophin Induction and Improvement in Muscle Function

The high efficiency of PPMO for dystrophin induction but relatively low LD50 call for a low-dose regimen for treating DMD. We hypothesized that repeated low-dose PPMO administration might be sufficient to induce functionally meaningful dystrophin expression. The mdx mice were therefore treated with PPMO at a dose of 1.5 mg/kg with biweekly intravenous injection for 1 year. Immunohistochemistry for dystrophin expression 2 weeks after the final injection showed <20% of muscle fibers expressing detectable dystrophin, most of them with weak signals in bodywide skeletal muscle (Figure 4A). The amount of dystrophin protein detected by Western blot ranged from 20% to 50% of muscle fibers expressing detectable dystrophin in nearly 100% of muscle fibers in bodywide skeletal muscle (Figure 4A). The amount of dystrophin protein detected by Western blot ranged from 20% to 50% of normal level in any skeletal muscle. This amount of protein was similar to that detected in muscle after a single intravenous injection of 1.5 mg/kg PPMO, suggesting no significant accumulation of protein. RT-PCR showed 15% to 50% exon 23 skipping in skeletal muscle (Figure 4B). The levels of dystrophin expression detected by Western blot ranged from 20% to 50% of normal levels (Figure 4, D and F). Histologically, treated skeletal muscle exhibited clearly detectable improvement, with reduced central nucleation and a more homogeneous population of fibers in size (Figure 5A); however, sporadic foci of degeneration and regeneration and monocyte infiltration remained in all muscles. Significant improvement was also detected in muscle function with grip force generation (Figure 5C). This finding was supported by a significant reduction in CK levels, compared with control mdx mice, although the CK levels were still higher than those of normal C57BL/6 mice (Figure 5D). Expression of dystrophin in cardiac muscle was clearly detectable in a proportion of fibers with weak and discontinuous membrane staining by immunohistochemistry, but was <5% of normal levels by Western blot (Figure 4, D–F). As described above for the group treated with 1.5 mg/kg PPMO, 6 mg/kg PPMO did not show clear differences in body weight, serum enzyme tests, or histological examination of lung, kidney, and liver, compared with control mdx mice (Figure 5). These results suggest that the effect of PPMO treatment at this dose can achieve measurable therapeutic outcome in skeletal muscle without detectable toxicity.

30 mg/kg PPMO Monthly Treatment Restores Near-Normal Levels of Dystrophin with Improvement of Muscle Pathology and Function

With the demonstration that the half-life of dystrophin induced by a single intravenous injection of 30 mg/kg PPMO is approximately 2 months, we designed a 1-year treatment of 30 mg/kg PPMO with monthly intravenous injection. The 1-year treatment was able to achieve dystrophin expression in nearly 100% of muscle fibers in bodywide skeletal muscle. Western blot detected >50% of normal levels of dystrophin protein in most skeletal muscles, although lower levels (as low as 25%) were also detected (Figure 4, E and G). Muscle pathology was clearly improved, with almost no degenerating fibers in any skeletal muscle examined (Figure 5A). The fibers within the same muscle became highly uniform, with no clear inflammatory cell infiltrations. Foci of degeneration and regeneration and monocyte infiltration, which remained in most muscles after lower-dose treatment, were absent in diaphragm. Consistently, CK levels were significantly reduced, compared with control mdx mice (Figure 5D). Muscle function as measured by grip force generation was significantly improved (Figure 5C). The levels of dystrophin expression in cardiac muscle were considerably lower than those detected in skeletal muscle, but was observed in >50% cardiac muscle fibers, and the amount of dystrophin protein reached 15% in cardiac muscle of all the PPMO-treated mice (Figure 4).

The mice under this dosage treatment showed no sign of abnormal body weight change when compared with the control mdx mice. No pathological change of the liver,
kidney, and lung was observed under H&E staining (Figure 5A). This finding was supported by serum tests, which showed normal levels of creatinine, total bilirubin, alkaline phosphatase, γ-glutamyltransferase, and blood urea nitrogen (Figure 5D). We also examined the levels of KIM-1 expression in the kidney of the PPMO-treated mice by Western blot. All kidneys from both the 1.5 mg/kg and 30 mg/kg PPMO-treated mice showed barely detectable levels of KIM-1 expression, similar to those from untreated mdx and C57BL/6 control mice (see Supplemental Figure S1 at http://ajp.amjpathol.org).

**Discussion**

Cationic peptides, also referred as cell-penetrating peptides, have been widely used for gene and oligonucleotide delivery. Successful applications of such peptides in
vivo, however, have been limited. One major limitation is toxicity.\textsuperscript{15,24–26} Data from previous studies in mouse models showed that doses inducing near-normal levels of dystrophin expression by a PPMO containing the cat-ionic peptide (RXRRBR)\textsubscript{2}XB sequence did not cause obvious short-term toxicity.\textsuperscript{15} The PPMO showed no toxic effect either at 20 mg/kg for six weekly injections in wild-type mice or at 30 mg/kg for 3 months of biweekly injections in \textit{mdx} mice.\textsuperscript{15,27} At these doses, adverse effects were detected in some mice, including an acute lethargy lasting up to a few hours after systemic injection. There was no clear toxicity in the kidney and liver, as assessed both by histology and by the levels of serum enzymes at the end of the PPMO intervention. Nonetheless, toxicity of PPMO has been reported as weight loss and tubular degeneration in kidney.\textsuperscript{26–28} Results from the present study confirmed that PPMO at 30 mg/kg by monthly injections caused only mild lethargy in some mice immediately after the systemic injection. However, further increase of the dose to 60 mg/kg led to death in some of the treated mice within 48 hours, and the remaining mice showed clear lethargy within 2 to 4 days. When the single dose was 120 mg/kg, >80% of the animals died. These findings suggest that the LD\textsubscript{50} of PPMO is approximately 85 mg/kg. All of the data together support the notion raised by Moultons\textsuperscript{27} that there seems to be a dose threshold for the toxicity of PPMO: below the threshold, acute toxicity cannot be clearly observed; above it, the severity of toxicity increases rapidly in a dose-dependent manner. The severity of toxicity is also suggested to be dependent on dose frequency.\textsuperscript{27}

One specific concern for PPMO is the potential chronic toxicity to kidney. The same peptide-conjugated PMO (AVI-5038) as used in the present study, but targeting human exon 50, was reported to cause tubular degeneration (although mild) in the kidneys of monkeys after four weekly injections of only 9 mg/kg.\textsuperscript{27} Elevated blood urea nitrogen levels were also reported in the rats treated with a similar peptide-conjugated PPMO.\textsuperscript{28} In this study, however, no clear kidney damage was indicated by histology.
or by tests of serum enzymes, including blood urea nitrogen and detection of KIM-1. Although the reasons for such discrepancies are not understood, two factors are most likely involved. In the present study, the kidney was examined 2 weeks after the last treatment, whereas in the other studies the serum and kidney were examined within 1 week after PPMO treatment. Our longer interval might have provided sufficient time for some kidney damage to recover. Perhaps more importantly, different species may respond to the drug differently, given that the other studies used three different animals. Nevertheless, the low LD$_{50}$ of the PPMO established in the present study and the possibly greater sensitivity of higher mammals (including humans) to the peptide raise serious safety concern for clinical use of the PPMO, especially for the repeated injections that are essential to maintain therapeutic levels of dystrophin in bodywide muscle in DMD patients. Thus, further studies in higher mammals with different dose regimes are essential before translation to the clinic, and the biggest challenge for PPMO as antisense drug chemistry is to determine safe and effective doses.$^{27}$

A threshold effect may also apply to the efficiency of exon skipping with dosing of PPMO. AVI-5038 targeted to skip human dystrophin exon 50 is currently in preclinical development for DMD patients. Initial efficacy of the PPMO in four healthy cynomolgus monkeys showed that weekly intravenous injection of 9 mg/kg for 4 weeks induced an average of 40%, 25%, and 2% exon-skipping effect in diaphragm, quadriceps, and cardiac muscle, respectively.$^{27}$ On the same schedule but at a lower dose (3 mg/kg), however, little exon-skipping product was detected. A similar trend was observed in the present study. Nearly all skeletal muscles expressed $\geq 20\%$ levels of dystrophin protein, as assessed by Western blot, and $>60\%$ fibers clearly stained for dystrophin by immunohistochemistry, with $\leq 5\%$ in cardiac muscle in mdx mice treated with 6 mg/kg PPMO. With the 1.5 mg/kg PPMO treatment, however, exon skipping was barely detectable by RT-PCR, and dystrophin protein was detected in $<$20% of muscle fibers. Similarly, dystrophin protein was barely detected in most muscles, although $\sim 5\%$ expression was detected in some skeletal muscles by Western blot. No dystrophin was detected in cardiac muscle. These results indicate a nonlinear relationship between the dose and the efficiency of dystrophin induction with systemically delivered PPMO. In clinical application, therefore, a threshold dose of PPMO may be required to achieve significant levels of dystrophin production and consequent functional improvement for DMD patients.

Results from the present study with three different dosing regimes provided a baseline for our assessment of a possible therapeutic window for PPMO treatment of DMD. Monthly treatment at a dose of 30 mg/kg was effective for maintaining near-normal levels of dystrophin in all muscles, with highly significant rescue effect on muscle pathology and function. Similar to what we observed previously in the study of short-term effects with biweekly PPMO administration,$^{16}$ this dosing regimen appeared to be safe for long-term administration without observable chronic toxicity, as indicated by normal levels of serum enzymes in liver and kidney. No pathology was revealed by histology of all skeletal muscles, cardiac muscle, liver, kidney, and lung, which was further supported by the normal behavior and life span of the treated mice. Nonetheless, despite the high efficacy associated with only mild lethargy immediately after the injection, this monthly dose is apparently too close to the LD$_{50}$ and must be tested in higher mammals before being considered for clinical application.

At the dose of 6 mg/kg with a biweekly injection, PPMO induced $>20\%$ dystrophin in all skeletal muscles. Despite clear variation in the distribution and levels of dystrophin expression between and within muscles, functional improvement was evident by the demonstration of serum CK levels and histopathology. Also, and importantly, this dose induced a significant amount (\textasciitilde 5\%) of dystrophin in cardiac muscle. This dose of PPMO is at least 10-fold less than the LD$_{50}$ and therefore could be considered as having potential for long-term clinical treatment of DMD.

Efficacy of antisense therapy for individual DMD patients depends on many other factors, especially the efficiency of individual antisense oligomers and the functionality of the truncated dystrophin created by exon skipping. AOs targeting individual human dystrophin exons need to be identified. Selection of AO targeting human and mouse dystrophin exons involves different cells and animal models. Thus, care must be exercised in extrapolating the effective dose obtained in the present study to clinical applications of PPMOs targeting human dystrophin exons. A more accurate estimation in exon-skipping efficiency of individual PMOs may become possible when efficiency from the first clinical trials of a PMO drug become available. This may be achieved by comparing new AO drugs with a PMO of known exon-skipping efficiency side by side in the same testing system or systems, preferably in both cell culture and in vitro animal model systemically.

The functionality of the truncated dystrophin protein created by skipping of a different exon must also be taken into account for establishing relevant therapeutic dose. Unfortunately, functionality of most of those truncated dystrophins remains to be determined. Such investigations are required if the therapy is to apply effectively to defined DMD populations; perhaps more important, such investigations are required in some cases to determine the choice of the exon or exons to skip for highest functionality of the restored dystrophin.

In summary, PPMOs as antisense drugs have demonstrated the highest efficacy for long-term treatment of DMD. The LD$_{50}$ of the PPMO in the present study was approximately 85 mg/kg, significantly lower than that of PMO without toxicity at doses of $\leq 3$ g/kg in mdx mice. Nonetheless, the low LD$_{50}$ may be mitigated by the high efficiency of the PPMO, permitting the safe use of lower doses that are still capable of achieving significant long-term therapeutic effect.
Acknowledgments

We thank AVI BioPharma for supplying the PPMO AVI-5038.

References