Long-term improvement in \textit{mdx} cardiomyopathy after therapy with peptide-conjugated morpholino oligomers\footnote{Morpholino oligomers.}

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Received 16 January 2009; revised 28 September 2009; accepted 5 October 2009; online publish-ahead-of-print 8 October 2009

Time for primary review: 23 days

Aims

The cardiomyopathy found in Duchenne muscular dystrophy (DMD) is responsible for death due to heart failure in \textasciitilde 30\% of patients and additionally contributes to many DMD morbidities. Strategies to bypass DMD-causing mutations to allow an increase in body-wide dystrophin have proved promising, but increasing cardiac dystrophin continues to be challenging. The purpose of this study was to determine if therapeutic restoration of cardiac dystrophin improved the significant cardiac hypertrophy and diastolic dysfunction identified in X-linked muscular dystrophy (\textit{mdx}) dystrophin-null mouse due to a truncation mutation over time after treatment.

Methods and results

Mice lacking dystrophin due to a truncation mutation (\textit{mdx}) were given an arginine-rich, cell-penetrating, peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) that delivered a splice-switching oligonucleotide-mediated exon skipping therapy to restore dystrophin in \textit{mdx} mice before the development of detectable cardiomyopathy. PPMO successfully restored cardiac dystrophin expression, preserved cardiac sarcolemma integrity, and prevented the development of cardiac pathology that develops in \textit{mdx}-null mice over time. By echocardiography and Doppler analysis of the mitral valve, we identified that PPMO treatment of \textit{mdx} mice prevented the cardiac hypertrophy and diastolic dysfunction identified in sham-treated, age-matched \textit{mdx} mice, characteristic of DMD patients early in the disease process, in as little as 5–6 weeks after the initiation of treatment. Surprisingly, despite the short-term replacement of cardiac dystrophin (<1\% present after 12 weeks by immunodetection), PPMO therapy also provided a durable cardiac improvement in cardiac hypertrophy and diastolic dysfunction for up to 7 months after the initiation of treatment.

Conclusion

These results demonstrate for the first time that PPMO-mediated exon skipping therapy early in the course of DMD may effectively prevent or slow down associated cardiac hypertrophy and diastolic dysfunction with significant long-term impact.

Keywords

Duchenne muscular dystrophy · Morpholino · Oligomers · Cardiomyopathy · Therapy · Exon skipping · Alternative RNA splicing

1. Introduction

Duchenne muscular dystrophy (DMD), a lethal and progressive muscle wasting disorder, is caused by deletions or mutations in the DMD gene, which disrupt the open reading frame and create premature termination of translation, resulting in the lack of dystrophin production. In contrast, Becker muscular dystrophy (BMD),...
a milder form, is caused by the deletions in DMD gene, which maintain the open reading frame, resulting in production of internally deleted but partially functional dystrophin. Thus, restoration of the open reading frame in DMD transcripts by antisense oligonucleotides that mediate targeted exon exclusion should create shortened transcripts, in which the reading frame is restored. These transcripts then generate truncated, partially functional dystrophin, which converts severe DMD to a milder BMD phenotype.

The potential of SSO-mediated exon skipping in dystrophin pre-mRNA has been investigated in primary X-linked muscular dystrophy (mdx) myoblast cultures and in the mdx mouse, a model of DMD, which carries a nonsense mutation in exon 23 of the DMD gene, preventing translation of dystrophin protein. The SSO targeted to a donor splice site of intron 23 or other splicing elements in exon 23 forces skipping of the exon, leading to internally deleted dystrophin production. Among oligonucleotide chemistries tested in mdx mice, systemically delivered phosphorodiamidate morpholino oligomers (PMOs) were particularly effective in exon 23 skipping and dystrophin expression at therapeutic levels in body-wide skeletal muscles of mdx mice, which led to functional improvement of skeletal muscles. Unfortunately, induction of exon skipping and dystrophin expression, if any, in heart was not detectable. Since all striated muscles, including cardiac muscle, are affected by DMD, and 30% of deaths in DMD patients are caused by heart failure, induction of dystrophin expression in cardiac muscle is critical for DMD treatment.

We previously reported for the first time that dystrophin expression was efficiently induced in the heart of mdx mice by arginine-rich cell-penetrating peptide conjugated PMO (PPMO), named AVI-5225 carrying (RXRRBR)₂XB peptide, where R is arginine, X is 6-aminohexanoic acid, and B is β-alanine. Effective exon skipping and sustained dystrophin expression were achieved not only in the heart but also throughout the body in skeletal muscle, leading to improvement in muscle integrity as shown by a significant decrease of serum creatine kinase (CK). In the present study, we examined the functional improvement of mdx cardiomyopathy in response to the efficient restoration of cardiac dystrophin protein using AVI-5225.

2. Methods

2.1 PPMO conjugates

PMOs were synthesized and purified at AVI BioPharma (Corvallis, OR) as previously described by Wu et al. The AVI-5225 PPMO, targeted to the 5’ splice site of mouse dystrophin intron 23 (5’-GGC CAA ACC TCG GCT TAC CTG AAA T-3’), was conjugated to B peptide (RXRRBR)₂XB, where R is arginine, X is 6-aminohexanoic acid, and B is β-alanine, at the 3’-end. PPMO 623-25-B, a negative control, was targeted to unrelated dystrophin human β-globin gene (5’-TGT TAT TCT TTA GAA TGG TGC AAA G-3’) and used for the sham-treated group. Both PPMOs were resuspended and diluted in sterile normal saline and stored at 4°C.

2.2 Experimental animals and PPMO treatment

All animal experiments were approved by Institutional Animal Care and Use Committee at the University of North Carolina and conform to the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Age-matched C57BL/10ScSn-Dmd<sup>mdx<sup>445</sup></sup>/J (X-linked muscular dystrophy (mdx)) and C57BL/10ScSn (C57BL) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were fed chow ad libitum. 0.3 mg per mouse per day (12 mg/kg) of PPMO conjugate was injected i.v. to 8–16 week-old mice in two cycles of four once daily injections with a 2-week interval (Figure 1). A sham PPMO 623-25-B targeted to unrelated, human β-globin gene was used in the short-term studies (Group 1) while a saline control was used in the long-term studies (Group 2). Mice were anaesthetized with isoflurane to the point they were non-responsive to toe pinch and then euthanized by cervical dislocation. All 47 mdx and 30 wild-type mice used successfully completed these studies.

2.3 RNA and protein analysis

The RNA and protein analysis was described in detail previously. Briefly, the level of exon 23-skipped transcript was analysed in total RNA extracted from tissue samples by nested RT–PCR with primers targeted to exons 21 and 25. The level of dystrophin restoration was evaluated in tissue total protein lysate by in-gel immunodetection. NCL-DYS2, a mouse monoclonal antibody against C-terminus of dystrophin (Novoceastra, Newcastle upon Tyne, UK), followed by IRDye 680-labelled goat anti-mouse IgG (LI-COR Biosciences, Lincoln, NE), were used. The gel was subsequently analysed by Odyssey infrared imaging system (LI-COR Biosciences).

An expanded Methods section is available in the Supplementary material online.

3. Results

3.1 AVI-5225 induced global dystrophin expression throughout the mdx heart

Systemic delivery of the PPMO AVI-5225, targeted to a donor splice site of mouse dystrophin intron 23, induces a high level of exon 23 skipping and sustained dystrophin expression body-wide in striated muscles, importantly in the cardiac muscle of the dystrophic X-linked muscular dystrophy (mdx) mouse. We therefore determined whether the induced dystrophin protein could produce functional benefit on the heart of AVI-5225-treated mdx mice.

mdx mice were treated with AVI-5225 by two cycles of four once daily IV injections at 12 mg/kg/day with a 2-week interval as outlined in Figure 1 (Group 1). Six weeks after the initiation of therapy, high levels of exon skipping and dystrophin induction were achieved extensively in all skeletal, smooth, and cardiac muscles (Figure 2 and data not shown). In the heart, the level of exon 23-skipped transcript determined by nested RT–PCR was about 80–90%, while no skipping product was observed in
sham-treated mdx mice, injected with the sham PPMO 623-25-B targeted to unrelated, human β-globin gene, or in wild-type C57BL mice (Figure 2A). The level of restored dystrophin protein in the hearts of AVI-5225-treated mdx mice analysed by in-gel immunodetection was about 30% of wild-type mice at 3 weeks after the last injection (Figure 2B). Immunofluorescence detection of dystrophin on heart sections showed that AVI-5225-induced dystrophin was expressed entirely throughout the heart of treated mdx mice and was properly localized at the sarcolemma of cardiac muscles (Figure 2C). As expected, no detectable dystrophin expression was observed in sham-treated mdx mice.

### 3.2 Rescued dystrophin in the mdx heart improved sarcolemma integrity

A lack of dystrophin in muscles of mdx mice and DMD patients results in plasma membrane instability and increased membrane permeability leading to leakage of intracellular contents into blood circulation.\(^{16,17}\) Serum CK and CK-MB isoenzyme are two hallmarks for membrane instability of whole-body muscles and heart specific muscle, respectively. As expected with increasing muscle damage, we identified that serum total CK (Figure 2D) and cardiac CK-MB (Figure 2E) increased with time in sham-treated mdx mice compared to wild-type controls. Both serum CK and cardiac CK-MB returned to wild-type levels in AVI-5225-treated mdx mice 5 weeks after the initiation of therapy (Figure 2D, E). We previously demonstrated that AVI-5225 treatment induces persistent increases in rescued dystrophin expression for up to at least 11 weeks after the last injection, after which it is no longer present in the heart.\(^{14}\) These results indicate that the sarcolemma of whole-body muscles was stabilized and that cardiac damage was significantly reduced by rescued dystrophin.

#### 3.2.1 Rescued dystrophin prevents cardiac hypertrophy and inhibits diastolic dysfunction that develops in mdx mice at ages 16–21 weeks (Group 1)

Increased cardiac mass and diastolic dysfunction has been consistently identified in the mdx model of DMD.\(^{18-20}\) To determine how therapy could help prevent the natural history of mdx cardiomyopathy, we analysed function at baseline (16 weeks of age) and 5 weeks after the initiation of therapy (21 weeks of age).
Figure 2 Global dystrophin expression in the heart of mdx mice induced by PPMO AVI-5225 prevents the loss of sarcolemma integrity 6 weeks after the initiation of therapy. (A) The level of exon 23-skipped mRNA was analysed by nested RT–PCR 3 weeks after the last injection. The upper band (445 bp) indicated by FL corresponds to the full-length dystrophin transcript, and the lower band (232 bp) indicated by Δ23 corresponds to the exon 23-skipped dystrophin transcript. The percentages of exon 23 skipping are shown. Samples A and B are from different mice. (B) In-gel western detection of total protein extracted from injected mdx heart. Dystrophin (DYS) was detected by the NCL-DYS2 monoclonal antibody. The percentages of dystrophin restoration compared with the average of two different wild-type mice are shown. (C) Immunofluorescence detection of dystrophin in the treated mdx heart 6 weeks after the start of therapy. Panels 1–4 are higher-magnification images of the regions shown in the full-view image of the AVI-5225- treated heart. Panel 1: septum; 2: anterior left ventricle wall (LV); 3: posterior LV; 4: lateral LV. C57BL: heart from normal C57BL mouse; Sham-treated: heart from PPMO 623-25-B-treated mdx mouse. Scale bar, 100 μm. (D) Significantly less serum CK (D) and CK-MB (E) is circulating in mdx mice after AVI-5225 treatment compared with age-matched mdx sham-treated controls. C57BL (n = 10), Sham-treated mdx mice (n = 11), and AVI-5225-treated mdx mice (n = 6). A one-way ANOVA was performed to determine significance, followed by a Holm-Sidak pairwise comparison to significance between groups, *P < 0.05 for comparisons to wild-type C57BL; †P < 0.05 for comparisons to sham-treated mdx mice.
High-resolution echocardiography was performed on conscious wild-type, sham-treated mdx mice, and AVI-5225-treated mdx mice to determine the efficacy of SSO treatment on these fundamental cardiac defects (Figure 3 and Table 1). By a multiple tests and measures, we identified that SSO treatment significantly improved the status and function of the heart in mdx mice.

Sixteen-week-old mice just prior to the development of the mdx cardiomyopathy were used to assess short-term PPMO therapy (Figure 3). Pre-treatment, mdx hearts did not differ from age-matched wild-type controls (Table 1, Figure 3A,B). No significant differences in wall thickness, systolic function (Table 1, Figure 3A), or diastolic function (Figure 3B) were identified at baseline before treatment. Five weeks after the induction of therapy (age 21 weeks), we identified that sham-treated mdx mice had significant increases in anterior and posterior wall thicknesses in both diastole and systole (AWTD/AWTS and PWTD/PWTS, respectively, in Table 1), represented in M-mode images (Figure 3C). Five weeks after the initiation of PPMO AVI-5225 therapy, mdx mice demonstrated a wall thickness nearly equivalent wild-type controls and baseline levels (Table 1), indicating that therapy prevented cardiac hypertrophy. This striking response to therapy is consistent with our previous study, which demonstrated increased cardiac dystrophin to ~5% of wild-type level in as little as one day after the four AVI-5225 daily injections. Persistence of dystrophin has been identified for a maximum of 11 weeks using this treatment (Day 1 ~90% correction; 11 weeks 1–2% correction). With the significant replacement of dystrophin by PPMO-treatment in these short-term experiments, we identified the nearly complete resistance against the mdx-associated cardiomyopathy if PPMO-therapy is given before characteristic phenotypic increases in cardiac mass and diastolic function occur.

Consistent with an increase in wall thickness determined by echocardiography, we identified an increase in echocardiography determined left-ventricular mass (LV mass) in mdx mice compared to wild-type mice (Figure 3D). This increase in cardiac mass was also identified by weighing the histologically fixed heart samples before processing. In mdx mice treated with AVI-5225 therapy, increased heart weight was not identified, and was nearly equivalent to age matched wild-type animals (Table 1). Likewise, 5 weeks after the initiation of treatment, both heart weight and LV mass/ body weight ratio determined by echocardiography (Figure 3D) were significantly less compared with sham treated mdx controls and comparable to age-matched wild type mice.

We identified that sham-treated mdx hearts had increased cardiomyocyte cross-sectional areas (Figure 3F, middle), compared with wild-type (Figure 3F, left) and AVI-5225-treated animals (Figure 3F, right), indicative of cardiac hypertrophy. Quantitatively, sham-treated mdx mice had a 63% increase in cross-sectional area (Figure 3G). In AVI-5225-treated animals, only small increases (16%) in cardiomyocyte cross-sectional area was identified compared with wild-type age-matched controls (Figure 3G). Histologically, rare patchy areas of fibrosis could be detected in both sham- and AVI-5225-treated mdx mice (Figure 4C–F), suggesting that improvements in cardiac hypertrophy and diastolic function were independent of improvements in the mild extracellular matrix changes. These studies demonstrate that the phenotype of cardiac hypertrophy and diastolic dysfunction we identified in 21-week-old mdx mice is significantly attenuated to nearly baseline levels after 5 weeks of treatment with the SSO.

A common method to identify diastolic function of the heart is the use of Doppler echocardiography to measure blood flow through the mitral valve. The E and A waveforms generated allow the determination of function by measuring the relative amount of flow through the mitral valve during systole and diastole. A decrease in the E/A ratio is a common marker of diastolic dysfunction that is identified in boys with DMD. Therefore, Doppler analysis of the mitral valve was performed to determine if SSO treatment improved diastolic function observed as an increase in the E/A wave ratio. Diastolic dysfunction was not observed at baseline 16-week-old mice (Figure 3B). While the diastolic function was not significantly decreased compared with wild-type controls, one mouse demonstrated inverted E/A waves (0.3), which decreased the overall mean (Figure 3B). This likely represents early subtle disease in a single mouse. Confirming previous observations that mdx mice have diastolic dysfunction early in life,10,21 we identified that sham-treated mdx mice had a significant reduction in the E/A wave ratio compared with wild-type control mice by 21 weeks of age (Figure 3E), indicative of diastolic dysfunction. In contrast, age-matched PPMO AVI-5225-treated mdx mice had cardiac function comparable to age-matched wild-type hearts (Figure 3E), consistent with previous studies of normal E/A ratios in healthy mice.23 This indicates SSO treatment prevents the mdx age-associated development of diastolic dysfunction if given prior to its development.

3.2.2 Rescue of dystrophin results in long-term reduction in cardiac hypertrophy ~7 months after the initiation of therapy (Group 2)

To determine the durability of the improvements in cardiac function induced by AVI-5225 treatment, we performed high-resolution echocardiography on conscious mice at baseline and after ~34 weeks of age (~7 months) after the initiation of SSO treatment (Table 1). As dystrophin replacement using AVI-5225 gives detectable levels of functional protein for up to 11 weeks, AVI-5225 treated mice did not have dystrophin for over 4 months (~17 weeks) in these long-term studies. The development of some cardiac hypertrophy in AVI-5225 treated mdx mice is evidenced by significant increases in anterior and posterior wall thicknesses (Table 1, Figure 3H) and cardiac LV mass/body weight ratios (Figure 3I). However, hypertrophy was significantly less than age-matched saline-treated mdx controls. As would be expected, these measures were not as close to wild-type controls as seen in the Group 1 short-term studies (Figure 3H) and were greater than measurements taken at baseline (Figure 3B, Table 1). The reduced cardiac hypertrophy in AVI-5225 treated mdx mice was paralleled by small, but not statistically significant improvement in diastolic function determined by E/A ratio (Figure 3J). Since the presence of rescued dystrophin expression is limited to ~11 weeks,14 these data suggest that transient expression of sustained dystrophin before the development of mdx cardiomyopathy is sufficient to maintain improvement and slow progression of mdx cardiomyopathy.
Figure 3  Treatment of mdx mice with AVI-5225 prevents the development of mdx-associated cardiac hypertrophy and diastolic dysfunction, which leads to significant improvements in long-term durability. No differences in cardiac wall thickness shown by representative M mode (A) or diastolic function determined by mitral valve Doppler analysis (E/A ratio) (B) are present in mdx and wild-type control mice at ages 8 or 16 weeks old. n.s. = not significant. (C) AVI-5225 treatment prevents the development of anterior and posterior wall thickening in mdx mice as evidence by representative M-mode; (D) LV Mass determined by echocardiography, *P < 0.001 vs. wild type; †P < 0.001 vs. sham-treated mdx mice; and (E) prevents the development of diastolic dysfunction in mdx hearts determined by mitral valve Doppler analysis, *P < 0.05 vs. wild-type; †P < 0.05 vs. sham-treated mdx. (F) Representative cross sectional micrographs of wild-type (left, n = 3), sham-treated mdx (middle, n = 3), and AVI-5225-treated mdx mice (right, n = 3) hearts, illustrating that cardiomyocytes from AVI-5225-treated mdx mice do not develop cardiomyocyte hypertrophy 5 weeks after the initiation of therapy. Arrows indicate representative cross-sectional areas. Scale bar, 10 μm. (G) Quantitative analysis of cardiomyocyte cross-sectional areas demonstrates this protection. n = 3 mice/group; each mean is calculated from 50 measurements from multiple sections from the three representative mice from each group. *P < 0.001 vs. wild-type; †P < 0.001 vs. sham-treated mdx mice. (H) Durable improvement in mdx mice ~7 months after AVI-5225 was identified by reduced anterior and posterior wall thickening as evidenced by representative M-mode; (I) LV mass determined by echocardiography, *P < 0.05 vs. wild-type; †P < 0.01 vs. sham-treated mdx. (J) Residual improvement in diastolic dysfunction determined by mitral valve Doppler analysis was identified as not statistically significant between wild-type or sham-treated mdx groups. *P < 0.05 vs. wild-type. A one-way ANOVA was performed to determine significance, followed by a Holm-Sidak pairwise comparison to significance between groups, with comparison groups identified above.
Table 1  High-resolution transthoracic echocardiography was performed on conscious on sham-treated, AVI-5225-treated mdx, and age-matched wild-type control mice at pre-treatment, 5 weeks of therapy, and 6 months after last treatment as described in Figure 1 (± SE).

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<th>Group 1</th>
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<tr>
<td></td>
<td>Wild-type n = 5, baseline 16 weeks old</td>
<td>Wild-type n = 10, 5 weeks after Rx initiation</td>
<td>Sham-treated mdx n = 11, 5 weeks after Rx initiation</td>
<td>AVI-5225-treated mdx n = 7, 5 weeks after Rx initiation</td>
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<td>Sham-treated mdx n = 5, baseline 10 weeks old</td>
<td>AVI-5225-treated mdx n = 5, baseline 8 weeks old</td>
<td>Wild-type n = 7, 10 months after Rx initiation</td>
<td>Wild-type n = 9, 7 months after Rx initiation</td>
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<tr>
<td></td>
<td>AVI-5225-treated mdx n = 7, 10 months after Rx initiation</td>
<td>Wild-type n = 9, 7 months after Rx initiation</td>
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<tr>
<td>BW (g)</td>
<td>22.4 ± 0.6</td>
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<td>27.9 ± 0.5</td>
<td>27.4 ± 0.4</td>
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<td>LV mass index (mg)</td>
<td>94.0±5.1</td>
<td>85.6 ± 2.5</td>
<td>132.1 ± 5.4</td>
<td>102.92 ± 3.7</td>
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<td>Heart weight/BW (mg/g)</td>
<td>4.81±0.13</td>
<td>6.13 ± 0.32</td>
<td>7.18 ± 0.03</td>
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<td>HR (b.p.m.)</td>
<td>625.0±12.9</td>
<td>624.1 ± 9.3</td>
<td>645.7 ± 10.6</td>
<td>632.0 ± 12.9</td>
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<td>AWTD (mm)</td>
<td>0.99±0.01</td>
<td>1.30 ± 0.01</td>
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<td>AWTS (mm)</td>
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<td>1.63 ± 0.03</td>
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<td>PWTD (mm)</td>
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<td>PWTS (mm)</td>
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<td>LVEDD (mm)</td>
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<td>LVESD (mm)</td>
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<td>EF%</td>
<td>87.9±0.6</td>
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BW, body weight; HR, heart rate; LV mass index [(ExLVD7d4 − LVED7d4) × 1.055]; ExLVD, external left-ventricular diameter; b.p.m., heart beats per minute; AWTD, anterior wall thickness in diastole; AWTS, anterior wall thickness in systole; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; LVEDD, left-ventricular end-diastolic dimension; LVESD, left-ventricular end-systolic dimension; FS, fractional shortening, calculated as (LVEDD − LVESD)/LVEDD × 100; EF%, ejection fraction calculated as (end Simpson’s diastolic volume—end Simpson’s systolic volume)/end Simpson’s diastolic volume × 100; ND, not determined; SND, statistics not determined as n = 2.

*P < 0.01 vs. wild-type.

**P < 0.05 vs. mdx sham.
4. Discussion

Skipping of exon 23 in dystrophin pre-mRNA and consequent restoration of dystrophin protein induced by the PPMO AVI-5225 prevented the disruption in cardiac sarcolemma integrity, cardiac hypertrophy, and the diastolic dysfunction in X-linked muscular dystrophy (mdx) mouse hearts. This was identified by histology, echocardiography, and Doppler analysis. Furthermore, we found that this transient replacement in dystrophin by AVI-5225 therapy attenuated the long-term onset and progression of cardiomyopathy.

These results were affected by a conjugate of arginine-rich cell penetrating peptide B to morpholino oligomer at relatively low dose of 0.3 mg per mouse (12 mg/kg). The key to the effectiveness of this compound appears to be the combination of a positively charged peptide with neutral oligomers that target pre-mRNA in a sequence specific manner.14,15,24,25 Similar peptides conjugated to negatively charged oligonucleotide were totally ineffective (data not shown). The importance of the B peptide is underscored by previous findings with unconjugated morpholino oligomer or 2'-O-methyl oligonucleotides in the same mdx mouse model. These unconjugated morpholino compounds restored dystrophin in skeletal muscles but not in the heart. Furthermore, much higher doses (up to 100 mg/kg) were required.8,11 Since cardiomyopathy is one of the causes of death in patients with DMD, therapies restricted to skeletal muscles are not adequate and paradoxically lead to the acceleration of cardiomyopathy in mouse models.26 Improvement of DMD cardiomyopathy in the mdx mouse was also accomplished by gene therapy methods systemically delivering microdystrophin gene or antisense RNA using adeno-associated virus (AAV) as a vector.27–31 The advantage of the current approach is that it is more akin to standard pharmacological treatment and therefore amenable to US Food and Drug Administration (FDA) accepted drug development pathway.

In the present study, we applied PPMO AVI-5225 therapy to mdx just prior to the development of detectable mdx cardiomyopathy. The application of PPMO AVI-5225 therapy to mdx mice resulted in the persistent splicing correction of dystrophin in the heart to levels nearing 79–91% (Figure 2A). Previously, this AVI-5225 correction was shown to be present by PCR for a maximum of 11 weeks (1–2% correction); protein was undetectable by 12 weeks.14 By treating mdx mice with AVI-5225 to replace dystrophin just prior to the development of cardiac hypertrophy and diastolic dysfunction, we were able to prevent these changes from occurring in the short term when the protein would still be detectable as shown in our Group 1 studies. That as little as 30% replacement of the dystrophin (Figure 2B) was so effective may be due to the uniformity of dystrophin protein expression throughout the heart (Figure 2C) in the absence of any inflammatory response as previously detailed.14,32 After treatment with

Figure 4  Treatment of mdx mice 6 weeks after the initiation of therapy does not alter the mild rare focal fibrosis. Representative heart sections from wild-type (A and B), sham-treated mdx (C and D), and AVI-5225-treated mdx (E and F) mice were analysed after Trichrome (A, C, E) or H&E (B, D, F) staining (20 x). While rare focal fibrosis is present in both mdx and AVI-5225-treated mdx mice, all three groups of mice are generally indistinguishable by histology.
the same peptide-coupled oligonucleotides used in the present study, no detectable antibodies were detected by ELISA.32 Histologic analysis of tissues from these mice confirmed a lack of inflammatory cells.32 Similarly, in the present study we did not identify any toxicity determined grossly by our observation that there was no morbidity or mortality throughout the course of the study. If these effects can be recapitulated in patients, drug treatment may be relatively infrequent and it can be anticipated that low levels of restored dystrophin will be sufficient to be therapeutic. This is further supported by clinical observations that BMD patients with levels of dystrophin as low as 20% of normal have relatively mild disease course.13,34

What was surprising in the present study, was that AVI-5225 therapy given to mice followed for up to 7 months after the initiation of therapy resulted in long-term improvements in cardiac hypertrophy and diastolic function compared with saline-treated mdx mice, a time point ~7 weeks after the replaced dystrophin would have been gone. These findings suggest that even transient replacement of dystrophin can significantly slow the progression of mdx-cardiomyopathy, but that continued replacement may be necessary for the progression to be completely attenuated. The effect of replacing dystrophin in established mdx-cardiomyopathy has yet to be determined.

In the present study, PPMO treatment to replace dystrophin ameliorated the development of cardiac hypertrophy determined by heart weight/body weight, LV mass/body weight, cardiomyocyte cross-sectional areas, and echocardiography analysis seen in mdx mice by 21 weeks of age. Dystrophin replacement also improved diastolic dysfunction, indicating a protection against the sequelae of pathologic cardiac hypertrophy. Previous reports have identified spontaneous cardiac hypertrophy in 29 week18 and 10-month35-old mdx mice. Consistent with our identification of cardiac hypertrophy developing at 16–21 weeks, recent studies have identified that 8 week mdx mice do not have cardiac hypertrophy18 and that cardiomyopathy is present at ~16 weeks.30 No previous study to our knowledge has identified the development of cardiac hypertrophy in mdx mice at 16–21 weeks, making this a novel clinical endpoint to study when testing DMD therapeutics in the mdx mouse.

A recent study by Wu et al.32 likewise found improvement in diastolic function using the same PPMO treatment to restore cardiac dystrophin. Since the cardiac wall thickness was not investigated, this previous study did not identify the significant improvements in cardiac hypertrophy detailed here. Furthermore, we did not observe systolic defects in mdx cardiac function as Wu et al.35 did, which is consistent with other investigators determination of function on conscious mice.18–20

In clinical application, the effectiveness of PPMO therapy will depend on the efficiency with which the dystrophin protein is restored in each DMD patient. Clinical heterogeneity of the DMD cardiomyopathy in patients indicates that the course of disease is mutation dependent.36 Considering that the DMD gene contains 79 exons and that mutations are detected throughout the gene, therapy may appear a daunting task, especially since exon skipping approach is sequence specific. However, it has been well established that a majority of DMD causing deletions are clustered around a hot spot exon 45–55 region. Analysis of deletion structure and frequency indicates that 5 SSOs targeted to exons in this region would provide treatment for over 50% of DMD patients. A single drug skipping exon 51 should be therapeutic for close to 20% of DMD deletion patients.37

It is anticipated that PPMO therapy would be initiated early in disease, and that, as in the present study, long-term efficacy may be achieved in younger children with DMD or in young adults before the establishment of significant cardiomyopathy. It is plausible that treatment of early dysfunction of boys with DMD, preceding the onset of dilated cardiomyopathy, may not only improve their current disease, but also protect against the development of systolic dysfunction and/or dilated cardiomyopathy.21 The effectiveness of PPMO therapy in more advanced disease remains to be tested. Because the mdx mouse does not progress to a lethal stage of disease, it would not be an adequate model for these studies, such as canine models of disease.38,39

A limitation to the current study is that age-matched untreated or saline-injected mdx mice were not used for Group 1 mice where hypertrophy was identified in 21 weeks old sham-oligo treated mice. The rapid hypertrophy related to the sham-oligo treatment cannot be completely ruled out, although cardiomyopathic changes have previously reported in young mdx mice this same age.30

Systemic administration of AVI-5225 restored dystrophin expression in skeletal muscle throughout the body, including diaphragm and cardiac muscle.14 This not only holds great therapeutic potential for the treatment of DMD, but also genetic cardiac diseases, such as familial hypertrophic cardiomyopathy, which affect as many as 1 in 500 people.40 Like DMD, these diseases affect children and adolescents and are due in part to truncation mutations of sarcomere proteins. Having the ability to improve the high prevalence of sudden cardiac death in FHC may prove ground breaking. Currently, two clinical trials are being conducted in DMD patients by using 2′O-methyl phosphorothioate and PMO (http://clinicaltrials.gov/ct2/show/NCT00159250?term=DMD&rank=5) to induce skipping of human dystrophin exon 51 restoring the in-frame transcript. Although the PPMO-B AVI-5225 showed great promising for clinical application, FDA mandated safety studies are required before this compound enters clinical trials.41

The mdx cardiomyopathy is a model of progressive disease, with younger mice at the ages used in this study showing few histologic changes,18 increased cardiac mass, and diastolic dysfunction.18–20 Similarly, patients with DMD can develop cardiac hypertrophy early in the course of their disease22 and diastolic dysfunction is a distinct finding in DMD-associated cardiomyopathy.23–45 AVI-5225 may provide a therapeutic approach to prevent and sustain the development of DMD cardiomyopathy by systematically replacing functional dystrophin to both skeletal muscle and heart to reduce the associated long-term morbidity and mortality.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.
Conflict of interest: N.J. and M.S.W. declare no conflicts of interest. P.S. is Executive Director of Preclinical Development, H.M.M. is Director of Discovery Research, and R.K. is Senior Vice President of Discovery Research and at AVI BioPharma, Inc.

Funding
N.J. was partially supported by the Royal Golden Jubilee (RGJ) scholarship from the Thailand Research Fund. This work was supported by NIH P01-GM059299 (to R.K.), the Children’s Cardiomyopathy Foundation Grant (to M.S.W.), and AHA Scientist Development Grant (to M.S.W.).

References