A Morpholino Oligomer Therapy Regime That Restores Mitochondrial Function and Prevents mdx Cardiomyopathy

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VISUAL ABSTRACT

HIGHLIGHTS

- DMD patients develop reduced myocardial metabolic activity and dilated cardiomyopathy due to the absence of dystrophin.
- Current clinical trials demonstrate patients receiving PMO therapy exhibit accumulation of functional dystrophin and improved ambulation; however, cardiac abnormalities remain.
- Utilizing the murine model of the disease (mdx), we identify a novel early-intervention PMO treatment regimen for the prevention of DMD cardiomyopathy.
- Pre-cardiomyopathic mdx mice were administered a nontoxic, long-term, high-dose PMO treatment regimen.
- Treated mdx mice exhibited accumulation of functional dystrophin, restoration of cardiac metabolic activity, and did not develop the cardiomyopathy.
**SUMMARY**

Current clinical trials demonstrate Duchenne muscular dystrophy (DMD) patients receiving phosphorodiamidate morpholino oligomer (PMO) therapy exhibit improved ambulation and stable pulmonary function; however, cardiac abnormalities remain. Utilizing the same PMO chemistry as current clinical trials, we have identified a non-toxic PMO treatment regimen that restores metabolic activity and prevents DMD cardiomyopathy. We propose that a treatment regimen of this nature may have the potential to significantly improve morbidity and mortality from DMD by improving ambulation, stabilizing pulmonary function, and preventing the development of cardiomyopathy. (J Am Coll Cardiol Basic Trans Science 2018;3:391–402) © 2018 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Duchenne muscular dystrophy (DMD) is a fatal X-linked disease that affects 1 in 3,600 to 6,000 live male births (1,2). It is caused by a lack of expression of dystrophin, an important cytoskeletal protein that plays a role in maintaining structural integrity in muscle cells (3). DMD patients exhibit muscle degeneration primarily in skeletal muscles (2). However, in addition to this, patients develop dilated cardiomyopathy that is associated with cytoskeletal protein disarray, contractile dysfunction, and reduced myocardial metabolic activity (4,5). Approximately 20% of DMD patient deaths result from dilated cardiomyopathy (6).

Approximately 70% of DMD-causing mutations are located between exons 45 and 55 (7). The effect of several therapeutic approaches on DMD cardiac pathology have been investigated, with varying degrees of efficacy reported (8). Effective therapy must be able to induce expression of dystrophin while improving function without inducing toxicity. We have utilized a nontoxic phosphorodiamidate morpholino oligomer (PMO) therapy that induces skipping of dystrophin exon 51, resulting in accumulation of truncated, functional dystrophin. Current clinical trials demonstrate improved ambulation and stable pulmonary function in DMD patients receiving this treatment (30 or 50 mg/kg/week, Phase 2b Eteplirsen Study 202, Sarepta Therapeutics, Cambridge, Massachusetts) (9,10). However, established cardiac abnormalities consistent with the DMD phenotype remain in these patients.

Using the murine model of Duchenne muscular dystrophy (mdx mice), we previously identified a novel mechanism by which absence of expression of dystrophin leads to myocardial metabolic dysfunction and compromised cardiac function in mdx hearts (11). This involves a structural and functional communication breakdown between the L-type Ca\(^{2+}\) channel (ICa-L) and mitochondria. We have demonstrated that in regulation of calcium-dependent mechanisms, the cardiac ICa-L plays an important role in regulating mitochondrial function through structural and functional communication via cytoskeletal proteins, independently of alterations in intracellular calcium (11,12). Myocytes isolated from mdx mice that exhibit cytoskeletal disarray due to the absence of dystrophin, exhibit impaired ICa-L regulation of mitochondrial function (11). Importantly, this lack of response occurs in both pre- and post-cardiomyopathic mdx mice, suggesting that metabolic dysfunction precedes development of the cardiomyopathy (11).

We have previously shown that long-term, low-dose treatment of pre-cardiomyopathic mdx mice with an antisense oligomer of the same PMO chemistry administered clinically to DMD patients (13), partially restores mitochondrial function...
in response to activation of \( I_{\text{Ca-L}} \) in cardiac myocytes isolated from treated mice (11). Because metabolic dysfunction precedes the \( \text{mdx} \) cardiomyopathy, we reasoned that early PMO treatment and subsequent restoration of metabolic function may prove beneficial in the prevention of DMD cardiomyopathy. Here, we treated \( \text{mdx} \) mice long term with a high concentration (120 mg/kg/week), yet non-toxic PMO dosing regimen that commenced prior to the development of the cardiomyopathy. This treatment regimen completely restored mitochondrial function and prevented subsequent development of the cardiomyopathy.

**METHODS**

**ANIMAL MODEL AND PMO TREATMENTS.** Male C57BL/10ScSn-Dmdmdx/Arc (\( \text{mdx} \)) and C57BL/10ScSnArc wild-type (\( \text{wt} \)) mice were used for all studies. Experiments were performed in myocytes isolated from 3 to 9 mice for each experimental group. Four- to 5-day-old \( \text{mdx} \) mice were treated with either 40 mg/kg PMO Mz23D (Sarepta Therapeutics), 3 × per week or 120 mg/kg PMO Mz23D once per week by subcutaneous injection for 3 weeks. In other studies, 24-week-old C57BL/10ScSn-Dmdmdx/Arc (\( \text{mdx} \)) mice were treated with 40 mg/kg PMO Mz23D 3 × per week for 19 weeks (M23D \( \text{wt} \)).

**ISOLATION OF VENTRICULAR MYOCYTES.** Animals were anesthetized with intraperitoneal injection of pentobarbitone sodium (240 mg/kg) prior to excision of the heart. Cells were isolated based on methods described (11,18). Detailed methods are provided in the Supplemental Methods.

**IMMUNOBLOTTING AND IMMUNOSTAINING.** Dystrophin in cardiac muscle was assessed by immunoblot with NCL-DYS2 (Novocastra Laboratories, Newcastle-upon-Tyne, United Kingdom) as described (15). Immunohistochemistry was performed on heart, diaphragm, and tibialis anterior cryosections using rabbit anti-dystrophin primary antibody (ab15277, 1:200 dilution; Abcam, Cambridge, United Kingdom) and Alexafluor 488 secondary antibody (goat anti-rabbit immunoglobulins, A1008, 1:400 dilution; Thermo Fisher Scientific, Melbourne, Australia). Detailed methods are provided in the Supplemental Methods.

**ECHOCARDIOGRAPHY.** Echocardiographic measurement of left ventricular function were performed on mice under light methoxyflurane anesthesia using an i3L probe on a Vivid 7 Dimension (GE Healthcare, Little Chalfont, United Kingdom) as previously described (20,21). Quantitative measurements represent the average of 24- 30-, 38-, and 43-week-old \( \text{wt} \) (\( n = 3 \)), \( \text{mdx} \) (\( n = 3 \) to 5), and PMO-treated \( \text{mdx} \) (\( n = 4 \) to 9) mice. Detailed methods are provided in the Supplemental Appendix.

**TABLE 1 RT-PCR Primers Used to Amplify Dystrophin Exons 20 to 26**

| Primer         | Sequence 5’-3’ | Expected Size (bp) |
|----------------|---------------|--------------------|
| Mouse exon 20-26 outer MExon20F | CAGATATTGCAATTGCTGAG | 900 |
| MExon26R | TTCTCTACCGGTGCTTCTCC | |
| Mouse exon 20-26 inner MExon20F | CCCAGTCTACCCATCACAGC | |
| MExon26R | CGTGCCTTTAAAGCTTCTTT | |

bp = base pairs; RT-PCR = reverse transcriptase polymerase chain reaction.
SERUM PARAMETERS OF KIDNEY AND LIVER TOXICITY FOLLOWING IN VIVO TREATMENT. Following treatment, mice were anaesthetized, terminal blood collected, and serum extracted to measure kidney and liver toxicity. Urea, creatinine, and alanine transaminase concentration was assessed using urea, creatinine, and alanine transaminase assay kits (BioAssay Systems, Hayward, California).

STATISTICAL ANALYSIS. Results are reported as mean ± SEM. Statistical significance was accepted at p < 0.05 using the Kruskal-Wallis or Mann-Whitney U test for nonparametric data (GraphPad Prism version 5.04, GraphPad, San Diego, California).

RESULTS

EFFECT OF TREATMENT OF PRE-CARDIOMYOPATHIC MDX MICE WITH A SHORT-TERM, HIGH-DOSE PMO TREATMENT REGIMEN. We previously demonstrated that treatment of pre-cardiomyopathic mdx mice with 10 mg/kg/week PMO for 24 weeks partially restored increases in $\Psi_m$ and metabolic activity in response to activation of $I_{Ca-L}$ in cardiac myocytes (11). Because the half-life of dystrophin is shorter in cardiac versus skeletal muscle (22), a higher PMO dose may induce more skipping of dystrophin exon 23 and accumulation of functional dystrophin in the heart. Therefore, we investigated the effect of a short-term, but high-concentration PMO treatment (120 mg/kg/week), on $\Psi_m$ and metabolic activity. First we treated 4- to 5-day old pre-cardiomyopathic mdx mice (11), with either 40 mg/kg PMO 3× per week for 3 weeks, or 120 mg/kg PMO once per week for 3 weeks. Cardiac uptake of PMO was then determined as evidence of exon skipping using RT-PCR. Treatment with both dosing regimens resulted in exon skipping (Figure 1A).

Next, we examined the efficacy of a short-term, high-dose PMO treatment regimen on alterations in $\Psi_m$ and metabolic activity induced by activation of $I_{Ca-L}$ in cardiac myocytes. Consistent with previous findings under calcium-free conditions, application of $I_{Ca-L}$ agonist BayK(–) elicited a significant increase in $\Psi_m$ assessed as changes in JC-1 fluorescence in myocytes from 4-week-old wt mice that was prevented with nisoldipine (Figure 1C) (11,12). No alteration was observed in age-matched mdx myocytes (Figures 1D and 1E). However, myocytes isolated from 4-week-old mdx mice treated with either PMO dosing regimen exhibited a significant increase in flavoprotein oxidation in response to BayK(–) (Figures 1D and 1E). Both treatment regimens rescued the response. BayK(–) did not significantly alter flavoprotein oxidation in wt or mdx myocytes (Figure 1E). Mitochondrial electron transport chain uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone increased flavoprotein signal confirming the signal was mitochondrial in origin (Figures 1D and 1E). These data indicate that treatment of pre-cardiomyopathic mdx mice with a short-term, high-dose PMO treatment regimen restores metabolic activity in the mdx heart.

EFFECT OF TREATMENT OF PRE-CARDIOMYOPATHIC MDX MICE WITH A LONG-TERM, HIGH-DOSE PMO TREATMENT REGIMEN. We investigated whether a long-term, high PMO treatment dose (120 mg/kg/week) would more effectively restore $\Psi_m$ and metabolic activity in mdx cardiac myocytes, compared with short-term treatment. The 24-week-old pre-cardiomyopathic mdx mice were treated with 40 mg/kg PMO M23D 3× per week for 19 weeks. Rather than 1 weekly dose, a triweekly treatment regimen was used because multiple injections may improve PMO distribution and prolong half-life (22). Cardiac uptake of PMO was then examined by assessing exon skipping using RT-PCR and detecting the presence of...
dystrophin on immunoblot and immunohistochemistry. This treatment regimen resulted in exon skipping (Figure 2A) and positive expression of dystrophin (Figures 2B and 2C).

We also examined the efficacy of a long-term, high-dose PMO treatment regimen on restoring $\Psi_m$ in isolated cardiac myocytes. Under calcium-free conditions, BayK(-) elicited a significant increase in $\Psi_m$.
in wt myocytes that was attenuated with nisoldipine (Figure 3B). No alteration was observed in age-matched mdx myocytes (Figures 3A and 3B). However, treatment of mdx mice with 40 mg/kg PMO 3× per week for 19 weeks completely rescued this response (Figures 3A and 3B). The response was attenuated with nisoldipine (Figure 3B). BayK(+) did not significantly alter $\Psi_m$ in wt or mdx myocytes (Figure 3B). Oligomycin induced a significant increase in JC-1 fluorescence in mdx myocytes demonstrating

(A) Reverse transcriptase polymerase chain reaction performed on cardiac muscle ribonucleic acid from PMO-treated mdx mice demonstrating exon 23 skipping (Δ 23 687 bp) (indicated by asterisk) and age-matched untreated mdx and wt control mice. The 22/23 541 bp amplicon represents an out-of-frame transcript missing exons 22 and 23 and has been previously reported (37,38). (B) Immunoblot performed on cardiac muscle from untreated wt (5%, 2%, and 1% dilutions), untreated mdx mice (undiluted), and PMO-treated mdx mice (undiluted) demonstrating presence of dystrophin (indicated by asterisk). (C) Immunostaining of heart cryosections from untreated wt and mdx mice and PMO-treated mdx mice demonstrating presence of dystrophin. Bars = 100 μm. PMO treatment: 40 mg/kg PMO 3× per week for 19 weeks; other abbreviations as in Figure 1.
functional electron transport (Figure 3B). Application of NaCN collapsed $\Psi_m$ in all myocytes demonstrating that the signal was mitochondrial in origin (Figure 3A). These data indicate that treatment of pre-cardiomyopathic mdx mice with a long-term, high-dose PMO treatment regimen completely restores the increase in $\Psi_m$ in response to activation of $I_{Ca-L}$.

We also investigated the efficacy of a long-term, high-dose PMO treatment regimen on metabolic activity. BayK(-) caused a significant increase in flavoprotein oxidation in myocytes from 43-week-old wt mice that was prevented with nisoldipine (Figure 3D). No alteration was observed in age-matched mdx myocytes (Figures 3C and 3D). However, myocytes isolated from 43-week-old mdx mice treated with PMO exhibited a significant increase in flavoprotein in response to BayK(-) that was attenuated by nisoldipine (Figures 3C and 3D). BayK(+) did not significantly alter flavoprotein oxidation in wt or mdx myocytes (Figure 3D). Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone increased flavoprotein signal in all myocytes confirming the signal was mitochondrial in origin (Figures 3C and 3D).

Metabolic activity was also assessed by measuring alterations in mitochondrial electron transport in intact cardiac myocytes, as formation of formazan from tetrazolium salt (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide). Consistent with previous findings, BayK(-) elicited a significant increase in metabolic activity in 43-week-old wt mice that was prevented with nisoldipine (Figure 4B) (11). The response was also attenuated with mitochondrial calcium uniporter (mitochondrial calcium uptake) blocker Ru360, but not with ryanodine receptor (sarcoplasmic reticulum calcium release) blocker dantrolene (Figure 4B). No alteration was observed in age-matched mdx myocytes (Figures 4A and 4B). However, myocytes isolated from 43-week-old mdx mice treated with PMO exhibited a significant increase in metabolic activity in response to BayK(-) that could be attenuated by nisoldipine or Ru360, but not dantrolene (Figures 4A and 4B). BayK(+) did not
significantly alter metabolic activity in wt, mdx, or PMO-treated mdx myocytes (Figures 4A and 4B). Oligomycin induced a significant decrease in metabolic activity in all myocytes demonstrating the myocytes were metabolically active (Figure 4B). These data indicate that treatment of pre-cardiomyopathic mdx mice with a long-term, high-dose PMO treatment regimen restores metabolic activity in the mdx heart.

**TREATMENT OF PRE-CARDIOMYOPATHIC MDX MICE WITH A LONG-TERM, HIGH-DOSE PMO TREATMENT REGIMEN PREVENTS THE DEVELOPMENT OF CARDIOMYOPATHY.** We examined the efficacy of a long-term, high-dose PMO treatment regimen on the development of cardiomyopathy. Serial echocardiography was performed on mdx mice before and after treatment with 40 mg/kg PMO M23D 3× per week for 19 weeks. Twenty-four-week-old mdx mice exhibited no significant difference in any echocardiographic parameter compared with untreated wt mice, indicating that these mice were pre-cardiomyopathic (Figures 5A and 5B). Consistent with the development of dilated cardiomyopathy, untreated mdx mice, compared with age-matched untreated wt mice, exhibited a significant increase in left ventricular end-systolic diameter (from 38 weeks) and left ventricular end-diastolic diameter (at 43 weeks) and a significant decrease in fractional shortening (from 38 weeks) (Figures 5A and 5B). PMO treatment significantly decreased left ventricular end-systolic diameter (from 38 weeks) and left
**FIGURE 5** Long-Term, High-Dose Treatment of 24-Week-Old *mdx* Mice With PMO Prevents Development of *mdx* Cardiomyopathy and Is Not Toxic

(A) Representative images of serial echocardiographic measurements from PMO-treated *mdx* mice and untreated *wt* and *mdx* mice pre- (24 weeks) and post-treatment (Rx; 43 weeks).

(B) Mean ± SEM of all echocardiographic measurements. *p < 0.05 compared with untreated age-matched *wt*; #p < 0.05 compared with untreated age-matched *mdx*. (C) Mean ± SEM of urea, creatinine, and alanine transaminase (ALT) concentrations in PMO-treated mice compared to untreated *mdx* mice. bpm = beats/min; FS = fractional shortening; HR = heart rate; IVDS = intraventricular septum in diastole; IVSS = intraventricular septum in systole; LVEDD = left ventricular end-diastolic diameter; LVESD = left ventricular end-systolic diameter; LVDPW = left ventricular posterior wall in diastole; LVSPW = left ventricular posterior wall in systole; PMO Rx = 40 mg/kg PMO 3x per week for 19 weeks; other abbreviations as in Figure 1.

| PMO mdx (n = 9) | wt (n = 3) | mdx (n = 5) |
|------------------|-----------|------------|
| **24 week (pre-Rx)** |           |            |
| LVEDD (mm)       | 3.98 ± 0.02 | 2.51 ± 0.01 |
| LVESD (mm)       | 1.29 ± 0.00 | 1.26 ± 0.06 |
| FS (%)           | 39.03 ± 0.38 | 36.37 ± 0.73 |
| LVDPW (mm)       | 0.90 ± 0.02 | 0.91 ± 0.04 |
| LVSPW (mm)       | 0.77 ± 0.01 | 0.75 ± 0.01 |
| IVDS (mm)        | 0.84 ± 0.01 | 0.84 ± 0.02 |
| IVSS (mm)        | 480 ± 31    | 480 ± 25    |
| HR (bpm)         | 487 ± 7     | 500 ± 9     |
| **30 week (6 weeks post-Rx)** |           |            |
| LVEDD (mm)       | 4.06 ± 0.03 | 2.54 ± 0.02 |
| LVESD (mm)       | 3.78 ± 0.26 | 3.75 ± 0.04 |
| FS (%)           | 0.93 ± 0.01 | 0.97 ± 0.04 |
| LVDPW (mm)       | 1.34 ± 0.02 | 1.30 ± 0.06 |
| LVSPW (mm)       | 0.79 ± 0.01 | 0.76 ± 0.01 |
| IVDS (mm)        | 0.84 ± 0.00 | 0.84 ± 0.01 |
| IVSS (mm)        | 470 ± 15    | 500 ± 9     |
| HR (bpm)         | 487 ± 7     | 500 ± 9     |
| **38 week (14 weeks post-Rx)** |           |            |
| LVEDD (mm)       | 4.09 ± 0.01 | 2.49 ± 0.03 |
| LVESD (mm)       | 3.96 ± 0.69 | 3.77 ± 0.56 |
| FS (%)           | 0.95 ± 0.01 | 0.97 ± 0.04 |
| LVDPW (mm)       | 1.22 ± 0.01 | 1.15 ± 0.05 |
| LVSPW (mm)       | 0.82 ± 0.00 | 0.84 ± 0.01 |
| IVDS (mm)        | 0.87 ± 0.00 | 0.88 ± 0.02 |
| IVSS (mm)        | 493 ± 24    | 510 ± 19    |
| HR (bpm)         | 493 ± 24    | 510 ± 19    |
| **43 week (19 weeks post-Rx)** |           |            |
| LVEDD (mm)       | 4.11 ± 0.00 | 2.62 ± 0.03 |
| LVESD (mm)       | 3.66 ± 0.75 | 1.00 ± 0.04 |
| FS (%)           | 1.25 ± 0.01 | 1.20 ± 0.04 |
| LVDPW (mm)       | 0.84 ± 0.00 | 0.84 ± 0.01 |
| LVSPW (mm)       | 0.86 ± 0.01 | 0.84 ± 0.00 |
| IVDS (mm)        | 487 ± 13    | 493 ± 7     |
| IVSS (mm)        | 487 ± 13    | 493 ± 7     |
| HR (bpm)         | 487 ± 13    | 493 ± 7     |

(A) Representative images of serial echocardiographic measurements from PMO-treated *mdx* mice and untreated *wt* and *mdx* mice pre- (24 weeks) and post-treatment (Rx; 43 weeks). (B) Mean ± SEM of all echocardiographic measurements. *p < 0.05 compared with untreated age-matched *wt*; #p < 0.05 compared with untreated age-matched *mdx*. (C) Mean ± SEM of urea, creatinine, and alanine transaminase (ALT) concentrations in PMO-treated mice compared to untreated *mdx* mice. bpm = beats/min; FS = fractional shortening; HR = heart rate; IVDS = intraventricular septum in diastole; IVSS = intraventricular septum in systole; LVEDD = left ventricular end-diastolic diameter; LVESD = left ventricular end-systolic diameter; LVDPW = left ventricular posterior wall in diastole; LVSPW = left ventricular posterior wall in systole; PMO Rx = 40 mg/kg PMO 3x per week for 19 weeks; other abbreviations as in Figure 1.
ventricular end-diastolic diameter (at 43 weeks) and significantly increased fractional shortening (from 38 weeks) to values comparable to those of age-matched untreated wt counterparts (Figures 5A and 5B). These data indicate that treatment of pre-cardiomyopathic mdx mice with a long-term, high-dose PMO treatment regimen prevents development of mdx cardiomyopathy.

**LONG-TERM, HIGH-DOSE PMO TREATMENT REGIMEN IS NOT TOXIC IN VIVO.** Following long-term, high-dose PMO treatment, serum was extracted and used to measure kidney and liver toxicity. Urea, creatinine, and alanine transaminase concentrations were measured. We found no evidence of toxicity following treatment of the mice (Figure 5C).

**DISCUSSION**

DMD patients exhibit progressive muscle degeneration primarily in skeletal and cardiac muscles. Advances in respiratory care have resulted in an increase in life expectancy for DMD patients over the last half-century; however, this prolongation of life has been accompanied by an increasing number of patients experiencing from an ultimately fatal dilated cardiomyopathy (23). The effect of several therapeutic approaches on DMD cardiac pathology have been investigated, with varying degrees of efficacy (8). This includes use of antisense oligonucleotides to alter exon or splice site selection in order to restore the correct dystrophin reading frame (24) and production of a truncated, but functional dystrophin protein.

Because approximately 70% of DMD-causing mutations are located between exons 45 and 55 (7), the majority of antisense oligonucleotides therapeutic development to date has focused on exon skipping within this region. It has been estimated that skipping exons 51, 45, and 53 could benefit 13%, 8.1%, and 7.7% of all DMD mutations, respectively (25). Two types of modified antisense oligonucleotides have been evaluated clinically for exon 51 skipping in DMD patients, including 2‘O-methylated phosphorothioate and PMO. Both have elicited recovery in skeletal and pulmonary function, but with limited ability to prevent progressive cardiac decline (8).

2‘O-methylated phosphorothioate oligonucleotides consist of a phosphorothioate backbone that contains internucleotide linkages that confer a negative charge, resulting in enhanced binding to circulatory proteins and an increase in plasma half-life (26). A phase 1 to 2a trial of this compound reported an improvement in the 6-min walk test in DMD patients 25 weeks following commencement of treatment (0.5 to 6 mg/kg for 5 weeks, followed by 6 mg/kg for 12 weeks) versus placebo, but by week 49, the difference was no longer significant (27,28). A subsequent phase 3 double-blinded placebo-controlled study involving prolonged treatment of DMD patients with 6 mg/kg/week (48 weeks) did not improve ambulation (6-min walk test) or secondary assessments of motor function (29).

Furthermore, adverse events including kidney and liver toxicity, likely to be off-target effects of the 2‘O-methylated phosphorothioate backbone, were reported in 46% of patients (27,28,30).

PMO consist of a 6-membered morpholine ring moiety, joined by phosphorodiamic acid linkages. Unlike phosphorothioates, the backbone carries no charge at physiological pH, reducing off-target effects (31). PMO are nontoxic and stable (32). A phase 2 randomized placebo-controlled study involving treatment of 8 DMD boys (7 to 13 years of age) with 30 or 50 mg/kg/week (24 weeks) demonstrated a significant increase in dystrophin production in these patients (Eteplirsen Study 201, Sarepta Therapeutics). With this, the placebo group joined the PMO treatment group. All 12 patients continued receiving PMO treatment in an ongoing extension study for over 3 years (phase 2b Eteplirsen Study 202, Sarepta Therapeutics) (9). At week 168, the continuously treated ambulatory patients continued to walk within 18% of their week-12 distance, whereas the placebo/delayed treatment cohort continued to walk within 23% of their week-36 distance (10). Pulmonary function remained stable in these patients, and the hallmark decline in respiration observed in DMD patients appears to have been repressed. Furthermore, no clinically significant treatment-related adverse events have been observed. However, cardiac abnormalities consistent with the DMD phenotype remain in these patients. Effective therapy must be able to improve cardiac function and prevent ultimately fatal dilated cardiomyopathy, without inducing toxicity.

The mdx mouse manifests a mild dystrophic phenotype relative to the human disease and is therefore not regarded as an ideal clinical model for DMD. However, it provides an excellent molecular model for the study of dystrophin function and novel therapeutics. Using the mdx mouse model of DMD, we previously identified that cytoskeletal disarray due to the absence of dystrophin results in impaired regulation of mitochondrial function by I$_{Ca-L}$ (11). Importantly, we identified that this decrease in myocardial metabolic activity occurs in pre-cardiomyopathic mdx mice and persists through to the development of the cardiomyopathy (11).
Cardiomyopathy in DMD patients shows variable onset and severity, with no clear correlation between genotype and phenotype. Conflicting reports on such an association are likely due to small sample size, variability in cardiac function analysis, clinical care and cardiac medications, and unverified consequences of the mutation on dystrophin transcript and/or protein (33–35). Patients with the same DMD genotype can show very different severity and age of onset of left ventricular dysfunction and, ultimately, survival (35). This confounds the interpretation of potential treatment-related effects. Although cardiomyopathy in the mdx mouse is relatively mild with late onset, this model has allowed us to investigate the mechanistic basis of metabolic dysfunction in the dystrophic heart and the consequences of partial restoration of a functional dystrophin. We have demonstrated that a long-term, low-dose PMO treatment regimen partially restores regulation of cardiac metabolic activity by $I_{Ca-L}$ (11).

Based on the success of current long-term, 30 to 50 mg/kg/week PMO trials on improving ambulation and pulmonary function, and the knowledge that the half-life of dystrophin is shorter in cardiac versus skeletal muscle (22), we reasoned that a higher PMO dose may induce a higher level of skipping of dystrophin exon 23 and expression of functional dystrophin in the mdx heart.

Initial studies indicated that treatment of precardiomyopathic mdx mice with a high PMO dose (120 mg/kg/week) for 3 weeks was sufficient to induce exon skipping and partially rescue alterations in $\Psi_m$ and metabolic activity in response to activation of $I_{Ca-L}$ (Figure 1) (11). Based on these findings, we proposed that a long-term, high-dose treatment regimen would result in a more complete restoration of $\Psi_m$ and metabolic activity. We find that administering precardiomyopathic mdx mice with 40 mg/kg PMO 3× per week for 19 weeks induced exon skipping and expression of dystrophin in the heart (Figure 2), completely rescued alterations in $\Psi_m$ and metabolic activity (Figures 3 and 4), prevented development of the cardiomyopathy, and, importantly, is not toxic in vivo (Figure 5). Consistent with previous studies, we find that PMO treatment results in accumulation of as little as 5% of normal dystrophin protein expression in mdx mice (Figure 2B) (36). Importantly, this is sufficient to result in functional improvement in the mdx heart (Figures 5A and 5B).

Using the mdx mouse model of DMD, we previously identified that cytoskeletal disarray due to the absence of dystrophin results in impaired regulation of mitochondrial function by $I_{Ca-L}$ (11). Additionally, we identified that metabolic dysfunction precedes development of mdx cardiomyopathy (11). Here we find that a long-term, high-dose yet nontoxic PMO treatment regimen that commences prior to the development of mdx cardiomyopathy completely restores metabolic function and, subsequently, prevents development of the cardiomyopathy. This study utilized the same PMO chemistry used in current clinical trials demonstrating that DMD patients receiving this treatment exhibit improved ambulation and stable pulmonary function (9,10). However, cardiac abnormalities consistent with the DMD phenotype remain in these patients. Based on our current findings, we propose that a long-term, high-dose PMO treatment regimen that commences prior to the development of cardiomyopathy may have the potential to concurrently improve ambulation, stabilize pulmonary function, and prevent cardiomyopathy in DMD patients.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Advances in respiratory care have resulted in an increase in life expectancy for DMD patients over the last half-century; however, this prolongation of life has been accompanied by an increasing number of patients suffering from an ultimately fatal dilated cardiomyopathy. Approximately 20% of DMD patient deaths result from dilated cardiomyopathy. Current clinical trials demonstrate that PMO therapy induces skipping of dystrophin exon 51 and accumulation of functional dystrophin, and that these patients exhibit improved ambulation and stable pulmonary function; however, cardiac abnormalities remain. Effective therapy must be able to improve cardiac function and prevent ultimately fatal dilated cardiomyopathy without inducing toxicity. Utilizing the same PMO chemistry as current clinical trials, we have identified a nontoxic PMO treatment regimen to restore metabolic activity and prevent mdx cardiomyopathy.

TRANSLATIONAL OUTLOOK: The efficacy of a long-term, high-dose PMO treatment regimen in DMD patients will now need to be determined. We propose that a treatment regimen of this nature may have the potential to significantly improve morbidity and mortality from DMD because in addition to improving ambulation and stabilizing pulmonary function, the treatment will prevent the development of cardiomyopathy in DMD patients.
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APPENDIX For supplemental material, please see the online version of this paper.