SUPPLEMENTAL INFORMATION

A morpholino oligomer therapy regime that restores mitochondrial function and prevents *mdx* cardiomyopathy

Brief Title: PMO therapy to prevent *mdx* cardiomyopathy

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Supplemental Methods

RNA preparation and RTPCR analysis

Muscle cryosections (4-6mg) cut from frozen tissue blocks, were used to extract total RNA from heart muscle using a MagMax-96 Total RNA Isolation Kit (Ambion, Melbourne, Australia) according to the manufacturer's protocol. RT-PCR was performed on 100 ng of total RNA for 40 cycles of amplification across exons 20-26 using a Superscript III One-step PCR system with Platinum Taq (Invitrogen, Melbourne, Australia) in a 12.5 µl reaction. The reaction volume was incubated at 55°C for 30 min before denaturation at 94°C for 2 min and 30 cycles of amplification of 94°C for 30 s, 55°C for 1 min, and 68°C extension for 2 min. A 2 µl sample from this reaction was then used as the template for secondary PCR amplification using 0.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, California) using an inner primer set with a 6 min denaturation included as the hot start, followed by 35 cycles of amplification of 94°C for 30 s, 55°C for 1 min, and 72°C extension for 2 min. Primers are listed in **Table 1**. Products were then electrophoresed on a 2% TAE agarose gel. Images were captured with a Fusion Fx Gel Documentation system (Vilber Lourmat, Marne La Valle, France).

Immunoblotting and Immunostaining

Dystrophin in cardiac muscle was assessed by immunoblot. Protein extracts were prepared from tissue cryosections (4.5 mg tissue per 150 µl treatment buffer). Samples (7.5 µg total protein) were loaded onto denaturing SDS – polyacrylamide electrophoresis (PAGE) gel with a 3% stacking gel and run at 30 mA for 2 h. Electrophoresis and western blotting were performed using NCL-DYS2 (Novocastra Laboratories, Newcastle-upon-Tyne, UK) (1), using a protocol derived from previously described methodology (2,3). Protein loading was

standardized according to myosin heavy chain expression, assessed by densitometry on a Coomassie blue stained gradient gel (1). Images were captured with a Fusion Fx gel documentation system (Vilber Lourmat, Marne La Valle, France).

Immunohistochemistry was performed on unfixed 6 µm heart, diaphragm and tibialis anterior cryosections from tissue snap frozen in liquid nitrogen-cooled isopentane. Serials sections were adhered to Superfrost Plus slides (Menzel-Gläser, supplied by ThermoFisher, Melbourne, Australia). Staining conditions for each antibody were as follows: Primary antibody (rabbit anti-dystrophin [ab15277], 1:200 dilution, Abcam) was incubated for 1 h at room temperature in PBT buffer containing 10% normal goat serum. Slides were washed in PBT and a secondary Alexafluor 488 antibody added (goat anti-rabbit immunoglobulins [A11008], 1:400 dilution, supplied by ThermoFisher, Melbourne, Australia). Fluorescence was assessed using a Nikon Eclipse 80i microscope at 20x magnification. All images were captured and processed using identical parameters (4,5).

Isolation of ventricular myocytes

Myocytes were isolated from *wt* and *mdx* mice. Animals were anesthetized with intraperitoneal injection of pentobarbitone sodium (240 mg/kg) prior to excision of the heart. Cells were isolated as previously described (6,7), based on methods described by O'Connell et al (8). Mouse hearts were excised, cannulated onto a Langendorff apparatus via the aorta and perfused with Krebs-Henseleit Buffer (KHB) containing (in mM): 120 NaCl, 25 $NaHCO_3$, 4.8 KCl, 2.2 $MgSO_4$, 1.2 NaH_2PO_4 and 11 glucose (pH = 7.35 with O_2/CO_2 at 37°C) for 4 min at 37°C. Hearts were then perfused with KHB supplemented with 2.4 mg/ml collagenase B for 3 min, followed by a further 8 min perfusion in the presence of 40 µM calcium. Following perfusion, hearts were placed in KHB supplemented with 10% fetal calf serum. Aorta and atria were removed and ventricles gently teased apart and triturated to

dissociate myocytes into suspension. Myocyte suspension was then spun at 500 RPM for 3 min, supernatant discarded, and myocytes resuspended in calcium free Hepes-Buffered Solution (HBS) containing (in mM): 5.3 KCl, 0.4 $MgSO_4.7H_2O$, 139 NaCl, 5.6 $Na_2HPO_4.2H_2O$, 5 glucose, 20 Hepes and 2 glutamine (pH = 7.4 at 37°C) in the presence or absence of 3 mM EGTA (for 0 mM calcium experiments). For calcium containing experiments, calcium was titrated back to achieve a final extracellular concentration of 2.5 mM. All experiments were performed in freshly isolated myocytes at 37°C.

Measurement of mitochondrial membrane potential (Ψ_m)

Fluorescent indicator 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 200 nM, ex 480 nm, em 580/535 nm, Molecular Probes, Eugene, Oregon) was used to measure Ψ_m in cardiac myocytes at 37°C as previously described (9). Cells were incubated in calcium-free HBS (0 mM calcium containing, supplemented with 3 mM EGTA and 200 nM JC-1) for at least 3 h prior to measuring changes in Ψ_m. Fluorescent signal was then measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Fluorescent images were taken every 2 min (50 ms exposure). Using Metamorph 6.3 580/535 nm ratiometric fluorescent signal was quantified by manually tracing myocytes and subtracting an equivalent background region that did not contain cells. Fluorescent values recorded over 6 min prior to and 14 min following addition of drugs were averaged and alterations in fluorescent ratios reported as a percentage increase from the basal average. 40 mM NaCN was added at the end of each experiment to collapse Ψ_m, confirming that the JC-1 signal was indicative of Ψ_m. Additionally, individual 580 nm and 535 nm wavelength signals were assessed in each experiment to determine whether the fluorescent indicator was accurately measuring Ψ_m.

Measurement of mitochondrial flavoprotein oxidation

Autofluorescence was used to measure flavoprotein oxidation in cardiac myocytes based on previously described methods (10-12). Fluorescence was measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope (ex 480 nm, em 535 nm). Fluorescent images were taken every 1 min (1.5 s exposure). Using Metamorph 6.3 fluorescent signal was quantified by manually tracing myocytes and subtracting an equivalent background region that did not contain cells. Fluorescent values recorded over 5 min prior to and 5 min following addition of drugs were averaged and alterations in fluorescent ratios reported as a percentage increase from the basal average. 50 μM Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was added at the end of each experiment to increase flavoprotein signal confirming the signal was mitochondrial in origin.

MTT assay

The basis for this assay is the reduction of yellow 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, Missouri) to purple formazan crystals by the electron transport chain within cardiac myocyte mitochondria (13,14). This reaction is dependent upon the presence of reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), and intact mitochondrial electron transport (15). An increase in formazan production (absorbance) indicates an increase in metabolic activity. Cardiac myocytes were treated with relevant drugs in 96 well plates. MTT was added to each well to a final concentration of 0.5 mg/ml and the rate of the increase in absorbance immediately measured using a spectrophotometer at 37°C (PowerWave XS, 570 nm, reference wavelength of 620 nm). The rate of the increase in absorbance in response to treatments was expressed as a percentage of the rate of the increase

prior to the addition of treatments. Each n represents number of replicates for each treatment group from myocytes isolated from a total of 9 *wt*, 6 *mdx* and 6 PMO treated *mdx* hearts.

Echocardiography

Echocardiographic measurement of left ventricular function were performed on mice under light methoxyflurane anesthesia using an i13L probe on a Vivid 7 Dimension (GE Healthcare, Little Chalfont, United Kingdom). Echocardiographic measurements were taken on M mode in triplicate from separate mice. Quantitative measurements represent the average of 24, 30, 38 and 43 week old *wt* (n = 3), *mdx* (n = 3-5) and PMO treated *mdx* (n = 4-9) mice. M-mode recordings were made at a sweep speed of 200 mm/s. Measurements of left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), fractional shortening (FS), left ventricular posterior wall in diastole (LVDPW), left ventricular posterior wall in systole (LVSPW), intraventricular septum in diastole (IVDS), intraventricular septum in systole (IVSS) and end diastolic diameter (EDD) were made. FS was calculated by the formula [(LVEDD-LVESD)/EDD] x 100.

Serum Parameters of Kidney and Liver Toxicity following in vivo Treatment

Twenty four week old *mdx* mice were treated with 40 mg/kg M23D three times per week for 19 weeks. At 19 weeks mice were anaesthetized and terminal blood collected. Serum was extracted and used to measure kidney and liver toxicity. Urea and creatinine concentration was assessed using QuantiChrom Urea and Creatinine assay kits respectively (BioAssay Systems, Hayward CA). Alanine transaminase (ALT) concentration was measured using an Alanine Transaminase assay kit (BioAssay Systems, Hayward CA). All assays were performed as per manufacturer’s instructions, using a spectrophotometer (PowerWave XS).

Supplemental References

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