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PRECLINICAL RESEARCH

Long-term blinded placebo-controlled study of SNT-MC17/idebenone in the dystrophin deficient *mdx* mouse: cardiac protection and improved exercise performance

Gunnar M. Buyse[1]*, Gerry Van der Mieren[2], Michael Erb[3], Jan D'hooge[4], Paul Herijgers[2], Erik Verbeken[5], Alejandro Jara[6], An Van Den Bergh[2], Luc Mertens[7], Isabelle Courdier-Fruh[3], Patrizia Barzaghi[3], and Thomas Meier[3]

[1]Department of Pediatric Neurology, University Hospitals Leuven, Herestraat 49, B-3000 Leuven, Belgium; [2]Department of Experimental Cardiac Surgery, University Hospitals Leuven, Leuven, Belgium; [3]Santhera Pharmaceuticals, Liestal, Switzerland; [4]Department of Cardiovascular Imaging and Dynamics, University Hospitals Leuven, Leuven, Belgium; [5]Department of Morphology and Molecular Pathology, University Hospitals Leuven, Leuven, Belgium; [6]Biostatistical Center KU Leuven, Leuven, Belgium; and [7]Department of Pediatric Cardiology, University Hospitals Leuven, Leuven, Belgium

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Aims Duchenne muscular dystrophy (DMD) is a severe and still incurable disease, with heart failure as a leading cause of death. The identification of a disease-modifying therapy may require early-initiated and long-term administration, but such type of therapeutic trial is not evident in humans. We have performed such a trial of SNT-MC17/idebenone in the *mdx* mouse model of DMD, based on the drug's potential to improve mitochondrial respiratory chain function and reduce oxidative stress.

Methods and results In this study, 200 mg/kg bodyweight of either SNT-MC17/idebenone or placebo was given from age 4 weeks until 10 months in *mdx* and wild-type mice. All evaluators were blinded to mouse type and treatment groups. Idebenone treatment significantly corrected cardiac diastolic dysfunction and prevented mortality from cardiac pump failure induced by dobutamine stress testing *in vivo*, significantly reduced cardiac inflammation and fibrosis, and significantly improved voluntary running performance in *mdx* mice.

Conclusion We have identified a novel potential therapeutic strategy for human DMD, as SNT-MC17/idebenone was cardioprotective and improved exercise performance in the dystrophin-deficient *mdx* mouse. Our data also illustrate that the *mdx* mouse provides unique opportunities for long-term controlled prehuman therapeutic studies.

Keywords Muscular dystrophy • Therapy • Cardiomyopathy • Hemodynamics • Heart failure • Animal model

Introduction

Duchenne muscular dystrophy (DMD) is the most common and devastating type of muscular dystrophy worldwide, affecting one in 3500 live male births.[1] This progressive and lethal X-linked myopathy is characterized by deficiency of dystrophin, a subsarcolemmal protein critical in membrane stabilization and prevention of contraction-induced cell membrane damage. Progressive striated muscle weakness and cardiomyopathy lead to severe disability and mortality of patients in their late teens to early twenties.

* Corresponding author. Tel: +32 16 34 38 45, Fax: +32 16 34 38 42, Email: gunnar.buyse@uzleuven.be

Following implementation of ventilatory assistance to treat respiratory failure, heart failure has become a leading cause of death in DMD.[2,3] In young DMD patients with still normal cardiac ventricular function at rest, reduced left ventricular contractile reserve (determined by inotropic stimulation with dobutamine) predicts later decline in cardiac function with age.[4]

Causative gene identification and pathophysiological insights have fuelled increasing therapeutic research efforts for DMD, of which the vast majority has been focused on the skeletal muscle involvement. Nevertheless currently no effective treatment exists, only corticosteroids have proved of some benefit but their long-term use is hampered by significant adverse effects. A major lesson from performed human trials that aimed to identify a disease-modifying treatment for DMD seems to be their apparently inevitable methodological shortcomings. Indeed, it is logic that the identification of a disease-modifying compound may require early-initiated (i.e. before onset of pathology) and long-term administration, but such type of therapeutic trial is not evident in human patients. We have performed such a trial of idebenone in the homologous dystrophin-deficient *mdx* mouse model of DMD,[5] based on the drug's potential to improve mitochondrial respiratory chain function and cellular energy production, as well as its potency to reduce oxidative stress.[6,7] It was anticipated that early-initiated and long-term idebenone-mediated blocking of these important downstream effectors of dystrophin-deficiency would result in a reduced disease state in treated *mdx* mice at old age. Other than facilitating presymptomatic initiation and veritable long-term administration of treatment, the mouse model allowed invasive *in vivo* haemodynamic studies for the assessment of cardiac contractility. Limitations of the study were the required use of anaesthesia (for cardiac assessments) and the multiple endpoint testing.

Methods

Animals

Male wild-type (C57BL/10ScSn) and dystrophin-deficient *mdx* (C57BL/10ScSn-*Dmd*^{mdx}/J) mice obtained from Jackson Laboratories were maintained at RCC Laboratory Services (Füllinsdorf, Switzerland). Sedentary mice (for cardiac assessments at age 10 months) from one litter were housed together from weaning to the end of the study period. Mice used for voluntary wheel running assessments (exercised mice) were housed individually in wheel cages from weaning to the end of the study period. All mice were kept under artificial light from 5 am to 5 pm and in darkness from 5 pm to 5 am throughout their life span. All procedures were performed in accordance with the Belgian and Swiss regulations and under the required licenses, approval of procedures was obtained from the KU Leuven committee on the use and care of animals.

Treatment protocol, treatment groups, and flow of animals

The molecule investigated in this study is idebenone: 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (research code: 'SNT-MC17'; International non-proprietary name (INN): 'idebenone'). A dose of 200 mg/kg bodyweight of either idebenone ('ide': SNT-MC17 from Santhera Pharmaceuticals, Liestal) or vehicle ('veh'; placebo) was given once daily by gavage from the age of 4 weeks until the age of 10 months in *mdx* ('*mdx*-ide' group, $n = 18$; '*mdx*-veh' group, $n = 14$) and wild-type mice ('wt-veh' group, $n = 10$). For wheel running performance (exercised mice), similar groups were treated from age 3 weeks until 12 weeks (wt-veh, $n = 8$; *mdx*-veh, $n = 10$; *mdx*-ide, $n = 17$). Animals were randomized per cage to the treatment groups. Litters of breeding cages were randomized to placebo (vehicle) and idebenone in an alternate fashion. All animals were included in the study between 17 April 2005 and 29 May 2005 (42 days). Vehicle consisted of 0.5% carboxymethylcellulose sodium salt (CMC, Fluka, Buchs, Switzerland) in water. To ensure accurate dosing of animals, idebenone was administered by gavage rather than by food mix. Since it was only feasible to administer animals once a day, a dose of 200 mg/kg/day was chosen as it represented a dose similar to the highest exposed animals (mice) in a therapeutic study in frataxin-deficiency.[8] During the course of the study one animal in the *mdx*-veh group died around 7 months and one animal in the wt-veh group had to be killed at 6 months due to a tumor-like swelling in the abdomen. From one animal in the *mdx*-ide group there were no serum-derived measurements (cardiac biomarkers) available at baseline as the serum sample was lost in process. For the cardiac haemodynamic data assessments at age 10 months, three experiments were excluded because of technical reasons (problems with catheter insertion; two animals from *mdx*-ide group, one animal from wt-veh group), and one experiment was excluded because of a technical complication (liver tear) with haemodynamic instability (one animal in *mdx*-veh group). All animals allocated to the wheel running experiment completed the experiment.

Echocardiography at age 10 months

Mice were anaesthetized by 2% isoflurane inhalation. After shaving their chest, the animals were positioned in a left decubitus position on a heating pad for body temperature maintenance. Animals were imaged using a Vivid7 Dimensions (GE Vingmed, Horten, Norway) equipped with a 96-element linear array transducer (i13L) transmitting at 14 MHz. Standard gray scale M- and B-mode images were acquired using a parasternal long- and short-axis view. Grayscale measurements were made off-line on a dedicated workstation (EchoPac, GE Vingmed). End-diastolic and end-systolic wall thickness of the anteroseptal (IVS) and inferolateral (PW) wall segments were measured together with the end-diastolic and end-systolic left ventricular internal diameter on both the M- and B-mode acquisitions from which fractional shortening (FS) was derived. From the average of the M- and B-mode assessment of ventricular morphology, LV myocardial volume (LVV), LV end-diastolic (EDV) and end-systolic (ESV) volumes were calculated using a half ellipsoid model of the LV. In this model, the long-axis dimension of the individual heart was taken into account by measuring it on the long-axis images. From these volumes LV ejection fraction (EF) was calculated. Finally, anteroseptal and inferolateral wall thickening was calculated as the change in thickness relative to the end-diastolic thickness and expressed as a percentage. All data were collected and analysed by two independent observers blinded to mouse type and randomization. For all measurements, the average value of both observers was used for statistical analysis.

In vivo cardiac haemodynamic measurements at age 10 months

Following anaesthesia for echocardiography, mice were allowed to recover completely for at least 3 days prior to haemodynamic assessment of cardiac contractility. For this, anaesthetized mice (intraperitoneal urethane 1200 mg/kg and alfa-chloralose 50 mg/kg) were tracheotomized and mechanically ventilated (Minivent 845; Hugo Sachs/Harvard Apparatus, March-Hugstetten, Germany). Body temperature was monitored with a rectal probe and maintained at 37°C

with a heating pad. A precalibrated four-electrode pressure-conductance catheter (1.4 Fr, SPR-839; Millar Instruments, Houston, TX) was inserted in the right carotid artery and advanced into the LV to measure instantaneous intraventricular pressure and volume (PV-loops).[9,10] After stabilization, baseline haemodynamic data were recorded. Subsequently LV preload was decreased by transient inferior caval vein occlusion (with cotton swab) while PV-loops (occlusion loops) were recorded, allowing to derive load-independent (intrinsic) contractility parameters. Afterwards, dobutamine was infused through a right jugular vein catheter at incremental doses of 1, 3, 10, and 30 ng/g/min each time for 2 min until a stable heart rate plateau was reached. During progressive dobutamine exposure, PV loops were recorded (allowing determination of contractile reserve). Next the dobutamine dose was stepwise reduced from 30 to 10 to 3 and to 1 ng/g/min, and each time occlusion loops were recorded. The parallel conductance attributed to tissues surrounding the LV cavity was estimated by bolus injection of 1.5 μl of 30% NaCl into the jugular vein. Blood conductance was determined at the end of each experiment using three precalibrated cuvettes, a conductance-volume calibration line was constructed with these cuvet data. Analysis was performed from each experiment with correction for parallel volume, and data were expressed in absolute volumes (PVAN software, Millar Instruments). All data are the average of at least five measurements during the experiment, each measurement representing at least 10 successive loops. Only technically acceptable loops (no arrhythmia, stable baseline) were included in the analysis for each experiment, which was performed blinded to mouse type and treatment groups.

Cardiac histology at age 10 months

After cardiac catheterizations, the animals were sacrificed, the hearts removed and fixed in a 6% formalin solution. The ventricles were cut into four slices parallel to the basis of the heart, and embedded in paraffin. Four-micron-thick histological sections were prepared in a standard way and stained with H&E and with Masson's trichrome for collagen. Sections were examined by light microscopy for the presence of inflammation and fibrosis, blinded to mouse type and treatment groups. The RV, and the septum, the anterior, lateral and posterior wall of the LV were assessed separately. The proportion of fibrosis was quantified by a conventional point counting method.[11] For each area, a total of 250 points were counted in three random fields, and the results were expressed as the proportion (in %) of the points hitting fibrosis. The amount of inflammation was scored from 0 (absent) to 3 (most severe case in our series) in five random fields per area. An average value was also calculated.

Striated muscle histology at age 10 months

The fibre diameter variability (minimal Feret's diameter variance coefficients) and the percentage of centralized nuclei in the diaphragm and the quadriceps muscle were analysed as previously described.[12]

Cardiac biomarkers

Cardiac troponin I (cTnI) levels were analysed in blood serum using the High Sensitivity Mouse Cardiac troponin-I Elisa Kit (Life Diagnostics, West Chester, USA). Blood samples were serially taken at ages 1, 6, and 10 months (retro-orbital sinus/plexus sampling; sample volume ~150 μL).

Computerized wheel running analysis

Voluntary exercise performance was monitored in parallel groups of mice from the age of 29 days (after 1 day acclimation to the monitoring equipment) until age 12 weeks. Voluntary running activity was measured with a computerized wheel system essentially as described previously.[13] Wheel revolution counts were recorded every 10 s. Since all mice run extensively during the night and showed only little and irregular daytime running, only night-time running was included into the analysis. Beside the total daily running distance, a subset of running events (10 s time windows) with a wheel revolution above a speed threshold equivalent to 1.75 km/h was analysed separately.

Statistical analyses

(i) Echocardiography and cardiac haemodynamic measurements: Analysis was performed using Statistica 7.1, with Kruskal–Wallis ANOVA and χ^2 for *post-hoc* analyses (including Bonferroni adjustments).[14] For the comparison of haemodynamic parameters at baseline (non-stress) conditions that could predict mortality during dobutamine stress Mann–Whitney *U* tests were used. (ii) Cardiac histology: In order to take into account the correlated nature of the data a first-order Generalized Estimating Equation (GEE) approach was employed using independent and exchangeable working correlations matrices. Marginal binary and ordinal logistic regression models were considered for the analysis of fibrosis and inflammation, respectively. For the analysis of inflammation, different models for ordinal data were intended. However, only proportional odds models were possible to be fitted due to sparseness of the sample. In each of the fitted models, the effect of the group, area, and group × area interaction term were included. The evaluation of the effect of the factors in the model was performed by using the Score-test for type 3 GEE analysis. Bonferroni adjustments to the significance levels for pairwise comparisons were performed when significant differences were found with the Score-test. Analyses were performed using SAS (9.1 for Windows). The GEE models were fitted using the procedure PROC GENMOD. (iii) Wheel running data: Analysis of the wheel running data (daily running distance, high speed running distance) integrating the whole analysed time period was done with a two-way ANOVA using the 'aov' method in the S-Plus software and with adapted (Bonferroni adjustment) significance levels for pairwise comparisons.

Results

In vivo cardiac haemodynamics at age 10 months: idebenone prevents diastolic dysfunction

The results of the comparison of the haemodynamic variables (under baseline conditions) between the different treatment groups at age 10 months are shown in *Table 1*. The heart weight (corrected for tibial length) was significantly higher in both *mdx* groups compared with the wild-type group, implying cardiac hypertrophy in *mdx* mice that was not corrected by idebenone treatment. Heart rates were similar in placebo-treated wild-type and *mdx* groups, but were higher in the idebenone treated *mdx* group. End-diastolic pressure, a measure of heart failure, was significantly increased in the placebo-treated *mdx* group, and corrected to wild-type levels in the idebenone-treated *mdx* group. In idebenone-treated *mdx* mice isovolumetric relaxation during diastole (Tau) was significantly faster than in placebo-treated *mdx* mice, and comparable with wild-type levels. Other load-dependent and -independent contractility measures under baseline conditions showed no further significant differences between the different groups.

Table 1 Comparison of haemodynamic parameters (mean ± SD) for wild-type vehicle-treated, *mdx* vehicle-treated, and *mdx* idebenone-treated mice at age 10 months

	WT-veh $n = 8$	mdx-veh $n = 12$	mdx-ide $n = 16$	ANOVA *P*-value
HW/TL (mg/cm)	72.2 ± 8.6	82.5 ± 8.8*	83.5 ± 9.9*	**0.022**
Parameters in steady state				
HR (b.p.m.)	501 ± 37	483 ± 36	532 ± 48**	**0.015**
Pmax (mmHg)	82.6 ± 7.5	80.6 ± 8.7	84.3 ± 11.8	0.621
Pes (mmHg)	73.7 ± 10.8	72.2 ± 9.5	77.7 ± 12.6	0.423
Ped (mmHg)	3.6 ± 1.4	5.4 ± 2.2*	3.4 ± 1.3**	**0.013**
Ved (μL)	30.1 ± 11.4	32.0 ± 7.5	32.1 ± 11.0	0.887
SV (μL)	16.1 ± 5.8	15.8 ± 3.6	15.9 ± 6.1	0.990
EF (%)	53.2 ± 11.7	47.6 ± 9.8	48.1 ± 11.1	0.483
CO (μL/min)	8149 ± 3189	7599 ± 1703	8639 ± 3846	0.689
SW (mmHg μL)	1096 ± 445	1021 ± 367	1159 ± 612	0.775
Ea (mmHg/μL)	5.6 ± 3.8	4.8 ± 1.0	5.5 ± 2.0	0.630
dP/dtmax (mmHg/s)	6349 ± 1005	5907 ± 1623	7466 ± 3099	0.209
dP/dtmin (mmHg/s)	−6257 ± 777	−5564 ± 1321	−6696 ± 1692	0.128
Tau (ms)	6.3 ± 0.9	7.0 ± 1.0	6.0 ± 0.9**	**0.032**
Parameters obtained after temporary preload reduction				
PAMP (mW/μL^2)	88 ± 50	62 ± 27	76 ± 41	0.325
Ees (mmHg/μL)	8.4 ± 0.9	8.0 ± 1.1	8.2 ± 1.2	0.721
PRSW (mmHg)	71.4 ± 8.3	70.1 ± 13.8	70.7 ± 14.9	0.978
EDPVR (mmHg/μL)	0.30 ± 0.17	0.42 ± 0.20	0.35 ± 0.14	0.260
dP/dt_EDV (mmHg μL/s)	303 ± 91	359 ± 203	296 ± 81	0.456
PVA (mmHg μL)	1328 ± 472	1310 ± 584	1281 ± 478	0.975
Efficiency (%)	58.1 ± 10.3	55.2 ± 9.8	61.3 ± 10.7	0.310

*$P < 0.05$ vs. WT-veh.
**$P < 0.05$ vs. *mdx*-veh.
HW/TL indicates heart weight corrected for tibial length; HR, heart rate; Pmax, maximum pressure; Pes, end-systolic pressure; Ped, end-diastolic pressure; Ved, end-diastolic volume; SV, stroke volume; EF, ejection fraction; CO, cardiac output; SW, stroke work; Ea, arterial elastance; PAMP, preload adjusted maximal power; Ees, end-systolic elastance; PRSW, preload recruitable stroke work; EDPVR, end-diastolic pressure–volume relationship; PVA, pressure–volume area.

Idebenone prevents dobutamine-induced cardiac failure in 10 month old mdx mice

Following baseline haemodynamic assessments, a dobutamine stress test was applied to assess cardiac contractile reserve (detection of subclinical cardiac failure) in the different treatment groups. Upon progressive challenge with the β-adrenergic receptor agonist dobutamine (incremental doses of 1, 3, 10, and 30 ng/g/min), already at low dobutamine doses placebo-treated *mdx* mice showed a 58% (7/12) mortality rate due to acute cardiac decompensation (*Figure 1*; lethal dobutamine doses: 1 ng/g/min for $n = 3$, 10 ng/g/min for $n = 3$, and 30 ng/g/min for $n = 1$). This cardiac failure was manifested by abrupt left ventricular dilatation and decreasing LV systolic pressures, and was not preceded by arrhythmias, indicating a primary heart failure based on diminished contractility. Cardiac pump failure during the dobutamine stress test was significantly prevented in *mdx* mice that had been treated with idebenone (*Figure 1*; reduction of heart failure and mortality to 19%, $P = 0.03$).

Given the pronounced differences in cardiac contractile reserve and mortality rate between idebenone- and placebo-treated *mdx* mice, we wanted to find parameters at baseline (non-stress) conditions that could predict mortality during the dobutamine stress test. Differences in all measured haemodynamic variables were analysed for mice that survived the stress test (pooled surviving animals from WT-veh, *mdx*-veh, and *mdx*-ide groups) vs. placebo-treated *mdx* mice that did not survive the stress test (*Table 2*). Non-surviving *mdx*-veh mice had a higher heart weight (cardiac hypertrophy) and showed reduced diastolic and systolic contractility at baseline conditions. Reduced values for the load-independent parameters PAMP (preload adjusted maximal power), Ees (end-systolic elastance), and PRSW (preload recruitable stroke work) in non-surviving mice indicate that it is a reduced intrinsic myocardial contractility that predicts lethal cardiac failure during dobutamine challenge.

Echocardiographic findings at age 10 months

The cardiac status of *mdx* and wild-type mice that had been long-term treated with placebo or idebenone was also evaluated by murine echocardiography at age 10 months (data summarized in *Table 3*). Morphological assessments showed significant differences (ANOVA) for end-diastolic diameters for the *mdx* mice. In *mdx*

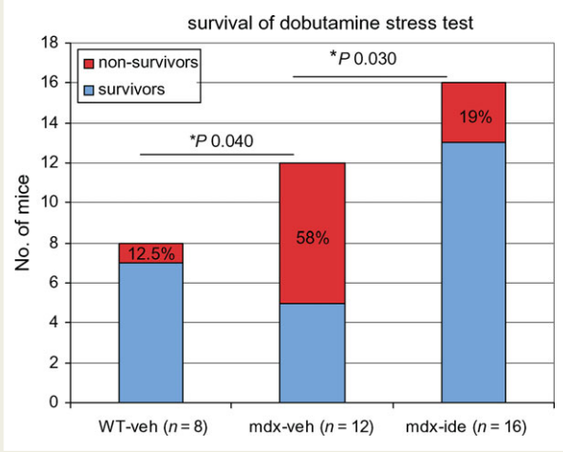

Figure 1 Idebenone significantly improves cardiac contractile reserve in *mdx* mice. Significant differences (Kruskal–Wallis ANOVA $P = 0.040$) in survival rate of dobutamine stress test protocol in 10 months old wild-type vehicle-treated, *mdx* vehicle-treated, and *mdx* idebenone-treated mice. Non-surviving mice died from acute heart failure based upon primary systolic contractile failure.

Table 2 Reduced diastolic and systolic contractility at baseline conditions (prior to inotropic challenge) predicts cardiac failure during dobutamine stress: comparison of baseline haemodynamic parameters (mean ± SD) of survivors (from all treatment groups) and non-survivors (from *mdx*-veh group only) of a physiological dobutamine stress test

	Survivors of full protocol, $n = 25$	Non-survivors (*mdx*-veh), $n = 7$	*P*-value
HW/TL (mg/cm)	78.0 ± 9.0	86.7 ± 9.4	0.032
Pmax (mmHg)	85.3 ± 9.6	79.1 ± 9.3	0.139
Pes (mmHg)	77.6 ± 11.1	71.9 ± 9.0	0.225
Ves (mL)	17.0 ± 6.5	23.9 ± 6.0	0.019
EF (%)	51.5 ± 8.3	41.5 ± 4.3	0.004
dP/dtmax (mmHg/s)	7249 ± 2487	5620 ± 1777	0.117
dP/dtmin (mmHg/s)	−6658 ± 1326	−5302 ± 1560	0.028
PAMP (mW/μL^2)	85.8 ± 39.4	45.1 ± 13.5	0.013
Ees (mmHg/μL)	8.46 ± 1.00	7.72 ± 1.03	0.094
PRSW (mmHg)	73.6 ± 11.6	64.0 ± 12.3	0.068
Tau (ms)	6.3 ± 0.9	7.0 ± 1.0	0.062

mice, idebenone treatment significantly improved end-diastolic volumes, suggesting improved diastolic filling properties. Whereas vehicle-treated *mdx* mice showed a tendency towards posterior wall hypertrophy (increased PWd), posterior wall thickness in idebenone-treated *mdx* mice was not different from wild-type mice although this did not reach statistical significance ($P = 0.095$). Grayscale function comparisons showed significant differences (ANOVA) for systolic anteroseptal wall thickening (IVS WT, IVS WT/PW WT) in both *mdx* groups. This anteroseptal wall hyperfunction in *mdx* mice could be compensatory for the hypertrophic posterior wall, the heart segment that also in human DMD is affected first. In *mdx* mice idebenone treatment showed a trend of reducing systolic anteroseptal wall thickening, but this was not significant (values in both *mdx* groups statistically different from wild-type levels).

Histological studies at age 10 months: idebenone reduces cardiac inflammation and fibrosis

Results of morphometric analysis of cardiac inflammation and fibrosis in 10-month-old wild-type and *mdx* mice (placebo or idebenone treated) are shown in *Tables 4* and *5*. For fibrosis (*Table 4*), GEE logistic regression analysis showed that the odd of developing fibrosis was, respectively, 15.80 (adjusted CI: 4.89–49.46, $P < 0.0001$) and 11.36 (adjusted CI: 3.50–35.71, $P < 0.0001$) times higher in the *mdx*-vehicle and *mdx*-idebenone groups when compared with the wild type-vehicle group. The odd of developing fibrosis was significantly 1.39 times higher (adjusted CI: 1.07–1.85, $P = 0.0062$) in the *mdx*-vehicle compared with the *mdx*-idebenone group. The absence of a significant group × area interaction term in the models ($P = 0.2048$ and $P = 0.1999$ under the independent and exchangeable model, respectively) suggests that is not possible to reject the hypothesis of null difference in the probability of developing fibrosis among the groups across the heart areas. The wild-type-vehicle group showed no inflammation whatsoever (score 0 for all mice in all heart regions) (*Table 5*). Because of that, the wild-type-vehicle group was removed from the analysis. GEE regression analysis showed a non-significant group × area interaction suggesting that the differences in the distribution of inflammation scores between the two groups are the same across the heart areas. The results indicate that the percentage of subjects in high inflammation scores is significantly lower ($P = 0.0037$) in the *mdx*-idebenone compared with the *mdx*-vehicle group.

In skeletal muscle (quadriceps) and diaphragm, histological assessments of fibre diameter variability and percentage of fibres with centralized nuclei showed no significant differences between the *mdx*-idebenone group and the *mdx*-vehicle group, neither in 10-month-old sedentary mice, nor in 12-week-old exercised (voluntary wheel running) mice (no further data shown).

Effects of idebenone on biomarkers reflecting myocardial degeneration

Serum levels of cardiac Troponin I, a marker reflecting degree of active myocardial degeneration, were measured in the different treatment groups at different ages (at age 4 weeks prior to initiation of treatment, age 6 months, age 10 months) (*Table 6*).

Table 3 Comparisons of echocardiography values (mean ± SD) for wild-type vehicle-treated, *mdx* vehicle-treated, and *mdx* idebenone-treated mice at age 10 months

	WT-veh, *n* = 9	mdx-veh, *n* = 13	mdx-ide, *n* = 18	ANOVA *P*-value
Morphology				
IVSd (mm)	0.79 ± 0.06	0.83 ± 0.10	0.84 ± 0.08	0.154
EDD (mm)	4.15 ± 0.21	3.83 ± 0.25*	3.9 ± 0.29*	0.022
PWd (mm)	0.70 ± 0.12	0.86 ± 0.14	0.79 ± 0.12	0.095
EDV (μL)	54.0 ± 6.4	44.6 ± 7.5*	47.4 ± 8.8	0.022
LVV (μL)	45.6 ± 3.9	47.5 ± 5.5	47.9 ± 5.6	0.522
Grayscale function				
HR (b.p.m.)	394 ± 79	454 ± 76	454 ± 86	0.252
FS (%)	24 ± 4	29 ± 8	27 ± 4	0.158
EF (%)	56 ± 7	63 ± 12	62 ± 7	0.158
IVS WT (%)	35 ± 7	50 ± 21*	46 ± 12*	0.022
PW WT (%)	40 ± 10	36 ± 14	34 ± 10	0.429
IVS WT/PW WT	0.92 ± 0.22	1.52 ± 0.72*	1.47 ± 0.64*	0.023

*Different ($P < 0.05$) from WT-vehicle.
LV, left ventricular; IVSd, end-diastolic anteroseptal wall thickness; EDD, LV end-diastolic diameter; PWd, LV inferolateral (posterior) wall thickness; EDV, LV end-diastolic volume; LVV, LV volume; HR, heart rate; FS, fractional shortening; EF, ejection fraction; IVS WT, LV anteroseptal wall thickening; PW WT, LV inferolateral (posterior) wall thickening.

Table 4 Degree of cardiac fibrosis (mean ± SD) in 10 month old mice [wild-type and *mdx*; vehicle ('veh') or idebenone ('ide) treated since age 4 weeks] (percentage of cardiac fibrosis by group and cardiac area)

Area	Group					
	WT-veh		mdx-veh		mdx-ide	
	n		*n*		*n*	
IVS	9	0.089 ± 0.267	13	2.677 ± 3.838	18	2.289 ± 2.249
LVAW	9	0.178 ± 0.211	13	1.138 ± 1.253	18	1.533 ± 2.144
LVLW	9	0.044 ± 0.133	13	0.738 ± 1.176	18	1.444 ± 2.852
LVPW	9	0.089 ± 0.176	13	2.523 ± 4.640	18	1.311 ± 1.734
RV	9	0.222 ± 0.406	13	3.415 ± 1.779	18	2.467 ± 1.560
All	45	0.124 ± 0.253	65	2.098 ± 2.994	90	1.809 ± 2.160

LV, left ventricular; IVS, interventricular septum; LVAW, LV anterior wall; LVLW, LV lateral wall; LVPW, LV posterior wall; RV, right ventricle.

Low levels were seen at age 4 weeks both in wild-type and *mdx* mice. Whereas cTnI levels remained low in wild-type mice at ages 6 and 10 months, at those ages levels were strongly elevated in *mdx* mice. Although at ages 6 and 10 months mean cTnI levels were lower in the *mdx*-idebenone compared with the *mdx*-vehicle group, indicating reduced myocardial degeneration, these differences were not statistically significant.

Idebenone improves voluntary wheel running performance

To evaluate if idebenone treatment improves the exercise performance of dystrophic *mdx* mice, voluntary wheel running performances of placebo (vehicle) treated wild-type mice and of placebo treated and idebenone treated *mdx* mice were recorded and analysed from the age of 29 days until age 12 weeks (*Figure 2*). Compared with vehicle-treated wild-type mice, daily running distances were significantly lower in the vehicle-treated *mdx* group. Idebenone treatment resulted in a significant and consistent improvement of daily running distances in *mdx* mice (*Figure 2A*). Cumulative running distance analysis revealed that idebenone treated *mdx* mice (mean 298 691 m) on average ran 57 507 metres more (95% CI: 1550–113 463, *t*-test: $P = 0.045$) during the 58-day study period than vehicle-treated *mdx* mice (mean 241 184 m) (*Figure 2B*). Further analyses revealed that these differences in daily and cumulative running distances were not due to differences in daily running time (data not shown), but due to significantly improved high speed running with idebenone treatment resulting in a greater distance run above a pre-set threshold (1.75 km/h) for running speed (*Figure 2C* and *D*).

Table 5 Degree of cardiac inflammation in 10 month old mice [wild-type and *mdx*; vehicle ('veh') or idebenone ('ide) treated since age 4 weeks] (distribution of inflammation score by group and cardiac area)

Area	Score	Group					
		WT-veh ($n = 9$)		mdx-veh ($n = 13$)		mdx-ide ($n = 18$)	
		n	%	*n*	%	*n*	%
IVS	0	9	100.00	0	0.00	0	0.00
	1	0	0.00	6	46.15	12	66.67
	2	0	0.00	6	46.15	6	33.33
	3	0	0.00	1	7.69	0	0
LVAW	0	9	100.00	3	23.08	11	61.11
	1	0	0.00	9	69.23	6	33.33
	2	0	0.00	1	7.69	1	5.56
	3	0	0.00	0	0.00	0	0.00
LVLW	0	9	100.00	6	46.15	11	61.11
	1	0	0.00	4	30.77	5	27.78
	2	0	0.00	3	23.08	2	11.11
	3	0	0.00	0	0.00	0	0.00
LVPW	0	9	100.00	5	38.46	9	50.00
	1	0	0.00	7	53.85	7	38.89
	2	0	0.00	1	7.69	2	11.11
	3	0	0.00	0	0.00	0	0.00
RV	0	9	100.00	0	0.00	1	5.56
	1	0	0.00	3	23.08	13	72.22
	2	0	0.00	10	76.92	4	22.22
	3	0	0.00	0	0.00	0	0.00
All	0	45	100.00	14	21.54	32	35.56
	1	0	0.00	29	44.62	43	47.78
	2	0	0.00	21	32.31	15	16.67
	3	0	0.00	1	1.54	0	0.00

A score of 0 equals no inflammation, a score of 3 equals maximal inflammation. LV, left ventricular; IVS, interventricular septum; LVAW, LV anterior wall; LVLW, LV lateral wall; LVPW, LV posterior wall; RV, right ventricle.

Discussion

The main finding of our study is that presymptomatic-initiated and long-term idebenone treatment significantly corrected (prevented) cardiac diastolic dysfunction, blocked the development of lethal acute heart failure during a dobutamine-mediated stress protocol (improvement of contractile reserve), reduced cardiac inflammation and fibrosis, and improved voluntary running performance in the dystrophin-deficient *mdx* mouse. As such, this study provides the first evidence ever for a potential therapeutic role of idebenone in dystrophin deficient muscular dystrophy. Whereas mice are not man and future human studies will have to show whether therapeutic studies in a homologous mouse model of dystrophin deficiency can be predictive for the human DMD situation, the strengths of the current study are the early initiation (in a presymptomatic stage) of treatment, its veritable long-term administration (far into adulthood for the mouse), and the extend of used *in vivo* outcome assessment methods (including invasive haemodynamic measurements for cardiac contractility assessment).

Limitations of this study are the required use of anaesthesia for cardiac assessments, and the multitude of endpoints being analysed (implying caution with the interpretation of statistically significant results). Whereas it seems that the anaesthesia may have reduced left ventricular contractility during the echocardiographic assessments (isoflurane anesthesia), this is expected to be only minor or modest for the haemodynamic assessments under urethane plus alfa-chloralose anaesthesia (as shown by the measured load-independent parameters). Furthermore, the anaesthesia was identical in all treatment groups of mice, which makes it unlikely that anaesthesia *in se* is a major determinant of the observed differences between the groups. Regarding the multitude of analysed endpoints, it was not possible for this study to have a trial design with a predefined primary outcome measure because of insufficient previous detailed knowledge about the cardiac phenotype in the *mdx* mouse.

Compared with wild-type mice, 10-month-old untreated *mdx* mice (placebo group) showed significant cardiac hypertrophy and diastolic dysfunction, the latter being prevented by idebenone treatment. It has been shown that acute cardiomyopathy and heart failure can be induced by cardiovascular stressors (dobutamine) in *mdx* mice.[15] Also, depressed cardiac contractile reserve has been shown in DMD patients with low-dose dobutamine stress echocardiography or angiography.[16] We therefore tested whether an acute dobutamine stress challenge *in vivo* could cause acute heart failure, and whether this phenotype could be blocked (prevented) by idebenone. Upon challenge with dobutamine, placebo-treated *mdx* mice developed acute cardiac decompensation with a 58% mortality rate. This heart failure during dobutamine stress was significantly blocked in *mdx* mice that had received idebenone treatment (reduction of mortality to 19%). These findings gain further significance from recent clinical studies in DMD patients where dobutamine-induced alterations in heart functions provided the single best indicator of age-related decline in cardiac function.[4] Our further analyses revealed indications that cardiac hypertrophy and reduced intrinsic myocardial contractility at baseline (non-stress) conditions were predictive for lethal cardiac failure during dobutamine challenge. This indicates that the protective ability of idebenone during stress results from preserved ventricular contractile properties and compliance. Besides these cardioprotective effects, we found that idebenone treatment was associated with significantly improved voluntary wheel running performances (increased daily and cumulative running distances). Remarkably, idebenone-treated *mdx* mice were capable of running faster and performed longer distances at high speed than placebo-treated *mdx* mice. At present it cannot be distinguished whether the improvements in exercise performance of these mice are linked to cardiac, skeletal muscle, and/or other changes.

The beneficial effects of idebenone can be explained by its ability to improve mitochondrial respiratory chain function and to reduce oxidative stress, pathways that have been implicated in the pathophysiology of dystrophin deficient muscular dystrophy. The absence of functional dystrophin protein causes sarcolemmal

Table 6 **Cardiac biomarkers (cTnI; mean ± SD) in wild-type and *mdx* mice (vehicle or idebenone treated) at ages 1 month (prior to initiation of treatment), 6 months, and 10 months [cardiac Troponin I levels (ng/mL)]**

Age	Group						ANOVA *P*-value
	WT-veh		mdx-veh		mdx-ide		
	n		*n*		*n*		
1 month	10	42 ± 15	14	31 ± 7	17	31 ± 8	0.7
6 months	9	42 ± 3	13	535 ± 177	18	392 ± 146	0.128
10 months	9	61 ± 8	13	401 ± 239	18	167 ± 28	0.254

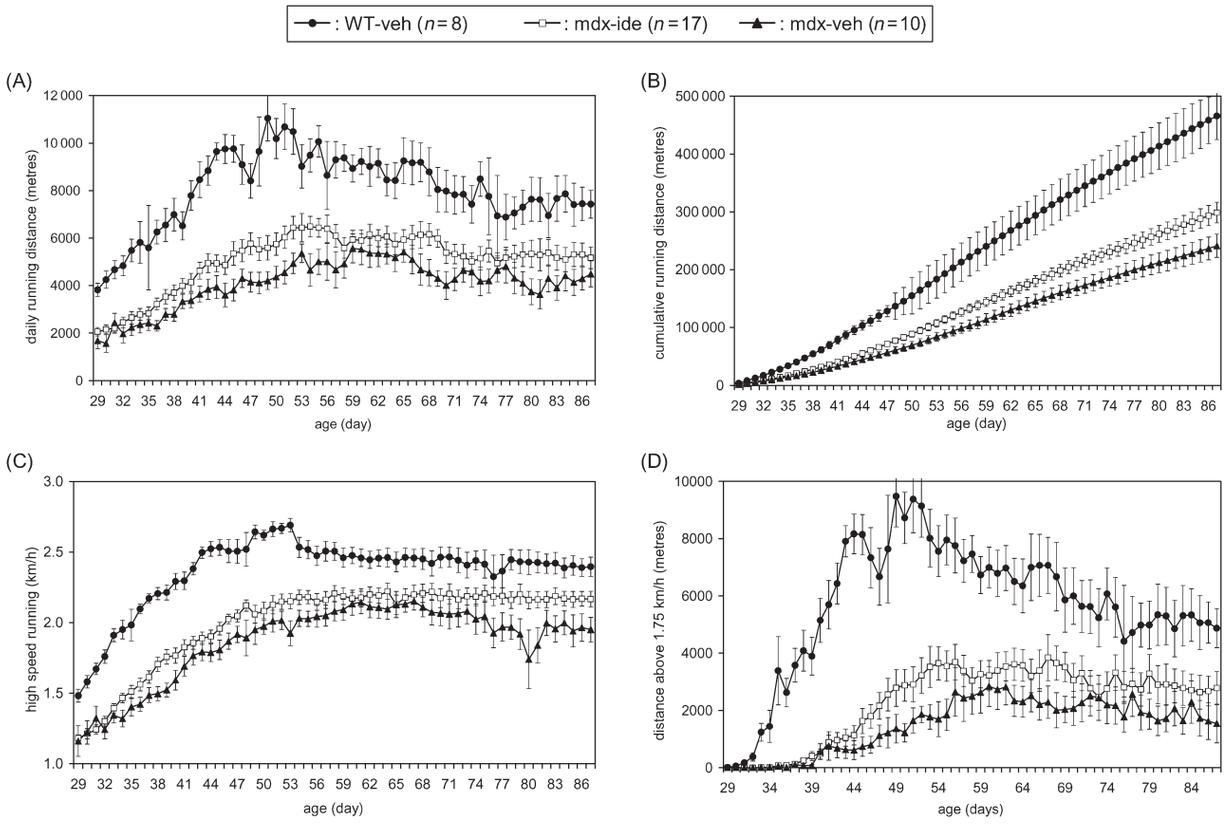

Figure 2 Idebenone significantly improves wheel running performance in *mdx* mice. Voluntary wheel running performance (mean ± standard error) in vehicle-treated wild-type, vehicle-treated *mdx*, and idebenone-treated *mdx* mice. (*A*) Average daily running distance. (*B*) Cumulative daily running distance. (*C*) Speed of running. (*D*) Running distance at high speed. ANOVA and pairwise comparisons showed significant differences between all groups as well as between *mdx* vehicle and *mdx* idebenone groups ($P < 0.001$) for the analyses presented in (*A*), (*C*), and (*D*). Data shown in (*B*) is a different graphical representation of the data shown in (*A*).

instability and initiates a cascade of biochemical events in skeletal and cardiac muscle that ultimately leads to disintegration of muscle proteins and cell death. Impaired mitochondrial oxidative phosphorylation and increased formation of reactive oxygen species have been reported in *mdx* skeletal muscle, and oxidative damage has been reported to be involved in the pathogenesis of the heart failure that occurs in *mdx* mice.[17–19] A recent study with *mdx* cardiomyocytes showed that excessive generation of reactive oxygen species is one of the key mechanisms that link the initial membrane fragility of the dystrophin-deficient cardiomyocyte to mitochondrial dysfunctions that precede cell death.[20] Increased oxidative stress and damage has also been reported in human DMD patients, where interactions between the primary genetic defect and disruptions in the normal production of free

radicals contribute to the pathophysiology.[21–23] Idebenone (2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone) has been shown to act as membrane-associated antioxidant that can prevent the formation of reactive oxygen species and reactive radicals.[6–7,24] Idebenone inhibits lipid peroxidation which protects cell membranes and mitochondria from oxidative damage. The molecule's optimized physicochemical properties favour uptake into cells and allow the incorporation into the mitochondrial membrane where it can facilitate the flux of electrons along the mitochondrial electron transport chain and increase cellular energy output which in turn protects mitochondria from malfunction and improves cellular survival.[25]

In conclusion, presymptomatic-initiated and long-term treatment with idebenone was cardioprotective and improved running performance in the dystrophin-deficient *mdx* mouse. We have therefore identified a novel potential therapeutic strategy for the homologous human DMD, a currently untreatable disease where the associated cardiomyopathy is responsible for early death of ~40% of patients. Our results therefore encourage studies with idebenone in human DMD patients. Such studies will also provide answers to the important question whether therapeutic studies in a homologous mouse model can be predictive for the human dystrophin-deficient situation. If so, our current study will gain further significance by demonstrating that the *mdx* mouse does provide unique opportunities for long-term controlled 'prehuman' therapeutic studies. Apart from its therapeutic findings, our study shows at a clinical level cardiac and motor deficits in the dystrophic mouse, and as such contributes to bridging the gap between laboratory scientists and clinicians in the field of muscular dystrophy.[26]

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References

1. Emery AEH. Clinical features. In: Emery AEH, ed. *Duchenne Muscular Dystrophy*. Oxford University Press; 2003. p26–45.
2. Muntoni F. Cardiomyopathy in muscular dystrophies. *Curr Opin Neurol* 2003;**16**: 577–583.
3. Finsterer J, Stollberger C. The heart in human dystrophinopathies. *Cardiol* 2003; **99**:1–19.
4. Bosser G, Lucron H, Lethor JP, Burger G, Beltramo F, Marie PY, Marçon F. Evidence of early impairments in both right and left ventricular inotropic reserves in children with Duchenne's muscular dystrophy. *Am J Cardiol* 2004;**93**:724–727.
5. Bulfield G, Siller WG, Wight PA, Moore KJ. X chromosome-linked muscular dystrophy (*mdx*) in the mouse. *Proc Natl Acad Sci USA* 1984;**81**:1189–1192.
6. Zs-Nagy I. Chemistry, toxicology, pharmacology and pharmacokinetics of idebenone: a review. *Arch Gerontol Geriatr* 1990;**11**:177–186.
7. Gillis JC, Benfield P, McTavish D. Idebenone. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in age-related cognitive disorders. *Drugs & Aging* 1994;**5**:133–152.
8. Seznec H, Simon D, Monassier L, Criqui-Filipe P, Gansmuller A, Rustin P, Koenig M, Puccio H. Idebenone delays the onset of cardiac functional alteration without correction of Fe-S enzymes deficit in a mouse model for Friedreich ataxia. *Hum Mol Genetics* 2004;**13**:1017–1024.
9. Georgakopoulos D, Mitzner WA, Chen CH, Byrne BJ, Millar HD, Hare JM, Kass DA. In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. *Am J Physiol* 1998;**274**:H1416–H1422.
10. Van den Bergh A, Flameng W, Herijgers P. Type II diabetic mice exhibit contractile dysfunction but maintain cardiac output by favourable loading conditions. *Eur J Heart Fail* 2006;**8**:777–783.
11. Weibel ER. *Stereological Methods*. Vol. 1. London, New York, Toronto, Sydney, San Francisco: Academic Press; 1979. p101.
12. Briguet A, Courdier-Fruh I, Foster M, Meier T, Magyar JP. Histological parameters for the quantitative assessment of muscular dystrophy in the *mdx* mouse. *Neuromusc Disord* 2004;**14**:675–682.
13. Hara H, Nolan PM, Scott MO, Bucan M, Wakayama Y, Fischbeck KH. Running endurance abnormality in *mdx* mice. *Muscle Nerve* 2005;**25**:207–211.
14. Siegel S, Castellan NJ. *Nonparametric Statistics for the Behavioral Sciences*. 2nd ed. New York: McGraw-Hill; 1988.
15. Yasuda S, Townsend D, Michele DE, Favre EG, Day SM, Metzger JM. Dystrophic heart failure blocked by membrane sealant poloxamer. *Nature* 2005;**436**: 1025–1029.
16. Wong BL, Mukkada VA, Markham LW, Cripe LH. Depressed left ventricular contractile reserve diagnosed by dobutamine stress echocardiography in a patient with Duchenne muscular dystrophy. *J Child Neurol* 2005;**20**:246–248.
17. Kuznetsov AV, Winkler K, Wiedemann FR, von Bossanyi P, Dietzmann K, Kunz WS. Impaired mitochondrial oxidative phosphorylation in skeletal muscle of the dystrophin-deficient *mdx* mouse. *Mol Cel Biochem* 1998;**183**:87–96.
18. Faist V, Koenig J, Hoeger H, Elmadfa I. Mitochondrial oxygen consumption, lipid peroxydation and antioxidant enzyme systems in skeletal muscle of senile dystrophic mice. *Pflügers Arch* 1998;**437**:168–171.
19. Williams IA, Allen DG. The role of reactive oxygen species in the hearts of dystrophin-deficient *mdx* mice. *Am J Physiol Heart Circ Physiol* 2007;**293**: H1969–H1977.
20. Jung C, Martins AS, Niggli E, Shirokova N. Dystrophic cardiomyopathy: amplication of cellular damage by Ca2+ signalling and reactive oxygen species-generating pathways. *Cardiovasc Research* 2008;**77**:766–773.
21. Rodriguez MC, Tarnopolsky MA. Patients with dystrophinopathy show evidence of increased oxidative stress. *Free Radic Biol Med* 2003;**34**:1217–1220.
22. Nakae Y, Stoward PJ, Kashiyama T, Shono M, Akagi A, Matsuzaki T, Nonaka I. Early onset of lipofuscin accumulation in dystrophin-deficient skeletal muscles of DMD patients and *mdx* mice. *J Mol Histol* 2004;**35**:489–499.
23. Tidball JG, Wehling-Henricks M. The role of free radicals in the pathophysiology of muscular dystrophy. *J Appl Physiol* 2007;**578**:327–336.
24. Suno M, Nagaoka A. Inhibition of lipid peroxidation by a novel compound, idebenone (CV 2619) in brain mitochondria and mode of action of the inhibition. *Biochem Biophys Res Commun* 1984;**125**:1046–1052.
25. Sugiyama Y, Fujita T. Stimulation of the respiratory and phosphorylating activities in rat brain mitochondria by idebenone (CV-2619), a new agent improving cerebral metabolism. *FEBS Letters* 1985;**184**:48–51.
26. Dubowitz V. Current and future therapy in muscular dystrophy: need for a common language between basic scientists and clinicians. *Acta Myol* 2004;**23**:5–9.