

RESEARCH PAPER

Investigation of Debio 025, a cyclophilin inhibitor, in the dystrophic *mdx* mouse, a model for Duchenne muscular dystrophyJ Reutenauer¹, OM Dorchies¹, O Patthey-Vuadens¹, G Vuagniaux² and UT Ruegg¹¹Laboratory of Pharmacology, Geneva-Lausanne School of Pharmaceutical Sciences, University of Geneva, Geneva, Switzerland and²Debiopharm SA Medical Affairs Department, Forum 'Après-demain', Lausanne, Switzerland

Background and purpose: Duchenne muscular dystrophy (DMD) is a severe muscle wasting disorder caused by the absence of the cytoskeletal protein dystrophin. This leads to muscle cell death accompanied by chronic inflammation. Cyclosporin A (CsA) is a powerful immunosuppressive drug, which has been proposed for DMD treatment. CsA also directly regulates the mitochondrial permeability transition pore (mPTP), which participates in cell death pathways through the inhibition of cyclophilin D. Here, we evaluated whether Debio 025, a cyclophilin inhibitor with no immunosuppressive activity, improves the dystrophic condition in a mouse model of DMD, through regulation of mPTP.

Experimental approach: The potency of Debio 025 to protect mouse dystrophic cells against mitochondria-mediated death was assessed by caspase-3 activity and calcium retention capacity assays. *Mdx*^{5Cv} mice (3-week-old) were treated daily by gavage for 2 weeks with Debio 025 (10, 30 or 100 mg kg⁻¹), CsA (10 mg kg⁻¹) or placebo. The effects on muscle necrosis and function were measured.

Key results: *In vitro* investigations showed protective effect of low concentrations of Debio 025 against cell death. Histology demonstrated that Debio 025 partially protected the diaphragm and soleus muscles against necrosis (10 and 100 mg kg⁻¹, respectively). Hindlimb muscles from mice receiving Debio 025 at 10 mg kg⁻¹ relaxed faster, showed alteration in the stimulation frequency-dependent recruitment of muscle fibres and displayed a higher resistance to mechanical stress.

Conclusions and implications: Debio 025 partially improved the structure and the function of the dystrophic mouse muscle, suggesting that therapies targeting the mPTP may be helpful to DMD patients.

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Keywords: dystrophic *mdx* mouse; cyclophilin D; mitochondrial permeability transition pore; cyclosporin A; myotube cell death; Duchenne muscular dystrophy; pharmacotherapy

Abbreviations: CK, creatine kinase; CRC, calcium retention capacity; CsA, cyclosporin A; CyPD, cyclophilin D; DMD, Duchenne muscular dystrophy; EDL, extensor digitorum longus; *mdx*, muscular dystrophy X-linked; mPTP, mitochondrial permeability transition pore; NRS, necrosis-regenerated surface; RT_{1/2}, time for half relaxation from the peak; STS, staurosporine; TTP, time to peak; wt, wild type

Introduction

Debio 025 (D-MeAla³EtVal⁴-cyclosporin) is a cyclophilin inhibitor with potent anti-human immunodeficiency and anti-hepatitis C virus activities (Noser *et al.*, 2006; Flisiak *et al.*, 2007). It has recently passed phase Ib and IIa clinical trials where it showed a strong anti-hepatitis C effect (Flisiak *et al.*, 2007). It binds to cyclophilin A (CyPA) at a nanomolar concentration and inhibits its prolyl *cis-trans* isomerase activity. However, unlike cyclosporin A (CsA), Debio 025

does not interact with calcineurin (Hansson *et al.*, 2004) and, therefore, is not immunosuppressive.

Duchenne muscular dystrophy (DMD) is a severe muscular dystrophy that affects approximately 1 in 3500 male births. The first symptoms appear in early childhood. The disease is characterized by progressive muscle wasting associated with respiratory complications and cardiac dysfunction leading to death by the age of 20–30 years. DMD is caused by the absence of the structural protein dystrophin that is encoded by the X chromosome (Xp21). Dystrophin is a large protein of 427 kDa that links the extracellular matrix through the transmembrane glycoprotein complex of dystroglycans to the intracellular F-actin network, thus providing mechanical stability to muscle cells during contraction (Petrof *et al.*, 1993).

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Although the gene encoding dystrophin was identified in 1987 (Hoffman *et al.*, 1987), the mechanisms leading to disease manifestation still remain unclear. The dystrophic condition is associated with a wide variety of cellular dysfunctions, including membrane instability (mislocation or absence of many of the dystrophin-associated proteins), deregulation in Ca^{2+} homeostasis, increased susceptibility to oxidative damage, enhanced proteolytic activity and apoptosis, and impaired energy metabolism (Blake *et al.*, 2002). Altogether, these events lead to muscle fibre death followed by infiltration of activated lymphocytes and macrophages, progressive replacement of muscle tissue by fibrotic and adipose tissue, and culminate in the loss of functional muscle mass.

Despite considerable efforts to replace the damaged gene by stem cell transfer or gene therapy (Nowak and Davies, 2004; Chakkalakal *et al.*, 2005), these approaches have not yet led to a cure mainly because of safety issues concerning vectors and limitations with targeting all the muscles of the body. To date, the main treatments that improve the life expectancy and the quality of life of DMD patients consist of surgery, kinesiotherapy, ventilatory assistance and pharmacological interventions. Currently, the only drugs used in DMD patients are the glucocorticoids, prednisolone and deflazacort (Muntoni *et al.*, 2002). Their action on inflammation, promotion of muscle-specific gene expression, correction of deregulated Ca^{2+} homeostasis (Metzinger *et al.*, 1995; Leijendekker *et al.*, 1996) and activation of the calcineurin/nuclear factor of activated T cells (NF-AT) pathway (St-Pierre *et al.*, 2004) might explain their therapeutic effects. However, in some cases, side effects inherent to this class of drugs were reported in treated DMD patients (Fisher *et al.*, 2005). Recently, with increasing knowledge of the mechanisms underlying the disease process, new therapeutic approaches have been proposed as palliative treatments for DMD, essentially based on investigations using the dystrophic *mdx* (muscular dystrophy X-linked) mouse model (Khurana and Davies, 2003; Chakkalakal *et al.*, 2005; Cossu and Sampaolesi, 2007). These include the treatment with creatine to increase muscle energetic balance and help with correcting Ca^{2+} deregulation (Pulido *et al.*, 1998; Passaquin *et al.*, 2002), specific calpain inhibitors to counteract the increased Ca^{2+} -dependent proteolytic activity (Lescop *et al.*, 2005), lipid peroxidation inhibitors to blunt nuclear factor- κB activation (Messina *et al.*, 2006), antioxidants such as green tea extract to counteract oxidative stress (Buetler *et al.*, 2002; Dorchies *et al.*, 2006), deacetylase inhibitors to induce muscle growth (Minetti *et al.*, 2006) or phosphodiesterase inhibitors to prevent ischaemia (Dorchies *et al.*, 2006; Rolland *et al.*, 2006; Asai, 2007). To block tissue infiltration by activated lymphocytes, anti-inflammatory and immunosuppressive drugs, such as glucocorticoids (Muntoni *et al.*, 2002) and CsA (Sharma *et al.*, 1993; De Luca *et al.*, 2005) have been tested in dystrophic mice and in DMD patients. In our view, a general immunosuppression with CsA is not a suitable treatment for DMD as the patients would need to be protected from infections, and also because CsA has significant adverse effects (Mason, 1990).

Another approach is to target apoptosis that may be primary or secondary. Primary apoptosis will remove damaged cells before the cell is disrupted and induce an

immune response. This is the classical apoptotic pathway that serves to protect the tissue as a whole. Secondary apoptosis generally occurs after primary tissue damage (necrosis) and is mostly caused by reactive oxygen species (Tidball and Wehling-Henricks, 2007). By comparing biopsies from healthy and DMD patients, the mitochondrial content was found to be similar, but more ultrastructural abnormalities such as aberrant morphology of the mitochondria were observed in DMD patients (Watkins and Cullen, 1987). Moreover, the Krebs cycle intermediate α -ketoglutarate is elevated only in the muscle of DMD patients, suggesting predominant oxidative metabolism (Sharma *et al.*, 2003). A specific decrease in all mitochondrial inner membrane enzymes, most probably as a result of Ca^{2+} overload, has been postulated to be the cause for the bioenergetic deficits in dystrophin-deficient skeletal muscle (Kuznetsov *et al.*, 1998). In addition to the established role of mitochondria in energy metabolism, regulation of cell death has emerged as a major function of these organelles (Bouchier-Hayes *et al.*, 2005; Orrenius *et al.*, 2007). Taking into account the key role played by mitochondrial Ca^{2+} handling in the control of cell death, these organelles are potential targets of impaired Ca^{2+} homeostasis in muscular dystrophy (Robert *et al.*, 2001). Calcium deregulation induces mitochondrial depolarization and apoptosis (Hajnoczky *et al.*, 2003). Indeed, depolarization of the inner mitochondrial membrane results in the opening of mitochondrial permeability transition pore (mPTP) and Ca^{2+} efflux from mitochondrial matrix through both the mPTP and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Bernardi, 1999).

The mPTP, the structure of which is still not established (Forte and Bernardi, 2005), behaves as a voltage-dependent, high-conductance and nonspecific megachannel of the inner mitochondrial membrane. In the fully open state, its apparent diameter is about 3 nm and the open-closed transitions of the pore are highly regulated by multiple effectors. Indeed, pore opening can be promoted by a high concentration of matrix Ca^{2+} levels, oxidative stress, pyridine nucleotide oxidation, thiol oxidation, alkalization or low transmembrane potential (Bernardi, 1999; Crompton, 1999). Persistent mPTP opening is followed by equilibration of ionic gradients and of species with molecular weight lower than 1.5 kDa, which may cause swelling, cristae unfolding and outer membrane rupture associated with the release of cytochrome *c* and pro-apoptotic factors (Petronilli *et al.*, 2001). Opening of the mPTP is inhibited by CsA after its binding to cyclophilin D (CyPD), a member of the cyclophilin family of *cis-trans* isomerases (Halestrap and Davidson, 1990). CyPD is involved in modulation of the mPTP affinity for Ca^{2+} , and conversely Ca^{2+} modulates the efficacy of mPTP inhibition by CsA (McGuinness *et al.*, 1990; Bernardi, 1992). The mPTP is activated early in apoptosis and its inhibition has been shown to protect against apoptosis (Bernardi *et al.*, 2001). Recently, it has been reported that mitochondria-mediated apoptosis/cell death can be reduced by CsA in liver diseases (Soriano *et al.*, 2004), in a model of Ullrich congenital muscular dystrophy (Angelin *et al.*, 2007), as well as in experimental models of ischaemia-reperfusion injury of the heart (Gateau-Roesch *et al.*, 2006) and of neuronal injury (Forte *et al.*, 2007).

Here, we investigated whether Debio 025 could prevent or reduce the massive necrotic episode, which affects skeletal

muscle of 3-week-old dystrophic mice. Because Debio 025 is capable of selectively and strongly inhibiting CyPD (Gomez *et al.*, 2007), we hypothesized that this compound would block the mPTP, prevent cell death and reduce muscle wasting. The advantage of Debio 025 over CsA lies in its increased affinity for cyclophilins and its very low calcineurin inhibitory activity (about 7000-fold less than CsA) (Hansson *et al.*, 2004). Indeed, calcineurin activity is required for the initiation of skeletal muscle differentiation (Friday *et al.*, 2000), and has been shown to be essential for successful dystrophic muscle regeneration (Chakkalakal *et al.*, 2004; Stupka *et al.*, 2004). In the current study, the use of CsA and Debio 025 allowed discrimination between effects mediated by the calcineurin/NF-AT pathway and direct CyPD effects that are likely to be linked to the inhibition of mitochondria-mediated cell death. Here, using *in vitro* and *in vivo* investigations, we have shown that the CyPD inhibitor Debio 025 has a therapeutic potential in the dystrophic mouse.

Methods

In vitro studies

Materials. Media and foetal calf serum were purchased from Invitrogen (Basel, Switzerland), except Ham's F10 nutrient mixture, and horse serum from Sigma (Buchs, Switzerland). Collagen type I and collagenase were from Sigma, dispase from Roche Molecular Diagnostics (Basel, Switzerland), and Matrigel from BD Biosciences. Other chemicals were from Sigma, unless stated otherwise.

Cell culture. Primary cultures of skeletal muscle cells were established from hindlimb muscles of 3- to 4-day-old newborn mice by collagenase–dispase digestion (100 and 2.4 U mL⁻¹, respectively). The cells were re-suspended in Ham's F10 supplemented with 20% foetal calf serum and 5 ng mL⁻¹ basic fibroblast growth factor (FGF2) (PePro-Tech, London, UK) and plated onto Petri dishes (Falcon; BD Biosciences, Basel, Switzerland) coated with collagen type I (3 µg cm⁻²) and Matrigel (1 µg cm⁻²). After 24 h, the medium was replaced by a 1:1 (vol:vol) mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F10, supplemented with 10% foetal calf serum and 2.5 ng mL⁻¹ FGF2 for another 24-h period. Then, primary cultures were used for the preparation of purified myoblasts. Myoblast separation was performed based on the expression of $\alpha 7$ integrin subunit on myoblast cell surface (Blanco-Bose *et al.*, 2001). Primary muscle cells were re-suspended with trypsin, incubated with a rat monoclonal anti- $\alpha 7$ integrin (a gift from Dr Randall Kramer, UCSF, USA), and then with magnetic beads coated with sheep anti-rat Ig (DynaBeads M-450; Dynal Biotech, Compiègne, France). Tagged myoblasts were purified with a magnetic device, and re-suspended in a 2:1 mixture of Ham's F10/DMEM supplemented with 10% foetal calf serum and 5 ng mL⁻¹ FGF2. The cells were plated (7000 cells cm⁻²) in both 35- or 60-mm diameter Petri dishes (Falcon; BD Biosciences) coated with a mixture of collagen type I and Matrigel as described above.

After 2 days, differentiation was induced by switching to DMEM containing 2% horse serum. The cells were kept in a humidified incubator gassed with 95% air–5% CO₂ at 37 °C.

Drug treatment. CsA, Debio 025 (Debiopharm, Lausanne, Switzerland), FK-506 and staurosporine (STS) (Alexis, Lausen, Switzerland) were prepared in dimethylsulphoxide (DMSO) as 10⁻² or 10⁻³ M stock solutions and frozen at -20 °C. Cultures were treated in a range from 1 nM to 10 µM using freshly thawed stock aliquots. The final DMSO concentration was 0.1%. Cultures receiving 0.1% DMSO without any test substance served as a control.

For toxicity assays, CsA or Debio 025 was added on either primary cultures or pure myotube cultures on day 4 of differentiation, the medium was refreshed daily by 50% until day 6 of differentiation, and cells were analysed on day 7. STS was added on pure myotube cultures on day 4 of differentiation for 24 h.

For caspase-3 activity assays, pure myotube cultures were pretreated with the compound (CsA, Debio 025 or FK-506) for 1 h on day 4 before being challenged with STS at 100 nM for 15 h.

Survival assay. Cell survival was assessed by the measurement of acid phosphatase activity (Connolly *et al.*, 1986). The cells were washed twice with phosphate-buffered saline, and lysed in 0.1 M citrate buffer pH 5.5 containing 0.1% Triton X-100 (Applichem, Axonlab, Le Mont-sur-Lausanne, Switzerland) and 1 mg mL⁻¹ *p*-nitrophenylphosphate as a substrate for phosphatase. After incubation for 20–30 min at room temperature, the reaction was stopped by the addition of 0.1 volume of 1 N NaOH and absorbance was read at 405 nm (Labsystem Multiscan MS, Allschwil, Switzerland). The yellow colour that appeared is considered proportional to the amount of cells in the sample. Results were expressed as a percentage of the value of the control culture.

Measurement of caspase-3 activity. Caspase-3 activity was measured in cell homogenates. The cells were washed with phosphate-buffered saline and scraped into lysis buffer (100 mM HEPES pH 7.4, 10% sucrose, 20 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS), 1 mM EDTA (Fluka), 200 mM NaCl, 10 mM dithiothreitol and protease inhibitor mixture), and repeatedly passed through a 26-gauge needle. The lysates were centrifuged at 14 000 *g* for 10 min and the supernatants were collected. Protein content was determined using the Bradford method (Bio-Rad, Reinach, Switzerland). Reaction mixtures (100 µL final) were prepared containing 100 µg protein, 20 µM of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) (Biomol, Anawa Trading, Zürich, Switzerland), 100 mM HEPES pH 7.4 and protease inhibitor mixture. After 10 min of stabilization, caspase-3 activity was measured by monitoring the proteolytic cleavage of DEVD-AMC for 5 min at 30 °C using a FLUOstar OPTIMA fluorimeter (BMG Labtech, Champigny sur Marne, France) (excitation 380 nm, emission 460 nm). Calibration curves were constructed using AMC (1 nM to 1 µM) instead of DEVD-AMC in the same reaction buffer. One unit of enzyme activity was defined as the amount of AMC

released per minute per microgram of protein. The caspase-3 inhibitor, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) (Biomol), was used at 10 μM as a control.

Isolation of mouse skeletal muscle mitochondria. Mouse skeletal muscle mitochondria were prepared as described previously (Madsen *et al.*, 1996) with slight modifications. Mice were killed by cervical dislocation and skeletal muscles (diaphragm and limb muscles) were rapidly removed and transferred into ice-cold isolation medium (250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA (Fluka), pH 7.4). Muscles were cleaned of visible fat and connective tissue, and minced with scissors. Muscle pieces were transferred to 20 mL of isolation medium supplemented with 0.2% BSA and Nagarse (Fluka) (0.2 mg mL⁻¹). After 2 min, muscles were homogenized using a motor-driven Teflon-glass potter, transferred to 80 mL of isolation medium supplemented with 0.2% BSA and centrifuged at 900 *g* for 6 min to pellet cellular debris. The supernatant was harvested and centrifuged at 10 000 *g* for 10 min. The resulting pellet was re-suspended in isolation medium and centrifuged at 7000 *g* for 6 min. The final mitochondrial pellet was re-suspended in 0.3 mL of the isolation medium at a final protein concentration of about 20 mg mL⁻¹ before being used for calcium retention capacity (CRC) assay. All procedures were carried out at 0–4 °C.

CRC. The extra-mitochondrial Ca²⁺ concentration was measured at room temperature in a 2 mL cuvette placed in a thermostated Perkin-Elmer LS-5B spectrofluorimeter (excitation 505 nm, emission 535 nm) equipped with magnetic stirring, using the Ca²⁺ fluorescent indicator Calcium Green-5N (Invitrogen) (Ichas *et al.*, 1997). For each measurement, a suspension of mitochondria corresponding to 0.5 mg per mL protein was prepared in a medium containing 250 mM sucrose, 10 mM 3-[*N*-morpholino] propanesulphonic acid, 10 μM EGTA, 1 mM ortho-phosphoric acid (Merck, VWR International, Dietikon, Switzerland), 5 mM glutamate, 2.5 mM malate, pH 7.4. Then, the test substance and subsequently the probe were added to the suspension at a final concentration of 1 μM each. After 1 min, Ca²⁺ concentration was raised stepwise by successive additions of 10 μL of 1 mM CaCl₂, one addition every minute. At the end of each measurement, the maximum fluorescence was checked by the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at a final concentration of 300 nM. Calibration of the signal was achieved by the addition of known amounts of Ca²⁺.

In vivo studies

Animal groups and drug treatment. All of the procedures involving animals were conducted in accordance with the Swiss Federal Veterinary Office's guidelines, based on the Swiss Federal Law on Animal Welfare, and were approved by the Cantonal Veterinary Office. The study was performed on dystrophic *mdx*^{SCv} mice (Im *et al.*, 1996). Breeding pairs of dystrophic *mdx*^{SCv} animals were kindly provided by Dr Serge Braun (Transgene, Strasbourg, France) with the agreement of The Jackson Laboratory (Bar Harbor, ME, USA). The colonies were thereafter maintained in our animal facility. Animals

were housed in plastic cages containing wood granule bedding, maintained with 10 h dark/14 h light cycles and unlimited access to food and water throughout the study. Roughly equal numbers of males and females were used in each group. Litters of 3-week-old animals were treated daily by gavage for 2 weeks and analysed at 5 weeks of age. Animals received CsA 10 mg kg⁻¹, Debio 025 10, 30, or 100 mg kg⁻¹. The substance suspensions were freshly prepared from a cremophor-based concentrate formulation provided by Debiopharm. The placebo group consisted of animals receiving the formulation alone. Substance and placebo solutions were prepared in a way to contain the same concentration of excipients (of which cremophor was at 4.1% final concentration), and were administered at 10 $\mu\text{L g}^{-1}$ of body weight. Treatments were performed at 12 \pm 2 h. Data from 7 to 9 animals per group were analysed.

Monitoring of mouse body weight and food intake. Mice were weighed every day. Animals were kept with the dam until weaning (28 days of age) and were isolated in separate cages thereafter. Known amounts of food pellets were delivered on day 28, and the amount of remaining pellets was determined on day 35 (day of death). Food consumption over this 7-day period was expressed as the mass of pellets consumed in gram per gram of body weight (on day 35) per day.

Isometric force recordings. At the end of the treatment period, animals were anaesthetized by i.p. injection of a mixture of urethane (1.5 g kg⁻¹) and diazepam (5 mg kg⁻¹). The Achilles tendon of the right hindlimb was exposed and linked to a force transducer coupled to a LabView interface for trace acquisition and analysis. The knee joint was firmly immobilized. Two thin steel electrodes were inserted into the triceps surae muscle (comprising the fast-twitch glycolytic gastrocnemius and plantaris muscles, and the slow-twitch oxidative soleus muscle). Muscles were electrically stimulated with 0.5 ms pulses of controlled intensity and frequency. The stimulation-recording protocol was performed as follows. After manual settings of optimal muscle length and optimal current intensity, a phasic twitch was recorded to measure the absolute phasic twitch force (P_t), the time to peak (TTP) and the time for half relaxation from the peak (RT_{1/2}). After a 3-min pause, muscles were subjected to a force–frequency test, where tetanic force was recorded using 200 ms bursts of increasing frequency (from 20 to 100 Hz by increments of 10 Hz) with one burst every 30 s. The strongest response was taken as the absolute optimal tetanic force (P_o). Finally, after another 3-min pause, muscles were submitted to a fatigue test for 5 min: frequency was set at 60 Hz, and the decrease in tetanic muscle force was recorded while 60 stimulation sequences were delivered, each consisting of a 2-s tetanic burst and a 3-s rest. The responses were expressed as a percentage of the maximal tension. Absolute phasic and tetanic forces were converted into specific forces (millinewtons per square millimetre of muscle section) after normalization for the muscle cross-sectional area. The cross-sectional area (in square millimetre) was determined by dividing the triceps surae muscle mass (in milligram), by the product of optimal muscle length (in millimetre) and *d*, the density of mammalian skeletal muscle ($d = 1.06 \text{ g mm}^{-3}$).

Plasma creatine kinase level. After the measurement of the muscle mechanical contractile properties, the mice were thoracotomized, received an intracardiac injection of heparin (approximately 30 U heparin per mL of blood) and the aorta was cut 20–30 s later. Whole blood was collected from the chest cavity in heparinized centrifuge tubes in a time window of 24 ± 4 h after the last administration of test substance. Fresh blood fractions were saved for creatine kinase (CK) determination. The blood was centrifuged at $3000g$ for 10 min at 4°C and plasma was stored at $2\text{--}6^\circ\text{C}$. CK determination was performed in four animals per group by spectrophotometric analysis using Catachem diagnostic kit (Investcare Vet, Middlesex, UK) according to the manufacturer's instructions within 48 h of plasma preparation.

Tissue sampling. Immediately after blood collection, the extensor digitorum longus (EDL), soleus, gastrocnemius and plantaris muscles were dissected bilaterally and weighed for determination of muscle mass. Heart, liver, kidneys and spleen were also collected on some animals and weighed. EDL, soleus muscles from the left leg and the left hemidiaphragm were embedded in Tissue Freezing Medium (Polysciences, Brunshwig, Basel, Switzerland), frozen in liquid nitrogen-cooled isopentane and stored at -80°C for histological analysis.

Morphometrical analysis. Transverse sections ($10\ \mu\text{m}$ thick) were prepared with a HM 560M cryostat (Microm, Volketswil, Switzerland) and collected on SuperFrost Plus slides. Whole muscle cross sections taken near the mid-point of the muscle were stained with haematoxylin–eosin according to classical procedures. Pictures were taken with a Spot Insight QE digital camera (Visitron Systems, Puchheim, Germany) coupled to an inverted microscope (Axiovert 200M, Zeiss, Feldbach, Switzerland). One cross section was analysed per animal. The total cross-sectional area and the necrosis-regenerated surface (NRS) were delineated using the Adobe Photoshop software. Then, the surfaces were calculated using

the MetaMorph software (Visitron Systems). Results were expressed as a percentage of the area occupied by necrotic and regenerated myofibres, and inflammatory cells to the total surface area. The samples were coded and analysed by an observer who was blinded to the study details.

Data analysis

The graphs were constructed and the data were analysed using the GraphPad Prism software (GraphPad, San Diego, CA, USA). Results were calculated as means \pm s.d. For clarity, data are shown as means \pm s.e.mean in figures. Statistical analysis was performed by using a two-tailed unpaired Student's *t*-test with the placebo *mdx*^{SCV} group used as a reference for comparison to any other group. Differences with *P*-values ≤ 0.05 were considered significant.

Results

Effect of Debio 025 on mitochondria-mediated death

CRC. Mitochondria isolated from both normal and dystrophic skeletal muscle were investigated with respect to their ability to withstand incremental challenges of Ca^{2+} using a CRC assay (Figure 1). No significant difference in CRC was found between mitochondria isolated from wild-type (wt) or dystrophic muscle. The CRC was $\sim 70\%$ higher when mitochondria were incubated with Debio 025 at $1\ \mu\text{M}$ compared with vehicle ($140\ \mu\text{M}$ versus $82\ \mu\text{M}$, respectively). Addition of CsA at $1\ \mu\text{M}$, used as a positive control, increased CRC to the same extent as Debio 025.

Survival assay. Test substances were added for 2 days on either primary cultures or pure myotube cultures (four independent experiments for each phenotype). None of the test substances ($1\ \text{nM}$ to $10\ \mu\text{M}$) altered the viability of pure myotubes as assessed by acid phosphatase activity (not shown). On primary myotube cultures, $10\ \mu\text{M}$ of either Debio 025 or CsA was slightly toxic (not shown). Of note,

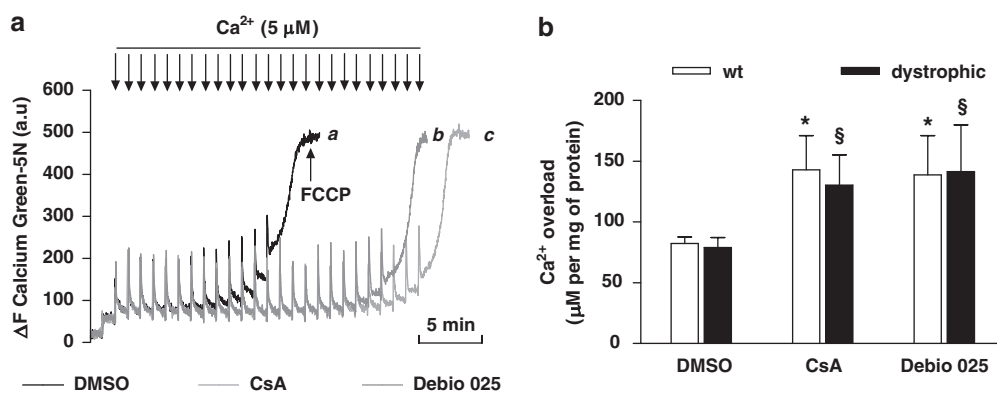


Figure 1 Effect of Debio 025 on the calcium retention capacity (CRC) of mitochondria isolated from mouse skeletal muscle. (a) CRC of isolated mitochondria from dystrophic mouse skeletal muscle. Mitochondria were repeatedly exposed to Ca^{2+} pulses. The ability of mitochondria to buffer Ca^{2+} was followed through the change in fluorescence of the Ca^{2+} indicator Calcium Green-5N. Muscle mitochondria were incubated with vehicle (trace a), $1\ \mu\text{M}$ Debio 025 (trace b) or $1\ \mu\text{M}$ cyclosporin A (CsA) (trace c). Pulses of $5\ \mu\text{M}$ CaCl_2 were added every minute (arrows). (b) Summary of four independent assays showing that the Ca^{2+} overload required to open the mitochondrial permeability transition pore (mPTP) was significantly higher in the presence of Debio 025 or CsA compared with control for both normal (wild type (wt)) and dystrophic muscle ($n = 4$). *Significantly different from wt + vehicle, §significantly different from dystrophic + vehicle ($P \leq 0.05$). Values represent means \pm s.e.mean.

dystrophic myotubes presented a 4.3-fold higher sensitivity to STS compared with normal myotubes ($IC_{50} \sim 7$ versus ~ 30 nM, respectively) (not shown).

Caspase-3 activity. Caspase-3 activity was measured in cell homogenates using a fluorogenic substrate (Figure 2). Basal level of caspase-3 activity was 2.3-fold higher in dystrophic myotubes as compared with normal myotubes. STS increased caspase-3 activity in both normal and dystrophic myotubes. Debio 025 used at 10 nM partially decreased STS-induced caspase-3 activity in dystrophic myotubes, whereas CsA and FK-506 were ineffective at this concentration. The caspase-3 inhibitor Ac-DEVD-CHO, used as a control, fully prevented the STS-induced caspase-3 activity.

Effect of Debio 025 on food intake, body weight, muscle and organ weight

Food intake and mouse body weight. Animals (3-week-old) were treated daily by gavage for 2 weeks with CsA (10 mg kg^{-1}), Debio 025 (10, 30, or 100 mg kg^{-1}). Data from eight animals per group were analysed. Food consumption over the second week of the administration period was expressed as the mass of pellets consumed in gram per gram of body weight (on day 35) per day. Food intake was similar

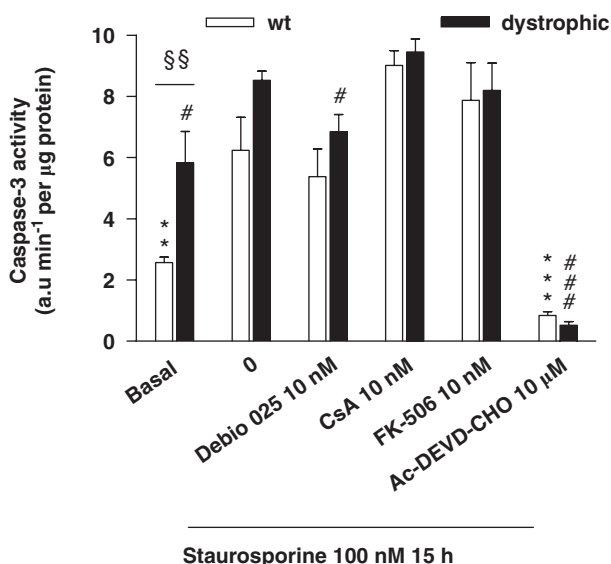


Figure 2 Caspase-3 activity assay. Basal and staurosporine (STS)-induced caspase-3 activity was assessed in normal (wild type (wt)) and dystrophic pure myotubes. Cells were pretreated for 1 h with the substances and then exposed to 100 nM STS for 15 h in the presence of the test substance. Caspase-3 activity was measured in cell extracts by monitoring the proteolytic cleavage of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) for 5 min at 30 °C. At the basal level, pure dystrophic myotubes presented a 2.3-fold higher caspase-3 activity compared with normal myotubes ($^{§§}P \leq 0.01$). STS increased caspase-3 activity in both normal ($^{**}P \leq 0.01$) and dystrophic ($^{\#}P \leq 0.05$) myotubes. Debio 025 significantly decreased STS-induced activity ($^{\#}P \leq 0.05$). The caspase-3 inhibitor *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), used as a control, abolished the STS-induced caspase-3 activity ($^{***}P \leq 0.001$, $^{####}P \leq 0.001$). ***** Significantly different from wt + STS, $^{\#,###}$ significantly different from dystrophic + STS ($n = 4$). Values represent means \pm s.e.mean.

in all groups of mice (from 0.22 ± 0.01 to 0.25 ± 0.01 g food consumed per gram body weight per day). Animals in all groups had normal gait throughout the study, and the body weight increased similarly (from ~ 8.8 g at day 28 to ~ 16.5 g at day 35).

Corrected weight of muscles and organs. Muscle weight (eight animals per group) was expressed in milligram per gram body weight. No differences were observed in the weight of EDL ($\sim 0.35 \text{ mg g}^{-1}$), gastrocnemius ($\sim 3.92 \text{ mg g}^{-1}$), plantaris ($\sim 0.50 \text{ mg g}^{-1}$) and soleus ($\sim 0.30 \text{ mg g}^{-1}$) muscles between the various groups of treatment. Treatment for 2 weeks with CsA at 10 mg g^{-1} or Debio 025 (10, 30 and 100 mg g^{-1}) did not alter the weight of heart, liver, kidney and spleen, collected from 5-week-old dystrophic mice.

Effect of Debio 025 on markers of muscle damage

Muscle histology. Data from 7 to 9 animals per group at 5 weeks of age were analysed. Normal and regenerated muscle fibres were readily identified by the presence of either peripheral or central nuclei after haematoxylin-eosin staining. Foci of necrotic fibres were characterized by the presence of infiltrated immune cells. The fractions of the muscle affected by necrosis, inflammation and regeneration are represented in Figure 3. Diaphragm muscles from placebo-treated mice exhibited $32.7 \pm 2.6\%$ of NRS, an index of muscle damage. In contrast, in mice receiving Debio 025 at 10 mg kg^{-1} , NRS dropped to $25.3 \pm 0.9\%$. This corresponds to

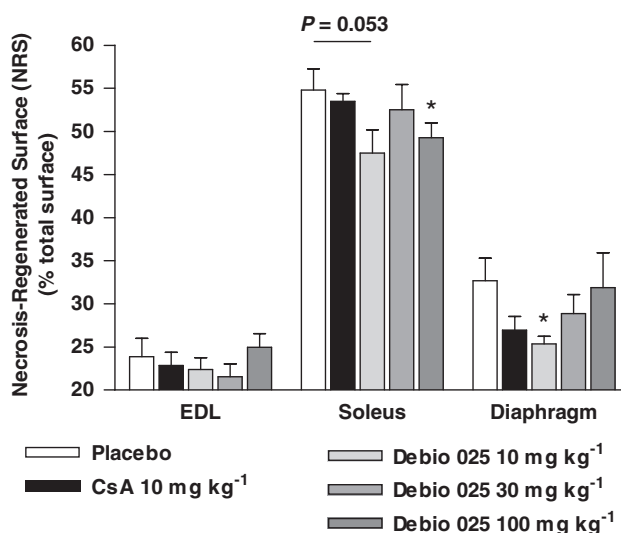


Figure 3 Effect of Debio 025 on the extent of necrosis and regeneration in dystrophic skeletal muscle. After a 2-week treatment with Debio 025, cyclosporin A (CsA) or placebo, whole transverse cross sections were prepared from extensor digitorum longus (EDL), soleus and diaphragm muscles, and stained with haematoxylin-eosin. The surface of the sections affected by necrosis and regeneration was measured and expressed as a percentage of total muscle section area (% necrosis-regenerated surface (NRS)). Diaphragm muscles from mice treated with 10 mg kg^{-1} of Debio 025 exhibited 29% of protection compared with placebo-treated mice. Debio 025 at 100 mg kg^{-1} significantly diminished necrosis in the soleus muscle compared with placebo ($^*P \leq 0.05$) ($n = 7-9$). Values represent means \pm s.e.mean.

29% of protection compared with placebo-treated mice. However, this effect disappeared at higher doses of Debio 025 (30 and 100 mg kg⁻¹). The slow oxidative soleus muscle was more affected with NRS values of 55.0 ± 2.4%. Debio 025 at 100 mg kg⁻¹ significantly diminished necrosis to 48.2 ± 1.5%. NRS values reached 23.9 ± 2.1% for the fast glycolytic EDL muscle of placebo-treated mice and were not changed by Debio 025 treatment.

Plasma CK level. Creatine kinase level was determined in four animals per group at 5 weeks of age, as CK activity is a well-established index of muscle damage. As expected, placebo-treated dystrophic mice presented high levels of CK (10 670 ± 3226 UL⁻¹). Although Debio 025 treatment at 10 mg kg⁻¹ tended to diminish CK levels (8790 ± 1153 UL⁻¹), statistical significance between groups was not reached (data not shown).

Effect of Debio 025 on isometric force parameters

Isometric force was recorded from 8 to 9 animals per group at 5 weeks of age.

Phasic twitch force and optimal tetanic force. The specific tension developed by triceps surae muscle is a parameter considered to be physiologically more relevant than absolute tension, which is influenced by changes in muscle weight and length. P_t (from 5.5 ± 0.6 to 6.9 ± 0.5 mN mm⁻²) and P_o (from 26.1 ± 1.6 to 32.6 ± 2.1 mN mm⁻²) were not significantly altered by any of the treatments (data not shown). As a result, the phasic-to-tetanic ratios remained unchanged (from 0.20 ± 0.01 to 0.24 ± 0.01).

Kinetics of contraction and relaxation. Time to peak and half relaxation time (RT_{1/2}) were determined from phasic twitch traces. TTP was ~17.5 ms in all groups and no significant alterations were seen in the TTP upon treatments (Figure 4a). The RT_{1/2} of mice treated with CsA (19.8 ± 0.6 ms) or Debio 025 at 10 mg kg⁻¹ (18.6 ± 0.7 ms) was significantly diminished compared with the placebo group (21.0 ± 0.3 ms), indicating that triceps surae muscles from these treated mice relaxed faster (Figure 4b).

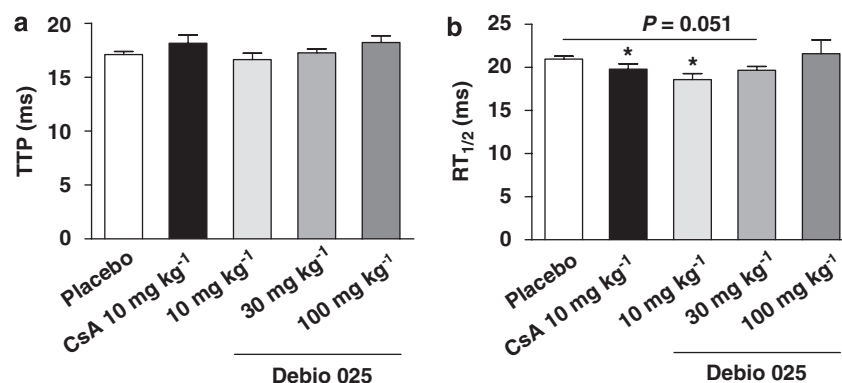


Figure 4 Kinetics of contraction and relaxation. Electrically evoked isometric contractions of the triceps surae were recorded. (a) Time to peak (TTP) and (b) time for half-relaxation from the peak (RT_{1/2}) were determined. TTP was similar in all groups. The triceps surae from mice treated with Debio 025 at 10 mg kg⁻¹ relaxed significantly faster compared with the placebo-treated mice (* $P \leq 0.05$) ($n = 8-9$). Values represent means ± s.e.mean.

Force–frequency relationship. Curves connecting tetanic tension output to stimulation frequency were established (Figure 5a). The fraction of maximal force developed by mice treated with 10 mg kg⁻¹ of Debio 025 at low stimulation frequencies (30–50 Hz corresponding to the preferential recruitment of slow-twitch fibres) was less (from 36.4 ± 1.8 to 64.3 ± 2.7%) than that of their placebo-treated counterparts (from 45.2 ± 2.5% at 30 Hz to 74.2 ± 3.8% at 50 Hz). This indicates a shift to the right of the force–tension relationship.

Resistance to mechanical stress. The resistance of triceps surae muscle to repetitive tetanization was assessed by a fatigue assay. As shown in Figure 5b, the tension abruptly decreased upon the first 15 tetanizations to reach a plateau over 150 s. The values measured at the end of the assay corresponded to the residual force after tetanus. At this stage, the contraction-induced damages within muscle were irreversible. The various groups presented similar profiles except for mice treated with Debio 025 at 10 mg kg⁻¹. These animals were significantly less fatigable than their placebo counterparts. Residual force at the end of the fatigue protocol was 25.4 ± 1.7% of maximal tension in the group with Debio 025 treated at 10 mg kg⁻¹ compared with 18.8 ± 2.4% in the placebo group. Thus, 10 mg kg⁻¹ Debio 025 made the dystrophic muscles ~37% more resistant to mechanical stress compared with untreated muscles.

Effect of agent used for the formulation. In the current *in vivo* study, cremophor was used to solubilize Debio 025 and CsA, which has been reported to have detrimental effects on mitochondria from muscle (Sanchez *et al.*, 2001). Therefore, we performed a study using six mice per group with a non-cremophor-based formulation to investigate this possibility. Both formulations gave similar results (not shown), which may be due to the fact that the cremophor concentration used was still below the toxic level.

Discussion

The basal level of activated caspase-3, an end point in the caspase activation cascade leading to cell death, was found to

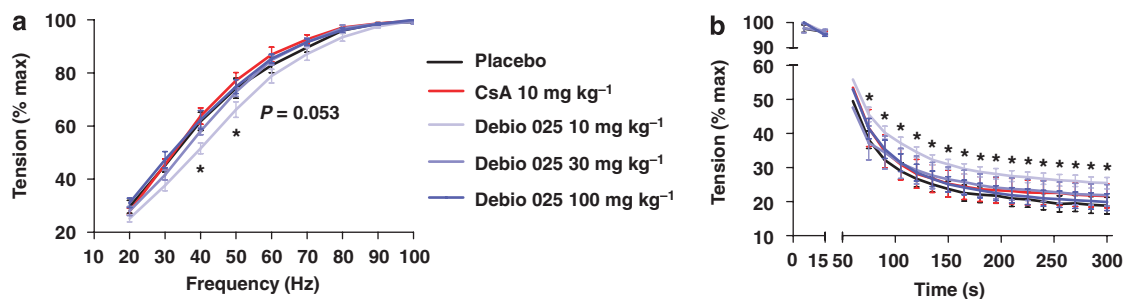


Figure 5 Force–frequency relationship and resistance to mechanical stress. Tension output was expressed as a percentage of maximal response. (a) Tetanic tensions elicited by stimulation of increasing frequency were recorded at 30-s intervals on mice. The fraction of maximal force developed by mice treated with Debio 025 10 mg kg⁻¹ at low stimulation frequency (30–50 Hz) that preferentially recruits slow-twitch fibres was less than in the placebo-treated counterparts. (b) Resistance to repetitive tetanizations was assessed. After a 2-week treatment with 10 mg kg⁻¹ of Debio 025, the triceps surae was significantly more resistant than in the placebo-treated counterparts (* $P \leq 0.05$). For both tests, the statistical significance of the test condition was calculated versus the placebo-treated mice ($n = 8–9$). Values represent means \pm s.e.mean.

be more than twofold higher in dystrophic myotubes than in normal myotubes. This is consistent with data obtained with muscle biopsies from DMD patients (Sandri *et al.*, 2001), suggesting that caspase-3 might be an appropriate target for the pharmacological treatment of DMD. STS is a potent protein kinase inhibitor with a broad spectrum of activities (Ruegg and Burgess, 1989), known to induce apoptosis through a mitochondrial pathway (Yang *et al.*, 1997) and to cause oxidative stress through generation of reactive oxygen species (Cai and Jones, 1998; Kruman *et al.*, 1998). Pure dystrophic myotubes were almost fivefold more sensitive to STS than normal myotubes, which contrasts with findings by Rando *et al.* (1998). Interestingly, Debio 025 decreased STS-induced caspase-3 activity in cultured muscle cells, whereas CsA and FK-506 were ineffective at the concentration examined.

It is generally accepted that degeneration of dystrophic skeletal muscle is associated with necrosis. However, several studies suggest that during the phase of acute muscle degeneration of *mdx* muscle, apoptosis precedes necrotic cell death (Tidball *et al.*, 1995). The occurrence of apoptosis was estimated to be around 0.5–2% of total nuclei in *mdx* muscle (Matsuda *et al.*, 1995; Sandri *et al.*, 1995; Tidball *et al.*, 1995). Moreover, dystrophin-deficient myotubes undergo apoptosis in mouse primary muscle cell culture after DNA damage (Sandri *et al.*, 1998). L-Carnitine, which plays a role in the transport of fatty acids into mitochondria, was described as a potential treatment for blocking apoptosis and preventing skeletal muscle myopathy in heart failure (Vescovo *et al.*, 2002). In addition, the anti-apoptotic Bcl-2 protein may regulate the mPTP, thus preventing the release of pro-apoptotic factors from mitochondria (Mignotte and Vayssiere, 1998), and overexpression of Bcl-2 has been reported to suppress Ca²⁺ activation of the mPTP (Murphy *et al.*, 2001). However, muscle-specific Bcl-2 overexpression did not ameliorate the dystrophic condition of *mdx* mice (Dominov *et al.*, 2005), suggesting that therapies designed to treat DMD by inhibition of apoptosis are not appropriate. Thus, whereas CsA upregulates the Bcl-2 protein in a calcineurin-dependent manner (Genestier *et al.*, 1995), it is likely not to be sufficient to completely prevent dystrophic cell death. In addition, investigations of CyPD-deficient mice indicated that CyPD is a regulator but not an intrinsic

component of the mPTP, which can form and open in the absence of CyPD (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005). Therefore, in contrast to the conclusions by others (Halestrap, 2005), Bernardi *et al.* (2006) stressed that the *in vivo* studies with CyPD knockout mice can only be interpreted in terms of the role of CyPD, not of the mPTP, in cell death.

Our results show that CsA (10 mg kg⁻¹) and Debio 025 (10, 30 and 100 mg kg⁻¹) treatments of 3-week-old dystrophic mice for 2 weeks did not alter the weight of heart, liver, kidney and spleen, known to be targets of CsA as reported by others (Stupka *et al.*, 2004). In this latter study, daily doses of 30 mg kg⁻¹ CsA were given *i.p.* to 2-week-old mice for 2 weeks. As suggested by De Luca *et al.* (2005), this dose is expected to totally inhibit calcineurin activity in skeletal muscle. Calcineurin is a Ca²⁺/calmodulin-activated serine/threonine protein phosphatase involved in a number of processes (Crabtree, 2001). Both pro- and anti-apoptotic roles have been reported for its activation (Baines and Molkentin, 2005), likely to reflect the highly specialized role that this protein plays in different tissues. In the heart, calcineurin directly regulates myocyte growth, yet its overexpression in skeletal muscle had no effect on growth or fibre size (Molkentin, 2004). In contrast, calcineurin functions as a critical regulator of fibre-type switch in skeletal muscle, enhancing the formation of slow-type fibres (Naya *et al.*, 2000). Calcineurin has also been implicated in regulating differentiation, developmental maturation and regeneration of skeletal muscle (Friday *et al.*, 2000; Hong *et al.*, 2002; Stupka *et al.*, 2004). Moreover, calcineurin activity has been shown to attenuate the dystrophic progression in *mdx* muscle (Chakkalakal *et al.*, 2004; Stupka *et al.*, 2006). Therefore, it appears that 30 mg kg⁻¹ of CsA is toxic to skeletal muscle, in particular to regenerating dystrophic skeletal muscle. The same dose of Debio 025 is not detrimental to this tissue. In addition, our results based on primary myotube cultures show that Debio 025 is not toxic at concentrations up to 1 μ M (that is, about 1000 times more than the IC₅₀ value).

Debio 025 at the low dose of 10 mg kg⁻¹ protected the dystrophic mouse diaphragm. This result is of clinical relevance as the diaphragm is the most severely affected muscle in *mdx* mice and is considered the most pertinent

muscle with respect to the human disease (Petrof *et al.*, 1993). Besides, the mild protection of the soleus muscle observed with Debio 025 treatment is probably due to the oxidative metabolism inherent to this type of muscle. Moreover, it is well established that the density of mitochondria in this slow-twitch oxidative muscle is higher compared with fast-twitch glycolytic muscle. Of note, other substances such as creatine, green tea extract or pentoxifylline were found to be ineffective to protect the soleus muscle of dystrophic mice (Buetler *et al.*, 2002; Passaquain *et al.*, 2002; Dorchies *et al.*, 2006).

However, CK activity, a well-known diagnostic marker for muscle injury, did not reach statistical significance between placebo and treated groups. This might be due to the surgical preparation prior to death, which through muscle damage may have attenuated a potential difference between placebo and treated mice.

The shortening of the time for relaxation by Debio 025 at 10 mg kg⁻¹ appears most interesting as dystrophic muscle, similarly to fatigued muscle (Allen *et al.*, 2008), is known to display marked slowing of relaxation as compared with normal muscle (Nicolas-Metral *et al.*, 2001). Substances beneficial to *mdx* mice normalize the relaxation rate to the one of control mice (Dorchies *et al.*, 2006). Both the faster relaxation and the alteration of the force–frequency relationship reported here suggest a change either in cytosolic Ca²⁺ handling or in the distribution of fibre types towards a faster phenotype.

The resistance to mechanical stress was improved with Debio 025 at 10 mg kg⁻¹, whereas CsA had no effect. These findings are in contrast with results by others showing a functional improvement in the *mdx* mouse using the same dose of CsA (De Luca *et al.*, 2005). This might be due to the use of a treadmill to accelerate muscle degeneration or to different ages and periods of treatment of the animals among studies.

In conclusion, our *in vitro* investigations showed a potent and efficacious protective effect of Debio 025 against mitochondria-mediated cell death. Daily oral treatment of 3-week-old dystrophic mice for 2 weeks with 10 mg kg⁻¹ of Debio 025 resulted in partial improvement of the structure and the function of the dystrophic mouse muscle, and higher doses did not yield stronger effects, indicating high potency but limited efficacy in this model and under our conditions. Considering the high affinity of Debio 025 for CyPD, lower doses of Debio 025, for instance 1–3 mg kg⁻¹, deserve to be investigated. While this paper was in the review process, a report was published showing similar results of Debio 025 injected s.c. at 50 mg kg⁻¹ day⁻¹ into *mdx* mice from 4 to 10 weeks of age (Millay *et al.*, 2008). Of note, mitochondria prepared from Debio 025-treated muscle were still protected in *in vitro* tests, presumably several hours after *in vivo* exposure to this compound, highlighting the strong affinity of Debio 025 for the mPTP target. This *in vivo* treatment also reduced fibrosis and, to a minor extent (5–10%), central nucleation of *mdx* muscle fibres. As outlined above, the dystrophic pathology results from alterations in multiple molecular pathways, which together contribute to muscle fibre death. Therefore, our findings suggest that selective CyPD inhibitors are potential candi-

dates for the symptomatic treatment of DMD and combinations with treatments targeting mechanical, immunological and signalling pathways should result in improved efficacy.

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Conflict of interest

The authors state no conflict of interest.

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