Evaluation of the therapeutic potential of carbonic anhydrase inhibitors in two animal models of dystrophin deficient muscular dystrophy

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Duchenne Muscular Dystrophy is an inherited muscle degeneration disease for which there is still no efficient treatment. However, compounds active on the disease may already exist among approved drugs but are difficult to identify in the absence of cellular models. We used the Caenorhabditis elegans animal model to screen a collection of 1000 already approved compounds. Two of the most active hits obtained were methazolamide and dichlorphenamide, carbonic anhydrase inhibitors widely used in human therapy. In C. elegans, these drugs were shown to interact with CAH-4, a putative carbonic anhydrase. The therapeutic efficacy of these compounds was further validated in long-term experiments on mdx mice, the mouse model of Duchenne Muscular Dystrophy. Mice were treated for 120 days with food containing methazolamide or dichlorphenamide at two doses each. Musculus tibialis anterior and diaphragm muscles were histologically analyzed and isometric muscle force was measured in M. extensor digitorum longus. Both substances increased the tetanic muscle force in the treated M. extensor digitorum longus muscle group, dichlorphenamide increased the force significantly by 30%, but both drugs failed to increase resistance of muscle fibres to eccentric contractions. Histological analysis revealed a reduction of centrally nucleated fibers in M. tibialis anterior and diaphragm in the treated groups. These studies further demonstrated that a C. elegans-based screen coupled with a mouse model validation strategy can lead to the identification of potential pharmacological agents for rare diseases.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive disorder affecting striated and cardiac muscles. It is caused by mutations in the dystrophin gene (1,2), one of the largest gene in the human genome. This gene encodes a large cytoskeletal protein called dystrophin that links the cytoskeleton to the extracellular matrix (3–5). DMD is the most prevalent X-linked recessive neuromuscular disorder, affecting 1 in 3500 male children.

There is still no curative treatment available against DMD. Current treatments for DMD are symptomatic and significantly improve longevity and quality of life but do little to prevent loss of muscle function (6). The standard care applied to DMD patients is prednisone therapy (7). Several therapeutic strategies have been developed in the past decade for which clinical trials have already been initiated: the reconstitution of dystrophin expression by replacing the mutated gene using adeno associated virus (AAV) vectors (8), repairing the endogenous gene by antisense-mediated

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RESULTS

Effect of MTZ and DCPM on C. elegans dystrophin-deficient muscle

We exploited a C. elegans model of dystrophin-dependent muscle degeneration in a screen of bioactive molecules to identify potential blockers of muscle degeneration. The relevance of such a screen has been previously demonstrated (24). During this screen, we observed by visual inspection that 7-day dys-1(cx18); hlh-1(cc561) adult animals grown on 0.5 mg/ml (2 mM) of MTZ or on 0.1 mg/ml (0.3 mM) of DCPM plates moved better than untreated animals, suggesting that the function of the body wall muscles in these animals was partially conserved. Examination of the musculature by phalloidin staining, which labels actin fibers, revealed a dramatic reduction of muscle degeneration in treated animals (Figs 1 and 2). Whereas the untreated dystrophic animals show 5–6 degenerating or dead muscle cells per pair of quadrants, the animals treated with MTZ or DCPM present only 1–2 such cells.

Dose-response experiments (Fig. 1) showed that the effect of MTZ and DCPM on muscle degeneration is optimal at 0.5 mg/ml and at 0.1 mg/ml in the medium, respectively (Fig. 1). The positive effect was qualitatively visible down to 0.1 mg/ml for MTZ and down to 0.01 mg/ml for DCPM. Concentration of drugs in the medium above 1 mg/ml of MTZ or 0.5 mg/ml of DCPM was toxic to the animals. The actual drug concentration in the animal is not known but is thought to be 100–1000× lower.

The muscle degeneration in the DMD worm model is caused by the synergy between dys-1(cx18) and hlh-1(cc561) mutations, in which the mild hlh-1(cc561) mutation serves as an amplifier of the dys-1(cx18) phenotype (17). Therefore, we wanted to verify that MTZ or DCPM were also active on the dys-1(cx18) mutation alone. The dose-effect experiments on dys-1(cx18) showed a decrease in muscle degeneration similar to that of the double mutant (Fig. 1).

The degeneration observed in the DMD worm model is activity- and time-dependent (17). Consequently, compounds that have a sedative effect or reduce growth rate also reduce muscle degeneration. To verify that MTZ and DCPM did not act this way, but had a real beneficial effect on muscle, we tested their impact on these parameters. Addition of 0.5 mg/ml of MTZ or 0.1 mg/ml of DCPM had no sedative effect on the locomotion and growth rate of dys-1(cx18) or wild-type worms compared with untreated animals (Fig. 3A and B). These results demonstrated that the gain obtained with MTZ and DCPM on muscle degeneration is not due to a sedative effect or to a reduced growth rate.

Effect of CA inhibition on C. elegans dystrophin-deficient muscle

Since we found that MTZ and DCPM can reduce muscle degradation in the dystrophic model of C. elegans, we further investigated the mode of action of these compounds. Sulfonamides are known to be strong inhibitors of human CAs (20). In C. elegans, this class of enzymes is poorly documented. The only documented CA is cah-4 (25,26). Consequently, we first inventoried CAs in C. elegans. A Blast
from 14 Homo sapiens sequences obtained from the NCBI website, revealed six putative CAs in the C. elegans genome. These genes are called cah-1 to 6. A maximum likelihood tree was constructed (Fig. 4). This tree shows that only cah-1 and cah-2 present a high homology with human CA-X and XI. The other worm CAs are too divergent to be matched with human CAs.

We hypothesized that the effect of MTZ and DCPM could be mimicked by knocking down one or several C. elegans cah genes. We tested this hypothesis by double-stranded RNA-mediated interference (RNAi) feeding experiments. RNAi is a useful method for gene inactivation in C. elegans (27). Four clones available from Geneservice Bank (III-2NO3, II-5LN09, X-7M16, X-6I20) allowed targeting of four of the six cah gene (cah-1, cah-2, cah-3 and cah-4). The last two clones were constructed for the purpose of this study. RNAi experiments with the six clones showed that only inhibition of the gene cah-4 significantly reduced muscle degeneration in the dys-1(cx18), hlh-1(cc561) strain (Fig. 5). RNAi experiments ran on gene combinations did not bring additional information, suggesting that cah-4 is solely responsible for the drug response to both MTZ and DCPM (Data not shown). Furthermore, cah-4 RNAi did not impact the activity or growth rate of worms, indicating that it is the cah-4 reduction per se which is responsible for the beneficial effect (Fig. 3A and B). Moreover, this experiment showed a partial restoration of dys-1, hlh-1 locomotion.

Synergy between sulfonamides and CA RNAi in C. elegans
To further elucidate the role of these genes in the response of sulfonamide, we tested for a cooperative effect between the presence of sulfonamide and the diminution of each cah transcripts. A dose–response experiment was conducted in presence or absence of cah dsRNA. No statistical difference was observed between the dose–response control and the dose–response coupled with the inhibition of cah-1, cah-2, cah-3, cah-5 and cah-6 transcripts (Fig. 6). The optimal concentration remains around 0.5 mg/ml in all conditions, and the sublethal MTZ concentration remains also unchanged (1 mg/ml). On the contrary, the inhibition of cah-4 transcripts in presence of MTZ or DCPM showed a dramatic shift of the dose–response curve to the left. The MTZ is lethal at 1 mg/ml in the medium for the negative control and this concentration goes down to 0.1 mg/ml when coupled with an inhibition of cah-4 transcripts. The optimal concentration to reduce degeneration goes down from 0.5 to 0.01 mg/ml when coupled to cah-4 RNAi. For DCPM, the toxic concentration is shifted from 0.5 to 0.05 mg/ml when coupled with cah-4 RNAi and the optimal concentration is shifted from 0.1 to 0.005 mg/ml.
These results suggest that the mechanism involved is common to both compounds, and further demonstrate that the protein CAH-4 is their target.

As a negative control the same experiments were performed with Imipramine, Nifedipine and Trimipramine. These drugs have been previously shown to reduce muscle degeneration in *C. elegans* but are not CA inhibitors (28). In presence of *cah*-4 dsRNA, their respective sublethal concentration remained unchanged (data not shown), thereby demonstrating that MTZ and DCPM interact with CAH-4 in a specific way.

**CAH-4 tissue distributions**

We then investigated the tissue expression of *cah*-4. To localize CAH-4, a plasmid carrying the putative endogenous promoter of *cah*-4 in frame with GFP was constructed and injected in worms. Fifty fluorescent worms (all stages) were analyzed under confocal microscopy. The GFP protein was localized at all stages in the excretory cell, in the nervous system (nerve ring, dorsal and ventral cord and motor neuron commissures), in body wall muscles and in the anal muscles (Fig. 7). This expression pattern is consistent with the effect observed.
Treatment of mdx mice with DCPM and MTZ. We then analyzed whether MTZ and DCPM would have an effect on the mouse mdx model of DMD. Sex- and age-matched (70-day) mdx mice were fed 120 days with drug containing food or control food with identical composition, and animals were then analyzed. For DCPM treatment, a low-dose
(15 mg/kg/day) or a high-dose (60 mg/kg/day) was administered, which is, respectively, 5× and 20× human average posology. For MTZ, 20× posology (86 mg/kg/day) was lethal, so we administered a low concentration which was 1× posology (4.3 mg/kg/day) and a high concentration which was 5× posology (21.5 mg/kg/day). Concentrations of drugs in food were calculated on the basis of that mdx mice eat ~15% of their body weight per day (L.S., unpublished results). All mice had similar weight at the end of the treatment suggesting that food intake was comparable. Two types of readouts were conducted: histology and force measurement.

Histology assessment of MTZ treated muscles. Muscles from drug-treated animals were histologically analyzed. Representative Wheat Germ Agglutinin (WGA-staining), for tibialis anterior (TA) muscles of MTZ high dosage group is shown in Figure 8. We performed Haematoxylin Eosin staining (HE-staining) to obtain a general impression of the histological appearance of both groups to be compared. No obvious differences of the histological appearance became visible for 5× MTZ treated muscles compared with the untreated group (data not shown). However, an automated analysis of the fiber type distribution revealed subtle differences (see below). In Figure 8A and B the Alexa 488 conjugated WGA-staining for sections from untreated and 5× MTZ treated mdx mice is shown. The WGA-staining allowed clear delineation of fiber boundaries for determination of minimal fiber diameters. Myosin Heavy Chain (MHC) double staining (Fig. 9A) provided information about the fiber type distribution, MHC isoforms I, and type IIa and IIb fibers, which are separately stained by the protocol applied. Both substances showed no effect on the fiber type composition (data not shown).

Automated histological analysis of muscle sections. In order to obtain a reliable and secured statistical analysis of pathology-relevant histological parameters of dystrophic muscle in mdx mice, a standardized and automated quantitative method was developed with S.CO Lifescience (München, Germany) (Fig. 9). Two adequate parameters for analysis of the histological status were chosen. The minimal Feret’s diameter of the fibers, and the percentage of centrally nucleated fibers (indicative of muscle regeneration) were determined (29).

Dystrophic muscle typically displays a higher variability of the muscle fiber diameter compared with wild-type muscle. The variance coefficient (VC) of all muscle fiber minimal Feret’s diameters of a given muscle cross-section provides a numerical value of the fiber size variability (29). The minimal Feret’s diameter is very robust against experimental errors such as the orientation of the sectioning angle. Regarding the second histological parameter, muscles of wild-type mice rarely contain centralized nuclei, whereas muscles of mdx mice show an average percentage of up to 66% of centrally nucleated fibers after the first crisis of degeneration and regeneration at around 7 weeks of age.

In this study, we examined about 3000 fibers of TA and diaphragm (DIA) muscles for each 5× MTZ treatment and untreated group covering both parameters. Automated histological analysis was performed only for the 5× MTZ treated group and its untreated control group, because quality of the muscles belonging to the DCPM group was not sufficient and thus analysis not possible.

The histological analysis of the TA muscle showed a significant decrease of the percentage of centrally nucleated fibers in the 5× MTZ treated group (MTZ: 57.4 ± 14.9; control: 65.1 ± 11.2; P = 0.03) (Fig. 10A), and a non-significant higher VC with Z = 487 in the treatment group compared with Z = 448 in the control group (P > 0.05). In accordance with the higher VC in the 5× MTZ-treated group, we were able to distinguish a shift of the minimal Feret’s diameter towards smaller fibers with diameters <20 μm (Fig. 10C) in the 5× MTZ-treated group (P = 0.03), whereas the number of fibers between 30 and 40 μm were higher in the untreated group (P = 0.02) (Fig. 10C).

In DIA no significant change in the percentage of centrally nucleated fibers between 5× MTZ treated group (72.1 ± 6.7) and untreated control group (75.1 ± 7.0) was observed (P = 0.085) (Fig. 10B) and the VC of the muscle fiber size distribution in diaphragm was slightly lower with Z = 425 in the treatment group compared with Z = 439 in the untreated group (P > 0.05). Moreover, a very similar fiber size distribution was observed in both groups (Fig. 10D).

Figure 8. Representative wheat germ agglutinin (WGA) of tibialis anterior (TA) muscle. (A and B) Alexa 488 conjugated WGA-staining of TA muscle of untreated (A) and 5× MTZ-treated (B) mdx mice. WGA binds to N-acetylgalactosaminyl and sialic acid residues at the border of the muscle fibers.
Muscle force measurements of mdx mice treated with MTZ and DCPM. The ability of therapeutic drugs to improve force generation was determined by isometric force measurement using different protocols able to distinguish between maximal force generation capability (tetanic force) and resistance to eccentric contraction. Treatment of mice with DCPM increased significantly the tetanic specific force. The 5× MTZ treatment group displayed a non-significant higher percentage of the mean force drop in DCPM treated mice (67 ± 14%) compared with untreated mdx mice (46 ± 15%). These results show that DCPM treatment improved the effective muscle strength, but was not able to increase muscle fiber resistance to exercise.

We further examined in mdx EDL muscle the maximal force generation using an eccentric contraction protocol with elongation of the muscle for 10% of its resting length (Lo). Treatment with DCPM at both concentrations, however, was not able to improve this parameter. On the contrary, results in Figure 11A show a significant (P = 0.034) higher percentage of the mean force drop in DCPM treated mice (282 ± 64; untreated: 198 ± 53; P = 0.034) (Fig. 11B). This improvement in muscle force generation was not seen in the second treatment group at lower concentration (data not shown).

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DISCUSSION

Caenorhabditis elegans as a model for drug discovery

Our findings confirm the use of C. elegans as a model organism for screening, identifying and characterizing potential lead pharmacological agents. In this study, we show the therapeutic potential of a previously unsuspected class of molecules for the treatment of DMD. One of the concerns of using the C. elegans model for drug discovery is the determination of the effective dose for a lead compound. The molecules used in this study were provided to the worms through the media. C. elegans has an outer exoskeleton (cuticle) which provides protection from environmental chemicals. Thus, the effective concentration absorbed through the cuticle and/or the intestinal lumen is not known and is approximately 100–1000× lower than in the medium. However, this limitation does not impede the use of C. elegans. Data have to be considered mostly in a qualitative manner.

Sulfonamide and CA in C. elegans

Sulfonamide is known to be strong inhibitors of several CA in humans. After identifying CAs in the worm, we knocked down their expression by RNAi experiments and we found that RNAi inhibition of cah-4 reduced muscle degeneration (Fig. 4). Since cah-4 reduction mimics the effect of sulfonamide treatment, it is likely that it is the target of the compounds (Fig. 5). Moreover, several sulfonamides including MTZ and DCPM have already been found to be active on CAH-4 with \( k_i \) values at 42 and 38 nm, respectively, and null mutants cah-4(tm2805) are lethal. We postulated that this lethality is due to the absence of CAH-4 activity since we could mimic it with a high concentration of MTZ (1 mg/ml) or DCPM (0.5 mg/ml). Furthermore, worms display an increased sensitivity to MTZ and DCPM when these treatments are coupled with cah-4 RNAi (Fig. 6). All together, these results suggest that the gain obtained in the C. elegans dystrophin-deficient model is due to an inhibition of CAH-4 activity by sulfonamide.

Furthermore, the cah-4::GFP reporter gene was localized in body wall muscles and in the nervous system. Although it cannot be formally demonstrated, it is likely that sulfonamides act directly on muscles for the following reasons: (i) Compounds did not reduce locomotion of dys-1 and wild-type worms, indicating an absence of a major effect on the
central nervous system (Fig. 3A), (ii) RNAi is poorly active on the nervous system of C. elegans (31), yet it is of considerable effect in our experiment.

MTZ and DCPM are known to induce metabolic acidosis and their mechanisms of action most likely involve changes of pH that alter the transmembrane potential and excitability (32). Lower pH values reduce potassium conductance of the cell membrane and have an effect on the activation and inactivation of sodium and calcium channels, respectively (33). On the other hand, acidification of the sarcoplasmic reticulum trans-chamber induced a reduction of the unitary conductance of the Ca\(^{2+}\) release channel (34). Since we found that calcium channel activity is a critical factor in the progression of dystrophin-dependent muscle degeneration in C. elegans (35), we hypothesize that an appropriate concentration of MTZ or DCPM could reduce Ca\(^{2+}\) transients and thus decrease muscle degeneration in C. elegans.

Effect of MTZ and DCPM on mdx mouse muscle

Despite the fact that muscle pathology for dystrophin-deficiency differs between the mdx mouse model and human patients, the mdx mouse model is the most important animal model for preclinical studies in DMD research (36,37). In the present study, we have applied recommended methods for pre-clinical analysis in DMD research. Readout parameters

Figure 10. Quantitative assessment of muscle histology. (A and B) Percentage of centrally nucleated fibers in TA muscle (A) and diaphragm (B) of untreated versus 5 × MTZ-treated mdx mice. Data expressed as mean ± SD; mean percentage of centrally nucleated fibers (n = 3000). (C and D) Fiber size distribution in TA muscle (C) and DIA (D) of untreated versus 5 × MTZ-treated mdx mice. * Different from control at P < 0.05.
included muscle force measurement on isolated muscle, and the histological examination including the percentage of centrally nucleated fibers and the VC of the fiber size which reflects the degeneration and regeneration processes.

Histological analysis of the TA muscle revealed a statistically significant decrease in the percentage of centrally nucleated fibers for 5\textsuperscript{\textsubscript{C2}}/MTZ treated mice, implying less degeneration/regeneration occurring during the treatment period. This result is particularly remarkable, since the treatment was initiated at an age of 70 days, a time point where intense degenerative and regenerative activity had already taken place (38–40).

Moreover, automated histological analysis revealed an increase in fiber size variability in the 5\textsuperscript{\textsubscript{C2}}-treatment group, which is not obvious by simple visual inspection of the corresponding HE sections. A detailed analysis of the fiber size distribution revealed the occurrence of more smaller and larger fibers, which typically arise during cycles of regeneration and degeneration, in this group. In addition, Neural Cell Adhesion Molecule (NCAM) staining for regenerating fibers clearly showed more NCAM positive fibers, corroborating the higher number of small fibers observed here (data not shown).

We then investigated the functional analysis of muscle force measurements of limb muscles. Significant differences between mdx and wild-type mice are easier observed in older mice (41). In the MTZ- and DCPM-high dosage groups an increase of the tetanic specific force led to an improvement of the maximal force. The increase in muscle force was not due to differences in muscle weight or size, since normalization of isolated muscles regarding to these two parameters occurred. The maximal force generation, but not the overall resistance of the muscle to a stimulated endurance protocol, was increased.

A possible mode of action of MTZ and DCPM could be through a reduction of reactive oxygen species, in particular by blocking the muscle-abundant CAIII (42–44). However, since CAIII is more than 10,000 times less sensitive to MTZ compared with other CA isoforms, this hypothesis is still a matter for conjecture (26).

Wetzel et al. analyzed the effects of CA inhibitors on muscle force generation and Ca\textsuperscript{2+}-transients. Inhibition of a sarcoplasmatic reticulum CA (SR-CA) isoform was suggested. Indeed, MTZ was shown to be a strong inhibitor of the membrane-bound CAIX and of the C. elegans cah-4, too, and it was stated that inhibition of only one SR-CA isoform does not affect muscle contraction, whereas inhibition of all three CAS IV, IX and XIV does (26,45). Thus inhibition of these CA isoforms could be implicated in the observed phenotypes in this study as well. Treatment of isolated muscle bundles with one particular inhibitor L-645151 or 6-ethoxylzolamide increased the peak force signals of twitches on EDL and Soleus and showed a prolonged T1/2 relaxation time. The authors suggested that CAS generate H\textsuperscript{+} outside the SR and supply the ions for the coupled Ca\textsuperscript{2+}–H\textsuperscript{+} bidirectional transport across the SR membrane and buffer incoming H\textsuperscript{+} ions.

**Figure 11.** Muscle force measurements of EDL from treated and untreated mdx mice. (A and B) Muscle force measurements for right EDL after 120 days treatment with 20\textsuperscript{\textsubscript{C2}} DCPM of 6-month-old male mdx mice (\(n_{\text{control}}=7, n_{\text{DCPM}}=6\)). (A) Mean force drop after eccentric stimulation (B) Mean tetanic specific force (TET specific) obtained by normalization of tetanic maximal force (TET max) with CSA (cross sectional area). (C and D) Muscle force measurements for EDL after 120 days treatment with 5\textsuperscript{\textsubscript{C2}} MTZ of 6-month-old female mdx mice (\(n_{\text{control}}=6, n_{\text{MTZ}}=5\)). (C) Mean force drop after eccentric stimulation of untreated and 5\textsuperscript{\textsubscript{C2}} MTZ-treated mdx mice (D) Mean TET specific obtained by normalization of TET max with CSA (\(P=0.05\)). Data expressed as mean ± SD. *Different from control at \(P<0.05\).
inside the SR by catalysis of the reverse reaction (46). Bruns et al. (47) showed an acceleration of the CO$_2$–HCO$_3^-$ reaction of ~1000-fold by the SR-CA. Catalysis by CAII reduced the half time from 7 s to ~7 ms. Inhibition of CAs by sulfonamides was shown to slow Ca$^{2+}$ release from the SR. Consequently, release and uptake of Ca$^{2+}$ as well as the rise time of twitches (increase in force) were prolonged, providing a plausible explanation for the increase in maximal tetanic force by CA inhibitors.

Despite a decrease in resistance of muscle fibers to eccentric contractions, the analyzed substances DCPM and MTZ showed efficacy in the mdx mouse and increased the specific tetanic muscle force, demonstrating that a C. elegans screen coupled with a mouse model validation strategy can conduct to the identification and characterization of potentials drugs against rare diseases. Differences in CA usage and model physiology likely account for differences of responses observed between the two models.

At this time, sulfonamide like MTZ and DCPM may not be pursued in further preclinical for treatment of DMD, but the development of isozyme-specific or at least organ-selective CA inhibitors may be highly beneficial to improve their efficacy against DMD and applied to other muscular dystrophies, as well as devoid of major side effects.

MATERIALS AND METHODS

Strains and culture conditions

Caenorhabditis elegans strains were cultured at 15°C on 6 cm Petri dishes containing NGM agar and a lawn of Escherichia coli OP50 unless stated otherwise. The dys-1(cx18); hlh-1(cc561) strain was grown at 15°C, which is the permissive temperature for the ts mutation hlh-1(cc561) (17). The wild-type N2 strain was obtained from the Caenorhabditis Genetics Center.

Pharmacological compounds

All pharmacological compounds were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Concentrated solutions of each drug were prepared by dissolving compounds in DMSO. C. elegans tolerates DMSO up to a final concentration of 2% vol/vol. Compounds were added to liquid NGM that had been autoclaved and cooled to 55°C of 2% vol/vol. Compounds were added to liquid NGM that had been autoclaved and cooled to 55°C. All pharmacological compounds were obtained from Sigma (St Louis, MO, USA). Concentrated solutions of each drug were prepared by dissolving compounds in DMSO. DMSO was shown to slow Ca$^{2+}$ release from the SR. Consequently, release and uptake of Ca$^{2+}$ as well as the rise time of twitches (increase in force) were prolonged, providing a plausible explanation for the increase in maximal tetanic force by CA inhibitors.

Locomotion rate and growth rate

To estimate the locomotion rate of worms, 7-day animals were scored for the number of body bends generated during an interval of 1 min. A body bend was defined as one complete sinusoidal movement from maximum to minimum amplitude and back again. The growth rate refers to the time from P0 egg to the first F1 egg (generation time). At last 30 worms were examined.

Phylogenetic analyses

To search for CA in C. elegans, a Blastp search was performed on Caenorhabditis elegans genome (taxid: 6239) with 14 Homo sapiens CAs sequences obtained from the NCBI website as query sequence. CA1 (NP_001729.1), CA2 (NP_000058.1), CA3 (AAH04897.1), CA4 (NP_000708.1), CA5 (NP_001730.1), CA6 (EAW71606.1), CA7 (EAW83046.1), CA8 (NP_004073.3), CA9 (NP_001207.2), CA10 (NP_064563.1), CA11 (NP_001208.2), CA12 (EAW77648.1), CA13 (NP_940986.1) and CA14 (NP_036245.1). The C. elegans sequences selected are: cah-1 (NP_498083.1), cah-2 (NP_495567.3), cah-3 (NP_510674.1), cah-4 (NP_510265.1), cah-5 (NP_509186.3) and cah-6 (NP_491189.1). A maximum likelihood tree was constructed with SEEVIEW version 4.0 (49), based on alignment using MUSCLE (Multiple Sequence Comparison by Log-Expectation) (50). The Anthopleura elegantissima CA sequence (AF140537_1) was used as an out-group. The bootstrap was performed with 500 replicates.

RNAi procedure

RNAi experiments were performed on NGM plates supplemented with 100 µg/ml ampicillin, 12.5 µg/ml tetracycline and 1 mM IPTG as previously described (51). The RNAi clones for cah-1, cah-2, cah-3 and cah-4 were obtained from the Geneservice library. RNAi for cah-5 and cah-6 were obtained by amplification of ~600 bp of genomic DNA of either gene and cloned into the RNAi feeding vectors L4440 (Courtesy of Dr Andrew Fire). Bacterial suspensions were used to seed RNAi plates. Five adult gravid worms were put on plate for one night and removed, so as the progeny were exposed to drug and RNAi from hatching to fixation. All animals were fixed and observed at day 7. As a control, pos-1 RNAi knockdown performed in parallel resulted in near 100% embryonic lethality.

Generation of cah-4::GFP construct and localization

The putative endogenous promoter of the cah-4 gene has been cloned in-frame with GFP in pPD95.67 (provided by A. Fire). To ensure the amplification of cah-4 promoter region, ~3 kb
upstream of the second ATG start codon of cah-4 have been amplified. Hermaphrodite from dpy-5(e907) strain (CB907) injected with 150 ng/μl pCEh361 (dpy-5 rescuing construct), 10 ng/μl of promoter-cah-4::GFP fusion PCR product. Plasmid microinjection was performed as described (52). Individual F1 wild-types were isolated and we retained a line that transmitted F2 transgensics. Transgenic worms with GFP fluorescence were selected, and the animals were viewed by fluorescence microscopy on a Zeiss LSM 510 Meta fluorescence confocal microscope.

Mice and treatment

mdx mice were obtained from Charles River Laboratories, Belgium. All experiments were conducted in accordance with the guidelines of the institutional animal care committee. Feeding started at an age of 70 days for mice with both 20 × (60 mg/kg/day) and 5 × (15 mg/kg/day) posology DCPM and 5 × (21.5 mg/kg/day) and 1 × (4.3 mg/kg/day) posology MTZ containing food. Control food consisted of the same compounds as the experimental food except the effective substances and did not show any effect on the weight, health and behavior of the mice.

Muscle force measurement

After feeding for 120 days Musculus extensor digitorum longus was removed and connected to an isometric force transducer (type GRA FT-03; FMI, Seeheim, Germany) (model MIO-0501 DC-Brückenmeßverstärker; ALFOS AG, Biel-Benken; FMI, Seeheim, Germany) coupled to a signal amplifier (model MIO-0501 DC-Brückenmeßverstärker; FMI, Seeheim, Germany) (model ISG-8834/1-S: FMI, Seeheim, Germany). Electrical field stimulation was generated by means of platinum electrodes on both sides of the muscle. Supramaximal stimuli with monophasic pulse duration of 1 ms were released through a computer-controlled electrical stimulator (model ISG-8834/1-S: FMI, Seeheim, Germany). Maximal isometric twitch force was assessed by three twitch stimulations. The maximal isometric tetanic force was measured stimulating the muscle at 125 Hz for 175 ms. Specific force was obtained by normalizing maximum tetanic force on total muscle cross-sectional area (CSA). CSA was calculated by following formula (53):

\[
\frac{\text{muscle mass}[\text{mg}]}{\text{fiber length}[\text{mm}]} \times 1.064[\text{mg/mm}]
\]

Measurements for eccentric contraction were performed in principle as described (54).

Histochemistry and immunohistochemistry

Eight-micrometer serial cross sections of M. tibialis anterior and diaphragm were stained with Haematoxylin–Eosin and sequentially with antibodies against type I myosin heavy chain and type II (IIa and IIb) myosin heavy chain for MHC-double staining, respectively. HE-staining was carried out according to standard protocols. The antibody against type I myosin heavy chain (NovoCastra, Newcastle upon Tyne, UK) was diluted 1:100 as well as the type II myosin heavy chain antibody (NovoCastra, Newcastle upon Tyne, UK). Each incubation of the sections with one of the two primary antibodies was followed by staining with the secondary antibody rabbit anti mouse Ig horseradish peroxidise (DAKO, Glostrup, Denmark) diluted 1:100. The different myosin heavy chain fiber types were visualized sequentially by different substrates 3,3′ diaminobenzidine (Sigma-Aldrich, Steinheim, Germany) showing a brown color (type I) and the substrate provided by the Vector SG visualization solution (Vector Laboratories, Burlingham, USA) showing a blue color (type IIa and IIb). After the first color reaction the sections were blocked with fetal calf serum before the second primary antibody was applied.

The fiber boundaries were stained by 100 ng/ml WGA conjugated to Alexa Fluor 488 (Molecular Probes, Leiden, Netherlands). Nuclei were stained with 10 ng/ml DAPI (Sigma, Steinheim, Germany). Finally, cover slips were mounted in DakoCytomation Fluorescent (DAKO, Glostrup, Denmark).

Quantitative assessment of muscle histology

Automatic quantitative analysis of muscle histology was performed with a special designed module for the image analysis system S.CORE by S.C.O LifeScience, Munich, Germany. Three different images of the observed section were taken in parallel: (i) Green fluorescent WGA staining to display the fiber boundaries, (ii) Blue fluorescent DAPI staining to show the nuclei, (iii) MHC-double staining to visualize all fibers which are positive for type I MHC or type IIa and IIb MHC, respectively. In the first image all membrane structures were subtracted from background, leading to a mask for the individual muscle fibers, and the minimal Feret’s diameter calculated.

This mask was superimposed first with the DAPI image to quantify the number of nuclei within each single muscle fiber and subsequently with the MHC-double staining to determine the fibers positive for type I MHC or type IIa and IIb MHC, respectively. The VC was calculated using the following formula:

\[
Z = 1000 \times \frac{\text{S.D. of muscle of fiber min} \cdot \text{Feret’s diameter}}{\text{Mean muscle fiber min} \cdot \text{Feret’s diameter}}
\]

Automated histological analysis was performed only for the 5 × MTZ treated group and the corresponding control group, because the quality of the muscles belonging to the DCPM group was not sufficient for analysis.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0 software (SPSS GmbH Software, Munich, Germany). All data sets were tested for normal distribution using the Kolmogorov–Smirnov test. Those data subsets that showed a normal distribution were analyzed performing a t-test. All others were tested with the Mann–Whitney test.

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