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Supplementary Materials for

Targeting protein homeostasis in sporadic inclusion body myositis

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This PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/8/331/331ra41/DC1)

Movie S1 (.mov format). Low-magnification serial block-face scanning electron microscopy of arimoclomol-treated and untreated mutant VCP mouse muscle.
Movie S2 (.mov format). High-magnification serial block-face scanning electron microscopy of wild-type VCP mouse muscle.
Movie S3 (.mov format). High-magnification serial block-face scanning electron microscopy of untreated mutant VCP mouse muscle.

Movie S4 (.mov format). High-magnification serial block-face scanning electron microscopy of arimoclomol-treated mutant VCP mouse muscle.

Supplementary Materials:

Materials and Methods

i) Primary muscle cultures

Sprague Dawley rats, aged postnatal day 0-2, were sacrificed by Schedule 1 methods. Hind limb muscles were retrieved following blunt dissection and separation of muscle from bone, then washed in Phosphate Buffered Saline (PBS) with 4% penicillin (100 units/ml) and streptomycin (100 μg/ml) (PenStrep), at 4°C. Muscle tissue was manually dissociated into smaller fragments then incubated at 37°C with 3 ml 0.1% collagenase II per 0.05 g of muscle for 45 minutes, triturating every 15 minutes. The resulting solution was filtered through a 100 μm and 40 μm nylon mesh. 5 ml of PBS was added to cell suspension and the suspension centrifuged at 480g for 10 minutes. Supernatant was removed and the pellet re-suspended in 1ml of muscle growth medium (20% Fetal Calf Serum (Life Technologies), 2% PenStrep and 0.5% Chick Embryo Extract (Sera Laboratories International) (in DMEM GlutaMAX-1). Satellite cells were seeded onto gelatinised cover-slips or appropriate tissue culture plates and incubated in 5% CO_2 at 37°C. Myotube differentiation was encouraged by substitution of the initial growth medium with differentiating muscle medium (10% Horse Serum (Life Technologies), 2% PenStrep and 0.5% Chick Embryo Extract in DMEM GlutaMAX-1). Culture medium was changed every 48 hours.

ii) Transfection of myocytes with β-APP

A DNA construct containing the full-length human β-APP gene of 2,312 bp was cloned into a pcDNA3.1+ plasmid (Invitrogen). The pcDNA3.1+ plasmid without the β-APP gene was used as an Empty Vector (EV) control. Plasmid transfection was induced 48 to 96 hours after

initial plating of cultures using Lipofectamine™ 2000 (Invitrogen) according to manufacturer's instructions (Invitrogen). Briefly, differentiating muscle medium was removed and replaced with Optimem ™ (Gibco Life technologies UK). This was subsequently replaced by a transfection solution prepared in Optimem™, comprising 1 μg DNA to 2 μl Lipofectamine 2000™. Cultures were incubated at 37°C for 5 hours with the transfection solution and subsequently replaced with differentiation medium.

iii) Exposure to Inflammatory Mediators

Upon differentiation of cultures to multinucleated myocytes, typically 96 hours after initial plating, inflammatory mediators TNFα (1-25 ng/ml), IL1β (1-20 ng/ml) and in some experiments IFN-gamma (1000 U/ml), were added at the corresponding concentration to muscle medium for 24 to 48 hours.

iv) Immunocytochemistry

Cells were fixed in chilled 1:1 methanol:acetone. Non-specific binding was blocked with 5% milk and 3% serum (species defined by secondary antibody) in 0.1% PBS-Triton X-100 for 1 hour at room temperature. The coverslips were incubated with the following primary antibodies for either 1 hour at room temperature or overnight at 4°C: Desmin D33 (1:100 Dako); β-APP (1:250 Invitrogen); Aβ-40 (1:200 Biosource); Aβ-42 (1:100 Biosource); C-terminal or N-terminal of TDP-43 (1:200 Proteintech); Ubiquitin (1:500 Genetex); HSP70 (1:200 Santa Cruz Biotech); NFκB (1:200 Abcam); p62 (1:100 BD Biosciences); Phosphorylated tau AT8 (1:500 Pierce Endogen) and LC3 II (1:1,000 Novus Biological). After washing, the cover-slips were incubated with the following secondary antibodies, as appropriate, for 2 hours at room temperature: anti-mouse Alexa568 (1:1,000 Invitrogen), anti-rabbit Alexa488 (1:500 Invitrogen); anti-mouse fluorescein (1:200 Vector Labs) and anti-

mouse Texas Red (1:200 Invitrogen). The coverslips were then stained with the nuclear stain, DAPI (1:2,000 Sigma-Aldrich) and mounted on slides using DakoCytomation Fluorescent Mounting Medium. The cells were then visualised using a Leica Fluorescent Microscope. At least three random fields of interest were analysed per cover-slip. The number of myocytes with ubiquitinated inclusion bodies, mislocalised TDP-43 or nuclear NFκB was determined by manual counting of at least 5 fields from 3 independent cultures.

v) Western blots

Cell lysates from cultured muscles and tissue homogenates from isolated TA muscles from VCP mice were obtained by homogenisation in RIPA buffer (2% SDS, 2 mM EDTA, 2 mM EGTA in 5mM Tris, pH 6.8). Samples were spun at 14,000 rpm for 15 minutes and supernatant collected. Protein concentration was determined using a Bio-Rad Protein assay system (Bio-Rad Laboratories).

Samples were then diluted in a 1:1 ratio with 2x Laemmli sample buffer (containing 5% 2-mercaptoethanol) and denatured by heating for 5 minutes at 95°C. Equal amounts of protein from each sample were loaded on acrylamide gels alongside a molecular weight marker (Bio-Rad). The concentration of acrylamide in the gel was dependent on the molecular weight of protein being investigated. Gels were run at 160 V for 1 hour. Proteins were then transferred onto a nitrocellulose membrane (Amersham) by running at 100V for 1 hour.

Blots were blocked for 1 hour in PBS+ 0.1% Tween 20+ 5% milk protein or TBS+ 0.1% Tween 20+ 5% bovine serum albumin (BSA) before incubating overnight at 4°C with appropriate primary antibody at 1:1,000 dilution. Primary antibodies used included β-APP (Invitrogen); Aβ-42 (Biosource); TDP-43 (Proteintech); HSP70 (Santa Cruz Biotech); MHC-

I (Abcam); NFκB (Abcam); LC3 II (Novus Biological); MHC-I (Santa Cruz Biotech) and phospho-IκBα (Cell Signaling Technology). Actin, GAPDH or α-tubulin were used as loading controls as appropriate. Following incubation in HRP-conjugated secondary antibodies (Dako, 1:1000; Thermo Scientific, 1:500) for 2 hours, blots were visualised using Amersham ECL reagent or Supersignal (Thermo Scientific) and then developed on Kodak film.

vi) Cell survival assays

Cell survival was assessed by means of either an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay or a lactate dehydrogenase (LDH) Assay. Protein concentration of homogenised cells from cultures was determined by Bio-Rad Protein assay.

A MTT working solution was prepared from 12 mM thiazolyl blue solution-PBS (Sigma 5655) diluted 1:10 with incubating medium. 50 µl of this working solution substituted existing cell culture medium in each well for 4 hours at 37°C. The resulting precipitate was dissolved in 100 µl of 0.4 M HCl and the absorbance measured at 570 nm by spectrophotometry to quantify the reduction of tetrazolium to formazan by mitochondrial dehydrogenase enzymes. Absolute readings were made relative to unstressed cultures as a percentage and inversed to obtain percentage cytotoxicity. Where cell survival was also assessed by an LDH Assay (Roche Applied Science Cytotoxicity Detection kit, Cat no. 11644793001), cultures in 96-well plates were centrifuged at 250*g* to pellet cellular material. The supernatant from each well was transferred onto a fresh plate. Assay reagents were added to wells according to manufacturer's recommendation and incubated for 15 minutes. The coupled enzymatic reaction produces a colorimetric result, which is then measured by spectrophotometry at 490 nm. Fresh muscle media in wells was used as low controls and

Triton X-100 treated wells were used as high controls. Cell survival assays were repeated in three separate cultures to verify the results.

vii) Determination of ER Stress

To examine ER stress, cytosolic calcium levels were examined using live confocal microscopy. Exposure to thapsigargin, a selective inhibitor of the SERCA, results in rapid depletion of ER calcium which can be detected as an increase in free cytosolic calcium that can be detected and quantified using the fluorescent dye Fluo4-AM. Cells plated and cultured in glass bottomed Petri dishes were incubated at 37°C in the dark for 30 minutes with 1 ml 5μM Fluo4-AM in calcium free standard recording solution (125 mM NaCl, 5 mM KCl, 1 mM $Na_3PO_4.12H_20$, 1 mM $MgSO_4$, 1 g/L D-glucose, 20 mM Hepes). Pluronic acid was added to permeate cells to the dye. After incubation, the Fluo4-AM was replaced by 1 ml recording medium. All steps were performed within a specialised incubator at 37°C, 95% air and 5% CO_2. After selecting a suitable region of interest, continuous imaging was initiated and a period of one to two minutes allowed from which a baseline level of fluorescence could be determined. Thapsigargin was then added to recording medium at a concentration of 1 μM. After 5-8 minutes, the maximal increase in fluorescence to thapsigargin was reached. Ionomycin (5 μM) was then added to recording medium. A maximal cytosolic calcium concentration to ionomycin followed within 2-3 minutes, which was subsequently used as a maximum calibration point. Regions of interest, from which the change in fluorescence during the course of each experiment could be determined, were drawn to correspond with myocytes. Fluorescence was converted to calcium concentration by the formula Ca^{2+} =Kd x (F/Fmax – 1/Rf) / (1 – F/Fmax) where Kd = 350 and Rf = 100 for Fluo4-AM, and Fmax = maximum fluorescence.

viii) Assessment of proteasomal function

Proteasome assays were conducted using a fluorogenic substrate which becomes proteolysed at the chymotrypsin-like catalytic site of the 26S proteasome complex. Cells harvested from T75 culture flasks were centrifuged at 350*g* for 5 minutes and the supernatant discarded. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM dithiothreitol, 2 mM ATP) to which glass beads were added. Cells were lysed for 1 hour on a rotator at 4˚C and 10 μg of the extracted protein was placed per well in a 96-well plate. Epoxomycin was added to control wells to inhibit proteasome activity. Total volume per well was normalized with lysis buffer without sucrose (reaction buffer). Next, 1 μl of fluorogenic substrate (Suc-Leu-Leu-Val-Tyr-AMC, Enzo Life sciences, Exeter UK) was added to each well and fluorescence over time measured using a plate reader at 30 second intervals.

ix) Histochemistry and immunostaining

Haematoxylin and Eosin (H&E) staining on 12 μm-thick muscle sections were performed using standard H&E staining protocol.

Fluorescent immunohistochemistry was performed on 12 μm cryosections of muscle which were incubated for 1 hour in blocking solution (10% normal goat serum in 0.1% PBS-Triton X-100) at room temperature. After blocking solution was removed, slides were washed in PBS and incubated in appropriate primary antibodies (rabbit anti-ubiquitin, Dako 1:500; rabbit anti-TDP-43, ProteinTech 1:500; rat anti-F4/80:PE conjugated, AbD Serotec 1:1,000) for 1 hour at room temperature. Following three washes with PBS, sections were incubated for 2 hours with an anti-goat secondary antibody conjugated to AlexaFluor488 or 568. Sections were then mounted with glass coverslips.

x) Electron microscopy

After fixation, muscle tissue were then cut into approximately 1 mm^3 blocks and further fixed in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for one hour and then processed for transmission electron microscopy (TEM) and serial block-face scanning electron microscopy (SBF SEM). All samples were prepared using the National Center for Microscopy and Imaging Research (NCMIR) method (59).

For TEM, 70 nm sections were cut using a UCT ultramicrotome (Leica Microsystems) and collected on formvar-coated slot grids. No post-staining was required due to the density of metal deposited using the NCMIR protocol. Images were acquired using a 120 kV Tecnai G2 Spirit TEM (FEI Company) and an Orius CCD camera (Gatan Inc.).

For SBF SEM, muscle tissue embedded in Durcupan resin was mounted on a pin and trimmed to a block face of less than 1 mm^2. Imaging was performed in a Sigma variable-pressure SEM (Carl Zeiss) equipped with a 3View 2XP ultramicrotome (Gatan Inc.) for automated serial imaging within the SEM chamber. Blocks were cut at a slice thickness of 50 nm, and imaging performed at 2 keV and 5 Pa with a pixel dwell time of 2 µs.

xi) Fibre area measurements

Tibialis anterior (TA) muscle cryosections from 3 mice per study group were cut at 12 µm thickness and stained for succinate dehydrogenase (SDH) according to published protocol (60). One image of the entire TA muscle section at 2.5x magnification was taken per mouse. Using a Wacom graphics tablet and pen, the perimeter of every fibre in the muscle section was drawn around. Image J software was used to record and analyse the data.

xii) Clinical Trial Inclusion and Exclusion Criteria

Patients fulfilling the following criteria were eligible for enrolment: 1) meet the Griggs diagnostic criteria for definite or probable IBM (53, 54) 2) muscle function adequate for quantitative muscle testing, with at least 8 of the following 16 muscle groups having a manual muscle testing (MMT) muscle grade ≥3- on the modified MRC scale – neck flexors, neck extensors, shoulder abductors, elbow flexors, elbow extensors, wrist flexors, knee extensors, knee flexors and ankle dorsiflexors, 3) age >50 years and 4) post-menopausal (no menses in >12 months) or status post-hysterectomy for females.

The presence of any of the following were exclusion criteria: 1) medical conditions: diabetes mellitus or patients taking anti-diabetic medications, chronic infection, chronic renal insufficiency, cancer other than skin cancer less than 5 years previously, multiple sclerosis or prior episode of central nervous system demyelination, or other chronic serious medical illnesses, 2) laboratory abnormalities: white blood cell count <3000/cm^3, platelets <100,000/cm^3, haematocrit <30%, urea >10 mmol/L, creatinine >150 μmol/L, symptomatic liver disease with serum albumin <30 g/L, prothrombin time or activated partial thromboplastin time greater than the upper range of control values, 3) currently taking riluzole, 4) women who were pregnant or lactating, 5) history of noncompliance with other therapies, 6) coexistence of other neuromuscular disease, 7) drug or alcohol abuse within last 3 months, 8) inability to give informed consent, 9) known bleeding disorder (e.g. haemophilia, Von Willebrand‘s Disease), 10) use of potentially nephrotoxic drugs and 11) prior difficulties with local anaesthetic.

xiii) Assessment of muscle strength in clinical trial

Muscle strength was assessed by manual muscle testing (MMT) and by maximum voluntary isometric contraction testing (MVICT) using the Quantitative Muscle Assessment (QMA) system designed by Computer Source, Atlanta, Georgia, USA (30, 57). MMT was performed on 26 muscle groups: neck flexors and extensors, shoulder abductors, elbow flexors and extensors, wrist flexors and extensors, hip flexors, extensors and abductors, knee flexors and extensors, and ankle dorsiflexors and plantar flexors. Muscle strength of each muscle group was graded utilizing a modified MRC score that was converted to a 13-point scale (grade 0=0, 1=1, 2-=1.67, 2= 2, 2+=2.33, 3-=2.67, 3=3, 3+=3.33, 4-=3.67, 4=4, 4+=4.33, 5-=4.67, 5=5), and the average MMT scores for each patient were calculated. QMA was performed to measure MVICT on 12 muscle groups: elbow flexors and extensors, knee flexors and extensors, ankle dorsiflexors and handgrip. Each muscle was tested twice and the maximum force generated by the patient from the two trials was recorded for each muscle group. The total summed score of strength in kilograms was computed for each patient. MVICT has been shown to be reliable and valid in several neuromuscular disorders, including IBM. Body composition was obtained using a standard dual-energy X-ray absorptiometry (DEXA) whole body scan to assess total body fat-free mass. DEXA has been used to measure lean body mass in previous neuromuscular diseases' studies, including IBM.

xiv) Determination of HSP70 and myosin levels in clinical trial muscle biopsies

For each patient, a muscle biopsy was performed at baseline and at the end of the treatment period (month 4). Muscle biopsy tissue from the USA was shipped to the UK and muscle tissue analyses were centralized and performed simultaneously, with all staff blinded for treatment allocation. Total human myosin content and HSP70 levels were determined in the muscle samples using ELISA assay kits (Enzo Life Sciences). Raw HSP70 and myosin values for each assay were first normalized to take into account the dilution factors. HSP70

content in each sample was then normalized for myosin content, so that for each sample HSP70 content was expressed in ng/100 ng myosin.

Sandwich ELISA assay kits for determination of total human myosin content (USCN Life Science Inc E86098Hu) and HSP70 (Enzo ADI-EKS-700B) were used. Prior to ELISA assays, all muscle biopsies were stored at -80˚C and all samples were processed together. Samples were first homogenized in the extraction reagent supplied with the HSP70 ELISA kit supplemented with protease inhibitors at an initial concentration of 0.5 g/ml using a hand held electronic homogenizer. Samples were then centrifuged at 21,000g for 10 minutes at 4˚C in a refrigerated centrifuge. Supernatants were transferred into new tubes. Protein concentration was determined using a Bio-Rad DC Protein Assay. Samples were then diluted to 0.5 mg/ml using sample diluent supplied with the HSP70 ELISA kit. Following this step, another protein assay was carried out to get an accurate reading of actual protein concentrations in each sample. Samples were then aliquoted and stored until ELISA assay was carried out.

HSP70 and Myosin assays were carried out using the same aliquots of each sample. For the HSP70 assay, samples were diluted 1 in 8 in sample diluent and 100 µl of this solution was loaded onto the ELISA plates in duplicates, alongside HSP70 standard wells (also in duplicates, ranging from 0.78 ng/ml to 50 ng/ml). For the determination of total myosin content, samples were diluted 1 in 32 using sample diluent supplied with the Myosin ELISA kit and also 100 µl samples were loaded onto ELISA plates in duplicates, alongside Myosin standard (also in duplicates, concentration ranging from 0.156 ng/ml to 10 ng/ml). Both assays were then carried out according to manufacturer's description. Once absorbance in each plate was measured, HSP70 and myosin content in each sample was determined using the standard curves in each assay and expressed as ng/ml. Raw HSP70 and myosin values for

each assay were first normalized to take into account the dilution factors. HSP70 content in each sample was then normalized for myosin content, so that for each sample HSP70 content was expressed in ng/100 ng myosin.

Supplementary Figures:

Fig. S1.

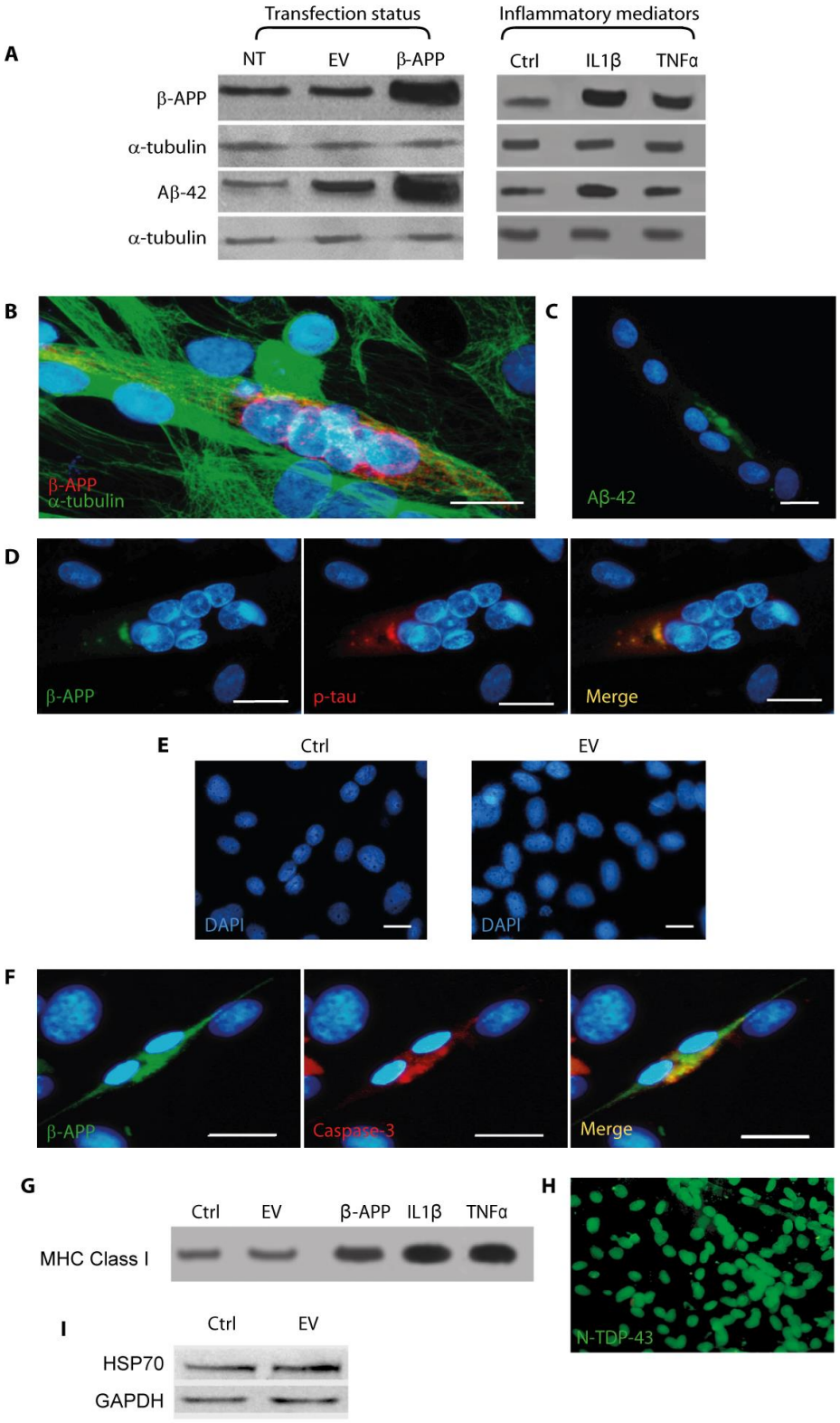

Fig. S1. Overexpression of β-APP and exposure to inflammatory mediators induce sIBM-like pathology in cultured myocytes.

(**A**) Western blot analysis of β-APP and Aβ-42 expression in myocyte cultures transfected with a β-APP plasmid or exposed to inflammatory mediators IL1-β or TNF-α. (**B**) Over-expression of β-APP in cultured myocytes following plasmid transfection with human β-APP gene. Formation of cytoplasmic inclusion bodies in myocytes immunoreactive for (**C**) Aβ-42 and (**D**) phosphorylated tau protein. (**E**) Untreated and empty vector treated myocyte cultures immunostained for β-APP and the nuclear marker DAPI (blue) shows there was no β-APP staining or formation of inclusions in control cultures. (**F**) Up-regulation of caspase-3 in myocytes following over-expression of β-APP. (**G**) Western blot analysis of MHC class I expression in myocyte cultures transfected with a β-APP plasmid or exposed to inflammatory mediators. (**H**) Nuclear localization of the N terminal portion of TDP-43 in β-APP plasmid transfected culture. (**I**) Western blot of HSP70 expression in untreated control and empty vector transfected control cultures. Scale bars= 10 μm

Fig. S2.

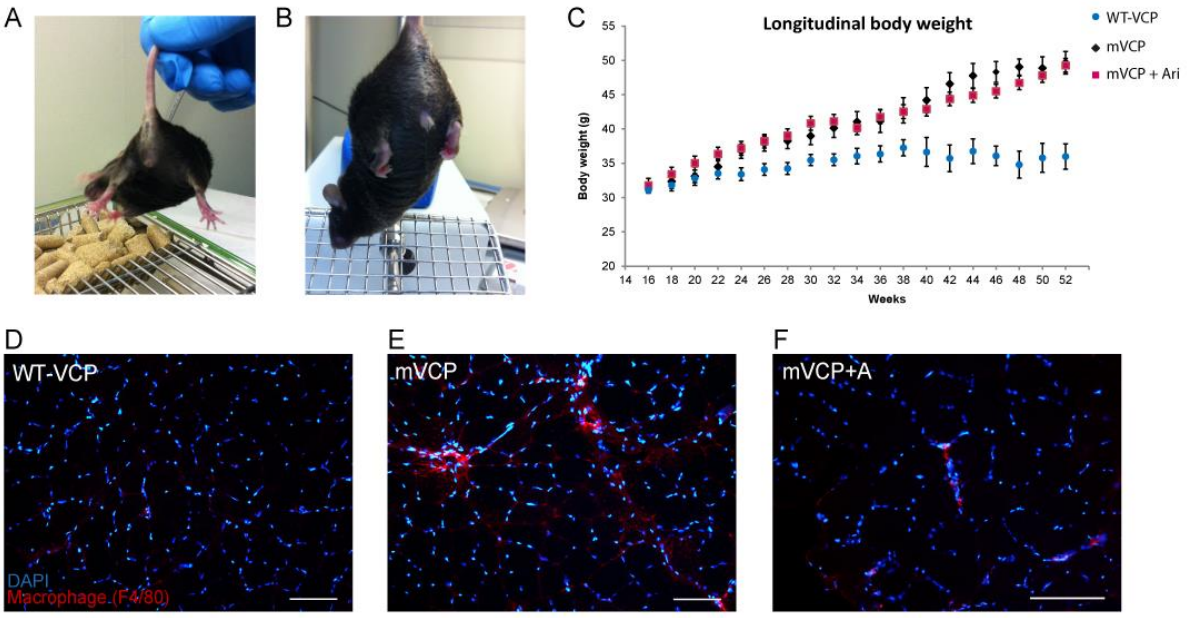

Fig. S2. Mutant VCP mice show further signs of pathology.

Hind-limb reaction upon suspension by tail observed in 7 month old mice. (**A**) Normal toe-spreading reflex in WT-VCP mouse. (**B**) Hind limb clenching response in mVCP mouse. (**C**) Graph of longitudinal body weight of WT-VCP mice (blue circles), mVCP mice (black circles) and arimoclomol-treated mVCP mice (red squares). (**D-F**) Cross sections of TA muscles from WT-VCP, mVCP and arimoclomol-treated mVCP mice immunostained for the macrophage marker F4/80 and the nuclear marker DAPI (blue). Error bars = SEM, scale bar = 50 µm

Fig. S3.

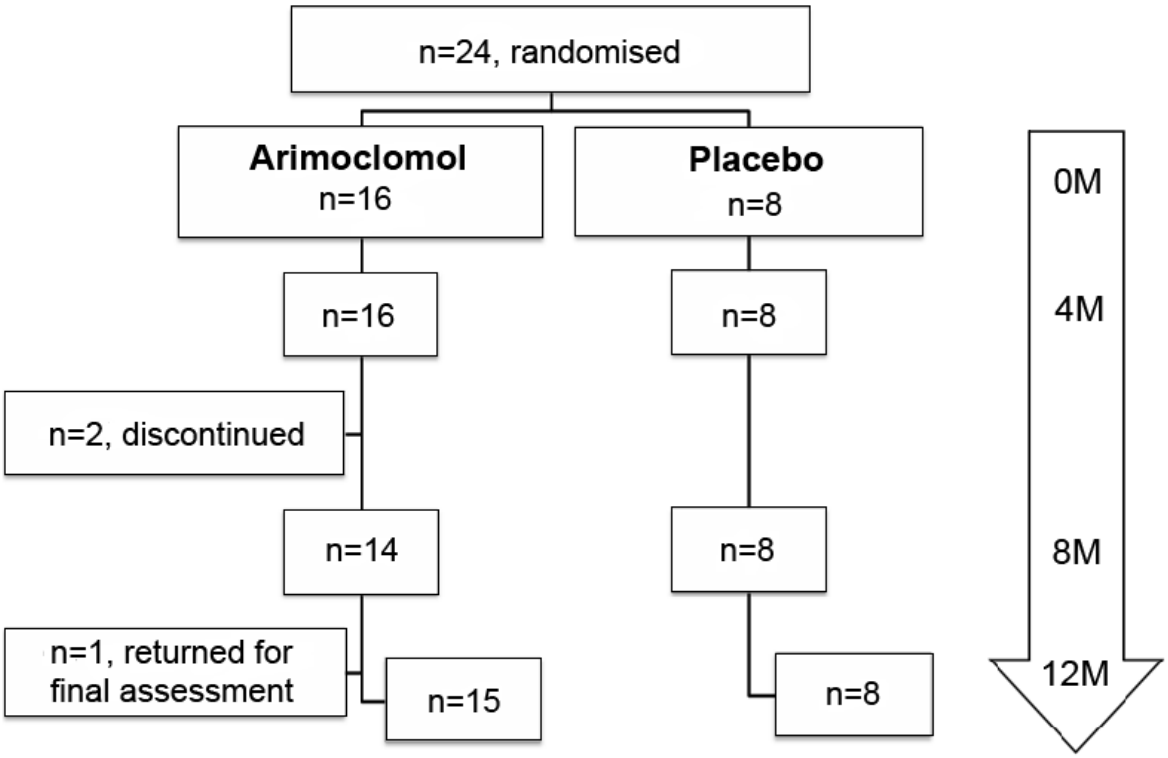

Fig. S3. Consort diagram of the patients participating in the clinical trial.

Supplementary Tables

Table S1. Baseline characteristics of the study population.

	All population (n=24)	Arimoclomol (n=16)	Placebo (n=8)	p-value*
Sex (male), n (%)	17 (70.8)	12 (75.0)	5 (62.5)	0.647
Age, mean ± SD	66.84 ± 7.49	65.85 ± 7.86	68.83 ±6.70	0.426
Ethnicity, n (%)				1.000
- White, not of Hispanic origin	22 (91.7)	14 (87.5)	8 (100)	
- Black, not of Hispanic origin	1 (4.2)	1 (6.3)	0	
- Native American	1 (4.2)	1 (6.3)	0	
Disease duration, mean ± SD	8.4 ± 4.3	7.7 ± 4.5	9.8 ± 3.5	0.298
Initial symptoms/signs, n (%)				1.000
- Swallowing problems	1 (4.2)	1 (6.3)	0	
- Proximal upper extremity involvement	1 (4.2)	1 (6.3)	0	
- Distal upper extremity involvement	4 (16.7)	3 (18.8)	1 (12.5)	
- Proximal lower extremity involvement	18 (75.0)	11 (68.8)	7 (87.5)	
- Distal lower extremity involvement	1 (4.2)	1 (6.3)	0	
IBM diagnostic criteria, n (%)				1.000
- Definite IBM	10 (41.7)	7 (43.8)	3 (37.5)	
- Probable IBM	14 (58.3)	9 (56.3)	5 (62.5)	
IBMFRS score (0-40), mean ± SD	26.6 ± 6.4	27.5 ± 7.0	24.6 ± 5.1	0.375
MMT average score (0-5), mean ± SD	4.2 ± 0.4	4.2 ± 0.5	3.6 ± 1.5	0.540
MVICT sum score, mean ± SD	119.4 ± 63.4	130.4 ± 70.4	94.4 ± 39.8	0.198
Right quadriceps femoris MVICT score, mean ± SD	7.7 ± 8.2	8.8 ± 9.2	5.4 ± 5.4	0.240
Left quadriceps femoris MVICT score, mean ± SD	6.8 ± 6.8	7.3 ± 7.2	6.0 ± 6.2	0.462
Right hand grip MVICT score, mean ± SD	13.6 ± 11.8	15.2 ± 13.6	10.4 ± 6.3	0.373
Left hand grip MVICT score, mean ± SD	11.6 ± 11.5	13.5 ± 13.2	7.8 ± 5.9	0.111
DEXA total body fat free mass (Kg), mean ± SD	47.1 ± 11.3	49.3 ± 9.7	42.6 ± 13.6	0.221

*Arimoclomol versus placebo. DEXA, Dual-energy X-ray absorptiometry; IBM, sporadic Inclusion Body Myositis; IBMFRS, Inclusion Body Myositis Functional Rating Scale; MMT, manual muscle testing; MVICT, maximum voluntary isometric contraction testing; SD, standard deviation.

Table S2. Summary of all adverse events over the course of 1 year.

MedDRA System Organ Class	Arimoclomol (16 patients)	Placebo (8 patients)
Blood and lymphatic system disorders	-	-
Cardiac disorders	Palpitations (*n*=1)	
Congenital, familial and genetic disorders	-	-
Ear and labyrinth disorders	Dizziness/tinnitus (*n*=2)	
Endocrine disorders	-	-
Eye disorders	Conjunctivitis (*n*=1), eye pain (*n*=1)	Dry eyes (*n*=1)
Gastrointestinal disorders	Constipation (*n*=4), throat irritation (*n*=4), loose stools (*n*=2), nausea (*n*=2), dry mouth (*n*=2), bowel movement problems (*n*=1), epigastralgia (*n*=1), gas pain (*n*=1), pyrosis (*n*=1), vomiting (*n*=1), geographic tongue (*n*=1)	Constipation (*n*=4), loose stools (*n*=4), painful parotids (*n*=2)
General disorders and administration site conditions	Weight loss (*n*=1), dizziness (*n*=1), loss of consciousness (*n*=1)	Fatigue (*n*=1)
Hepatobiliary disorders	-	-
Immune system disorders	-	-
Infections and infestations	Sinus infection (*n*=2), upper respiratory tract infection (*n*=7), lower respiratory tract infection (*n*=2), erysipelas (*n*=1), tooth infection (*n*=1)	Tooth infection (*n*=4), upper respiratory tract infection (*n*=3), cellulitis (*n*=1), leg ulcer infection (*n*=1)
Injury, poisoning and procedural complications	Fall/contusion (*n*=23), post-biopsy pain (*n*=3), post-biopsy fatigue (*n*=1)	Fall/contusion (*n*=9), post-biopsy pain (*n*=1), pruritus in biopsy scar (*n*=1), finger cut (*n*=1)
Investigations	Hyponatremia (*n*=2), high thyroxine levels (*n*=1)	Spinal stenosis (*n*=1), herniated disk (*n*=1)
Metabolism and nutrition disorders	-	-

Musculoskeletal and connective tissue disorders	Musculoskeletal pain (*n*=10), cramps (*n*=1), rheumatoid arthritis flare (*n*=1), heat and soreness of proximal lower limbs (*n*=1)	Musculoskeletal pain (*n*=2)
Neoplasms benign, malignant and unspecified (including cysts and polyps)	-	-
Nervous system disorders	Headache (*n*=7), worsening of restless leg syndrome (*n*=1), paresthesia (*n*=1)	Headache (*n*=3), paresthesia (*n*=1), stroke (*n*=1)
Pregnancy, puerperium and perinatal conditions	-	-
Psychiatric disorders	-	-
Renal and urinary disorders	Hematuria (*n*=1)	-
Reproductive system and breast disorders	-	Decreased libido (*n*=1)
Respiratory, thoracic and mediastinal disorders	Cough (*n*=2)	Cough (*n*=1)
Skin and subcutaneous tissue disorders	Rash (*n*=2), rosacea (*n*=1), insect bite with erythema (*n*=1), cold sores (*n*=1)	Rash (*n*=1)
Social circumstances		
Surgical and medical procedures	Tooth extraction (*n*=1), sinus surgery (*n*=1), solar lentigines removal (*n*=1)	Tooth extraction (*n*=1)
Vascular disorders	Hypertension (*n*=3), edema (*n*=2)	Hypertension (*n*=3), edema (*n*=3)
Average number of adverse events (AEs) per patient	**AEs = 6.8/patient**	**AEs = 6.5/patient**

Table S3. Mean changes (±SD) in secondary outcome measures throughout the study period.

Outcome Variable	Arimoclomol change	Placebo change	*P*-value
IBMFRS score			
4M (n=16+8)	-0.34±1.38	-0.88±1.16	0.239
8M (n=14+8)	-0.68±1.58	-2.50±3.31	0.055
12M (n=15+8)	-2.03±2.68	-3.50±3.35	0.538
Average MMT score			
4M (n=15+8)	-0.04±0.19	-0.12±0.20	0.561
8M (n=13+8)	-0.12±0.22	-0.26±0.27	0.147
12M (n=14+7)	-0.21±0.21	-0.35±0.20	0.232
MVICT sum score			
4M (n=14+8)	0.46±12.11	-0.30±14.49	0.633
8M (n=13+8)	7.20±19.65	-1.71±17.80	0.347
12M (n=14+8)	-1.21±20.76	0.52±17.98	0.946
Right quadriceps femoris MVICT score			
4M (n=14+8)	0.33±2.37	0.33±1.49	0.495
8M (n=13+8)	0.21±1.65	-0.09±2.14	0.942
12M (n=14+8)	-0.44±2.18	-0.00±1.89	0.453
Right hand grip MVICT score			
4M (n=14+8)	0.76±2.74	0.50±2.46	0.608
8M (n=13+8)	1.26±2.63	-0.54±1.86	0.064
12M (n=14+8)	1.21±3.70	-0.24±2.94	0.339
Left quadriceps femoris MVICT score			
4M (n=15+8)	0.65±1.26	-0.20±1.22	0.302
8M (n=13+8)	0.31±1.77	-0. 19±1.41	0.664
12M (n=14+8)	-0.44±1.28	-0.07±2.35	0.707
Left hand grip MVICT score			
4M (n=14+8)	0.92±2.44	1.201±2.47	0.946
8M (n=13+8)	1.09±2.43	1.43±3.38	0.638
12M (n=14+8)	1.04±3.10	0.52±2.22	0.891
DEXA total body fat free mass percentage, mean ± SD			
4M (n=15+8)	1.3±1.3	1.9±2.8	0.949
12M (n=14+8)	-2.0±3.8	-1.0±2.0	0.339
HSP70 levels (ng/100ng myosin), mean ± SD			
4M (n=15+8)	-110.72±757.40	-34.70±336.35	0.466

DEXA, Dual-energy X-ray absorptiometry; HSP, heat shock protein; IBM, sporadic Inclusion Body Myositis; IBMFRS, Inclusion Body Myositis Functional Rating Scale; MMT, manual muscle testing; MVICT, maximum isometric voluntary contraction testing; SD, standard deviation.

Supplementary Movies

Movie S1. Low-magnification serial block-face scanning electron microscopy of arimoclomol-treated and untreated mutant VCP mouse muscle. This movie shows 98 of 400 images collected for the untreated (mVCP) and arimoclomol-treated (mVCP+A) muscle samples using a 30 μm aperture. Horizontal frame width and total volume of WT-VCP are 68.6 μm and 24,480 μm^3, respectively; mVCP+A, 70.7 μm and 23,059 μm^3, respectively. Scale bar = 10 μm.

Movie S2. High-magnification serial block-face scanning electron microscopy of wild-type VCP mouse muscle. This movie shows 100 serial images using a 20 μm aperture. Horizontal frame width and total volume of the sample are 21.0 μm and 2,205 μm^3, respectively. Scale bar = 2 μm.

Movie S3. High-magnification serial block-face scanning electron microscopy of untreated mutant VCP mouse muscle. This movie shows 100 serial images using a 20 μm aperture. Horizontal frame width and total volume of the sample are 19.8 μm and 1,960 μm^3, respectively. Scale bar = 2 μm.

Movie S4. High-magnification serial block-face scanning electron microscopy of arimoclomol-treated mutant VCP mouse muscle. This movie shows 100 serial images using a 20 μm aperture. Horizontal frame width and total volume of the sample are 21.0 μm and 2,205 μm^3, respectively. Scale bar = 2 μm.