HHS Public Access

Author manuscript

Sci Transl Med. Author manuscript; available in PMC 2016 September 30.

Published in final edited form as:

Sci Transl Med. 2016 March 23; 8(331): 331ra41. doi:10.1126/scitranslmed.aad4583.

Targeting Protein Homeostasis in Sporadic Inclusion Body Myositis

Mhoriam Ahmed[1,2,#], **Pedro M. Machado**[1,#], **Adrian Miller**[1,2,#], **Charlotte Spicer**[1,2,#], **Laura Herbelin**[3], **Jianghua He**[4], **Janelle Noel**[4], **Yunxia Wang**[3], **April L. McVey**[3], **Mamatha Pasnoor**[3], **Philip Gallagher**[5], **Jeffrey Statland**[3], **Ching-Hua Lu**[1,2], **Bernadett Kalmar**[1,2], **Stefen Brady**[1], **Huma Sethi**[6], **George Samandouras**[6], **Matt Parton**[1], **Janice L. Holton**[1], **Anne Weston**[7], **Lucy Collinson**[7], **J. Paul Taylor**[8], **Giampietro Schiavo**[1,2], **Michael G. Hanna**[1,*], **Richard J. Barohn**[3,*], **Mazen M. Dimachkie**[3,*,¶], and **Linda Greensmith**[1,2,*,¶]

[1]MRC Centre for Neuromuscular Diseases, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK

[2]Sobell Department of Motor Neuroscience and Movement Disorders, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK

[3]Department of Neurology, University of Kansas Medical Center, 3901 Rainbow Blvd, Mail Stop 2012, Kansas City, Kansas 66160, USA

[4]Department of Biostatistics, University of Kansas Medical Center, 3901 Rainbow Blvd, Kansas City, Kansas 66160, USA

[5]Department of Health, Sport & Exercise Science, the University of Kansas, Lawrence, Kansas 66045-7567, USA

[6]Victor Horsley Department of Neurosurgery, National Hospital for Neurology & Neurosurgery, University College London Hospitals, Queen Square, London WC1N 3BG, UK

[¶]Correspondence should be addressed to: Professor L. Greensmith, Sobell Department of Motor Neuroscience and Movement Disorders, MRC Centre for Neuromuscular Diseases, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK. Phone: +4402034484280. l.greensmith@ucl.ac.uk, Dr. M. M. Dimachkie, Professor of Neurology, Director of Neurophysiology Division and of Neuromuscular Section, Department of Neurology, University of Kansas Medical Center 3901 Rainbow Blvd, Mail Stop 2012, Kansas City, KS 66160, USA. Phone: +19135886970. mdimachkie@kumc.edu.

[#]These authors contributed equally to this paper

[*]Co-Senior Authors

Author contributions

M.A., A.M. and L.G. designed the *in vitro* experiments. M.A. and L.G. designed the *in vivo* experiments and M.A. and C.S. performed the experiments and data analyses. J.P.T generated and characterized the VCP mice. P.M., A.M., L.H., Y.W., A.L.M., M.P., P.G., J.S., S.B., M.P., J.H., M.G.H., R.J.B., M.M.D. and L.G. designed the clinical trial. P.M. performed the clinical trial assessments in the UK. L.H., Y.W., A.L.M., M.P., R.J.B. and M.M.D. performed the clinical trial assessments in the US. J.H. and J.N. conducted data analyses of the trial data. M.M.D., A.L.M., H.S. and G.S. performed the trial muscle biopsies. C-H.L. and B.K. performed the heat shock protein measurements in the trial muscle tissue. A.W. and L.C. carried out the electron microscopy analyses and G.S. undertook their interpretation. M.G.H, R.J.B. and M.M.D. supervised the clinical trial. L.G. supervised the *in vitro* cell model and *in vivo* preclinical trial experiments and heat shock protein measurements in the muscle tissue. M.A., P.M., C.S and L.G. wrote the drafts of the manuscript and all authors provided scientific input to the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

LG became an unpaid consultant to Orphazyme Aps (the owner of Arimoclomol) after completing this study. The other authors declare no competing interests.

[7]The Francis Crick Institute, Lincoln's Inn Fields Laboratory, 44 Lincoln's Inn Fields, London WC2A 3LY, UK

[8]Department of Cell & Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105-3678, USA

Abstract

Sporadic inclusion body myositis (sIBM) is the commonest severe myopathy in patients over age 50. Previous therapeutic trials have targeted the inflammatory features of sIBM, but all have failed. Since protein dyshomeostasis may also play a role in sIBM, we tested the effects of targeting this feature of the disease. Using rat myoblast cultures, we found that up-regulation of the heat shock response with Arimoclomol reduced key pathological markers of sIBM *in vitro*. Furthermore, in mutant valosin-containing protein VCP mice, which develop an inclusion body myopathy (IBM), treatment with Arimoclomol ameliorated disease pathology and improved muscle function. We therefore evaluated the safety and tolerability of Arimoclomol in an investigator-lead, randomised, double-blind, placebo-controlled, proof-of-concept patient trial and gathered exploratory efficacy data which showed that Arimoclomol was safe and well tolerated. Although Arimoclomol improved some IBM-like pathology *in vitro* and *in vivo* in the mutant VCP mouse, we did not see statistically significant evidence of efficacy in this proof of concept patient trial.

Introduction

Sporadic inclusion body myositis (sIBM) is the commonest idiopathic inflammatory myopathy (IIM) occurring in patients over 50 years of age (1-7), but no treatment is available. The prevalence of sIBM differs between different populations and ranges between 1 and 71 individuals per million (8-13). sIBM is distinguished from other IIMs by early asymmetric finger flexor and knee extensor weakness leading to loss of hand function and propensity to fall, and resistance to immunosuppressive therapy. However, any skeletal muscle may be affected including oesophageal and pharyngeal muscles. Late-stage disease is characterized by significant morbidity, including motor disability, swallowing failure and poor quality of life. Death in IBM is related to malnutrition, cachexia, aspiration, and respiratory failure (1-7).

Although the aetiology of sIBM remains uncertain, the varied pathological findings observed in patient muscles have driven a number of hypotheses, including viral infection, accumulation of toxic proteins, autoimmune attack, myonuclear degeneration, endoplasmic reticulum (ER) stress and impairment of autophagy and proteasomal function (14-18). Muscle biopsies from sIBM patients typically show several pathological features broadly described as either inflammatory or degenerative. Inflammatory features include endomysial infiltration by mononuclear cells, which surround and invade non-necrotic muscle fibres and overexpression of major histocompatibility complex class I (MHC-I), which is not constitutively expressed by skeletal muscle. The degenerative features of sIBM include the formation of rimmed vacuoles and inclusion bodies containing a range of proteins including β-amyloid precursor protein (β-APP), heat shock proteins (HSPs), phosphorylated tau (p-Tau), p62 and the cytoplasmic mislocalisation of RNA-binding proteins including transactive

response DNA binding protein 43 (TDP-43), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and hnRNPA2B1 (19-22).

Despite lack of any experimental evidence, protein accumulation could theoretically play a role in triggering inflammation in IBM muscle. For example, amyloid oligomers can induce key components of sIBM pathology, at least *in vitro*, so that β-APP overexpression and consequent accumulation impairs innervation by co-cultured neurons (23), causes mitochondrial dysfunction (24), and *in vivo*, results in calcium dyshomeostasis (25). Conversely, inflammation may induce the onset of degenerative features through exposure to inflammatory cytokines and increased nitric oxide production (26). In muscle biopsies of sIBM patients, expression of APP mRNA correlates with inflammation and expression of mRNAs of chemokines and IFN-γ. Furthermore, unlike muscles of inflammatory myopathies, in sIBM muscles, inflammatory mediators co-localize with β-amyloid deposits within myofibres. *In vitro*, exposure of human myotubes to IL-1β causes upregulation of APP with subsequent aggregation of β-amyloid. These findings suggest there is a link between the production of pro-inflammatory mediators and β-amyloid-associated degeneration in sIBM muscle (27).

Previous clinical trials in sIBM have only tested agents directed at the inflammatory component of pathology and all were ineffective (3, 4, 28-31). Whether the degenerative aspect of IBM is primary to the pathogenesis or not, it very likely plays a role in the deleterious effects in muscle, and may be a potential therapeutic target. Protein homeostasis (proteostasis) is essential for normal cellular functions (32) and in conditions of cellular stress, proteins can become unfolded or misfolded, leading to their aberrant aggregation (33). Protein misfolding is normally controlled by endogenous chaperone proteins (34) that prevent aberrant protein-protein interactions and promote correct protein folding. Heat shock proteins are a family of ubiquitously expressed protein chaperones, which are up-regulated following stress-induced activation of the heat shock response, an endogenous cytoprotective mechanism. Since the heat shock response declines with advanced age (35), upregulation of the heat shock response in disorders in which there is evidence of protein mishandling, such as sIBM, may be of therapeutic value.

Arimoclomol is a pharmacological agent that co-induces the heat shock response (36) by prolonging the activation of Heat Shock Factor-1 (HSF-1) (32), the main transcription factor that controls heat shock protein expression, thus augmenting heat shock protein levels (36-39). Importantly, Arimoclomol only acts on cells under stress in which HSF-1 is already activated, and does not induce the heat shock response in unstressed cells (40) thereby avoiding the side effects associated with widespread non-targeted heat shock response activation. Indeed, previous examination of the effects of Arimoclomol in mice has shown that treatment with Arimoclomol has no detectable effect on non-stressed cells, and there was no increase in heat shock protein expression in any tissue studied (including spleen, heart, brain, spinal cord, nerve and muscle).

In this study, we took a 3-step translational approach to test whether heat shock response augmentation may be a potential therapy for sIBM. We first established robust *in vitro* models representing the degenerative and inflammatory components of sIBM. Primary

rodent myotube cultures were induced to overexpress human β-amyloid precursor protein (β-APP) by transfection of the wild-type human gene, or cultures were exposed to the inflammatory cytokines interleukin 1β (IL1β) and tumor necrosis factor α (TNFα). In these models, muscle cells developed several key IBM-like pathological features, which were used as outcome measures to examine the therapeutic potential of Arimoclomol in IBM. Having established the beneficial effects of Arimoclomol *in vitro*, we next tested its ability to ameliorate sIBM-like pathology in mice overexpressing mutant valosin-containing protein (VCP; p97 in mouse). These transgenic mice model the degenerative disorder Multisystem Proteinopathy (MSP), which has a phenotypic spectrum that includes Inclusion Body Myopathy, Paget's Disease of the bone, and frontotemporal dementia (41). Although these mice are a model of the genetic rather than sporadic form of IBM, they do develop progressive muscle weakness and pathological hallmarks of inclusion body myopathy. In addition, we also undertook a randomized, double-blind, placebo-controlled (RDBPC), proof-of-concept trial of Arimoclomol verses placebo for the treatment of sIBM in human patients. The aim of this trial was to evaluate the safety and tolerability of Arimoclomol and to gather exploratory efficacy data.

Results

Arimoclomol improves sIBM-like pathology in myoblast cultures

We first established accurate *in vitro* models of sIBM–like pathology in which primary rat myoblast cultures were either transfected with β-APP or exposed to inflammatory cytokines. For both cell culture models, we first undertook a series of titrating experiments to determine the optimal conditions; the effects of different DNA and lipofectamine ratios or different cytokine concentrations on β-APP and Aβ40 and Aβ42 expression was examined, at different stages *in vitro*.

Transfection of primary rat myocytes with full-length human β-APP increased the expression of β-APP and its toxic cleavage product amyloid beta-42 (Aβ-42), and led to the formation of cytoplasmic inclusion bodies, a hallmark of sIBM, containing β-APP, ubiquitin and TDP-43, amongst other proteins (Fig.1A-C; Fig.S1A-E). Treatment with Arimoclomol post-transfection, significantly reduced the formation of cytoplasmic inclusions ($p<0.01$; $n=3$; Fig.1A-C). Additionally, β-APP overexpression increased the expression of MHC-I, a characteristic feature of sIBM muscle and the proapoptotic protein caspase-3 (Fig.S1F). Exposure of cultured rat myocytes to IL1β and TNFα was also associated with elevated expression of β-APP and Aβ-42, although in the absence of inclusion body formation. Increased MHC-I expression was also reproduced in the cytokine-exposed cultures (Fig.S1G). Similar results were obtained with IFN-γ.

Overexpression of β-APP or exposure to inflammatory mediators resulted in cytoplasmic mislocalisation of the C-terminus of TDP-43 from the nucleus (Fig.1D-G), which is a key pathological feature of sIBM patient muscle. This mislocalisation was almost completely prevented by treatment with Arimoclomol (Fig.1D-G). Thus, whereas only 1.4±0.6% of rat myocytes showed mislocalised TDP-43 in control cultures, overexpression of β-APP induced TDP-43 mislocalisation in 52.2±5.3% of myocytes, which was reduced to only

2.4±0.53% following treatment with Arimoclomol for 24 hours post-transfection (P<0.001; n=3).

Treatment with inflammatory mediators also induced TDP-43 mislocalisation in rat myocytes, which was also reduced by Arimoclomol treatment, from 47.4±2.8% to 24.8±2.0% in myocyte cultures exposed to IL1β (P<0.05) and 59.9±9.0% to 29.8±2.1% in TNFα-exposed cultures (P<0.05). In addition, exposure to inflammatory cytokines also increased the overall level of TDP-43 expression, and this was also reduced by treatment with Arimoclomol (Fig.1H). The N-terminus of TDP-43 remained in its nuclear location under all conditions studied (Fig.S1H).

Although the extent of TDP-43 mis-localisation induced by β-APP overexpression and exposure to inflammatory mediators was similar, Arimoclomol was more effective in preventing TDP-43 mis-localisation in the β-APP overexpression model. This difference most likely reflects the underlying cause of TDP-mislocalisation in the two models, where stress induced by protein misfolding is the key pathological trigger in β-APP overexpressing cells. Arimoclomol directly reduces levels of misfolded proteins and cell stress by upregulating the heat shock response. The mode of action of Arimoclomol in the inflammatory models is less direct, and may involve a general reduction in cell stress and consequently NF-κB activation, as shown in Fig. 1I-K.

β-APP overexpression also activated the NF-κB cascade, most likely as part of a non-specific stress response, as shown by nuclear translocation of the NF-κB subunit p65, observed in 43.1±6.2% of myocytes in β-APP transfected cultures compared to 8.8±1.2% in control cultures (P<0.01; Fig.1I, K). Treatment with Arimoclomol reduced this to 23.6±4.2% (P<0.05). Unsurprisingly, exposure to inflammatory cytokines also activated the NF-κB cascade, from 4.6±1.3% and 9.1±1.4% in control cultures to 88.1±6.3% and 38.9±2.9% (P<0.001) in IL1β-treated and TNFα-treated cultures, respectively (Fig.1J, K). Arimoclomol reduced this effect to 41.2± 6.8% (P<0.05) and 24.3± 3.1% (P<0.05), respectively.

Arimoclomol augments HSP70 expression and improves cell survival

Overexpression of β-APP and exposure to inflammatory mediators resulted in a significant increase in HSP70 expression, which increased by 3.7 and 2.3 fold of control following β-APP overexpression and treatment with IL1β, respectively (p<0.05, n=3). Arimoclomol increased HSP70 expression further, by 2.4 and 2.1 fold in β-APP and IL1β treated cultures, respectively (Fig.2A-C). There was no difference in HSP70 levels in untreated controls and empty vector treated cultures (Fig. S1 I). β-APP overexpression and exposure to inflammatory mediators also resulted in significant cell death, which was reduced by treatment with Arimoclomol (p<0.05; n=3; Fig.2D, E).

Arimoclomol attenuates ER Stress and protein mishandling

β-APP overexpression and exposure to inflammatory mediators increased endoplasmic reticulum (ER) stress in cultured rat myocytes, as determined by measurement of intracellular calcium ions, where reduced cytosolic [Ca^{2+}] was indicative of ER stress. In β-APP over-expressing cells exposed to the ER stressor, thapsigargin, cytosolic [Ca^{2+}] was

significantly lower than in controls ($p<0.05$; n=3; Fig.3A). β-APP overexpression also induced upregulation of expression of the ER stress mediator CHOP (Fig.3B). This β-APP-induced disruption in ER calcium ion handling was prevented by treatment with Arimoclomol, where cytosolic [Ca^{2+}] was significantly increased to the levels observed in control cultures ($p<0.05$, n=3; Fig.3A), accompanied by a decrease in CHOP expression (Fig.3B).

Exposure to the inflammatory cytokines IL1β and TNFα also resulted in a significant reduction in cytosolic [Ca^{2+}] (Fig.3A) and an increase in the expression of CHOP (Fig.3B). Treatment with Arimoclomol restored cytosolic [Ca^{2+}] to control levels and reduced the expression of CHOP ($p<0.05$; n=3; Fig.3A, B).

Aberrant expression of the shuttle protein p62 (Sequestosome 1) was observed in β-APP overexpressing cells (Fig.3C). This protein targets misfolded proteins for degradation and was observed in cytoplasmic aggregates in cultured rat myocytes, suggesting altered protein handling. Assessment of proteasomal function demonstrated a significant decrease in proteasomal activity following β-APP overexpression ($p<0.05$; n=3), however, this was not improved by treatment with Arimoclomol (Fig.3E). Autophagic degradation was assessed by examination of the autophagosome marker LC3 II, which increased by 1.28-fold in β-APP overexpressing cells compared to controls. However, this was reduced to 0.74-fold the expression level of controls in Arimoclomol-treated cultures, indicating a reduction in autophagic degradation, possibly due to a reduction in mis-folded proteins ($p<0.05$; n=3; Fig.3D, F).

Arimoclomol ameliorates IBM-like pathology in mutant VCP mice

Arimoclomol has clear beneficial effects in cellular models of sIBM. We therefore next tested the efficacy of Arimoclomol *in vivo* in mutant VCP mice. These mice model Multisystem Proteinopathy (MSP) and develop characteristic hallmarks of inclusion body myopathy (IBM). Mutant VCP mice were treated with Arimoclomol from 4 to 14 months of age. By 14 months, there was a significant decrease in muscle force in mutant VCP mice ($p<0.0001$; n=10), as assessed by longitudinal analysis of grip strength as well as acute *in vivo* physiological assessment of isometric muscle force (Fig. 4A-C). The loss of muscle force observed at 14 months was prevented by treatment with Arimoclomol. Grip strength was significantly greater in treated mutant VCP mice than untreated mice ($p=0.0175$; n=10; Fig.4A-C). There was also an improvement in the toe-spreading reflex observed in Arimoclomol-treated mutant VCP mice (Fig.S2A, B). However, Arimoclomol had no effect on the abnormal increase in body weight observed in mutant VCP mice (Fig.S2C). Histopathological analysis of hind-limb muscles of mutant VCP mice confirmed the presence of significant IBM-like pathology including myofibre atrophy, increased endomysial connective tissue, the presence of degenerating fibres and vacuoles as well as hypertrophic fibres (Fig 4D). All of these pathological findings were reduced in Arimoclomol-treated mutant VCP mice (Fig.4D-I; Movie S1). In addition, mutant VCP mouse muscles showed evidence of inflammatory cell infiltration as well as upregulation of major histocompatibility complex I (MHC-I) and the phosphorylated form of the NFκB substrate, IκBα. (Fig. 4E-G). There was no evidence of inflammatory infiltrates in

Arimoclomol-treated mutant VCP mouse muscles and levels of MHC-I and IκBα were reduced (Fig.4E-G). In particular, treatment with Arimoclomol dramatically improved mitochondrial morphology and reduced sarcoplasmic reticulum swelling and vacuole number in mutant VCP mouse muscle fibres compared to controls (Fig.4H; Movie S2-S4). Furthermore, the macrophage infiltration observed in mutant VCP mouse muscle was also reduced in Arimoclomol-treated mice (Fig. S2D-F). Arimoclomol-treated mutant VCP mouse muscles also showed an decrease in the muscle fibre diameter compared to untreated mutant mouse muscle ($p<0.0001$; n=3; Fig.4I). These beneficial effects of Arimoclomol were accompanied by an increase in HSP70 expression in mutant VCP mouse muscles (Fig. 4J), a decrease in the expression of ubiquitin (Fig.5A-C) and a reduction in cytoplasmic mis-localisation of TDP-43 ($p=0.01$; n=3; Fig.5D-F).

Clinical trial of safety and tolerability of Arimoclomol for the treatment of sIBM

We next undertook a randomized, double-blind, placebo-controlled (RDBPC) proof-of-concept trial of Arimoclomol versus placebo for the treatment of sIBM. The primary aim of the trial was to evaluate the safety and tolerability of Arimoclomol, but exploratory efficacy data were also gathered. Sixteen sIBM patients were randomized to receive Arimoclomol and eight to receive placebo. The duration of the treatment period was 4 months, and the follow-up continued for a further 8 months after treatment ceased, with an overall trial duration of 12 months. At 4 months (end of the treatment phase) all sIBM patients were still participating in the trial. At 8 months, two patients had discontinued the study (due to travel difficulties), but one of these patients returned for the final assessment at 12 months (Fig. S3). Baseline clinical and demographic characteristics were similar between groups (Table S1).

There were no significant differences between treatment groups regarding the rate, type and severity of adverse events (Table S2). There were 8 adverse events in the placebo group and 14 in the Arimoclomol group, the most common being gastrointestinal (see Table S2 for details). In the Arimoclomol group, one serious adverse event was reported as a result of persistent high blood pressure requiring overnight hospitalization in a patient with known poorly-controlled hypertension, in whom the first trial muscle biopsy was identified as a stressful event. Blood pressure normalized after adjustment of the patient′s anti-hypertensive medication and kept within the normal range throughout the remainder of the trial. Hypertensive episodes were also observed in two placebo patients, under similar circumstances, although these cases did not require hospitalization. Two cases of hyponatremia and one case of high thyroxine levels were observed in the Arimoclomol group, however these were transient, asymptomatic and did not require treatment. The episode of hematuria in the Arimoclomol group was also limited and did not require treatment. All infections resolved with standard treatments, with or without antibiotics, and did not require hospitalization. Ocular toxicity and arrhythmia were not observed in any study subjects.

Clinical trial secondary outcomes

Overall, there was no statistically significant difference in the secondary outcome measures favouring Arimoclomol. However, there were trends in favour of Arimoclomol, but these

will require a formal large-scale patient trial powered for efficacy to be assessed further. Physical function and muscle strength decline rates over time were numerically higher in the placebo group compared to the Arimoclomol group (Fig. 6; Table S3). At 8 months, there was a trend favoring the Arimoclomol group, with a *P*-value of 0.055 for change in the IBM Functional Rating Scale (IBMFRS) score (-0.68±1.58 vs. -2.50±3.31), 0.147 for change in the average Manual Muscle Testing (MMT) score (-0.12±0.22 vs. -0.26±0.27), and 0.064 for change in the right grip Maximum Isometric Voluntary Contraction Testing (MVICT) score (1.26±2.63 vs. -0.54±1.86). No differences were seen for changes in the other MVICT scores, changes in dual-energy X-ray absorptiometry (DEXA) fat free mass percentage, or changes in myosin-adjusted HSP70 expression in muscle (Table S3).

Discussion

Here, we examined the effects of targeting the heat shock response in sIBM. In sIBM patients, Arimoclomol was found to be safe and well tolerated, with a trend of a slower decline in muscle strength and physical function compared with placebo control sIBM patients, although the trend was not significant. Although we did not observe statistically significant clinical efficacy or significant morphological changes in the repeat muscle biopsies taken from Arimoclomol-treated patients, studies both in cellular models *in vitro* and in an *in vivo* mouse model which recapitulates many features of sIBM in muscle showed that the pathological and functional deficits associated with sIBM were ameliorated by Arimoclomol in these model systems. *In vitro*, both β-APP overexpression and exposure to inflammatory cytokines induced degenerative sIBM-like pathology in rat myocytes, including an increase in the formation of ubiquitinated inclusion bodies. Treatment with Arimoclomol reduced inclusion body formation, indicating an improvement in protein handling. This was most likely due to upregulation of the heat shock response, in particular enhanced HSP70 expression. Both models also recapitulated the increase in mis-localised TDP-43 observed in sIBM patient myofibres. TDP-43 is cleaved by caspase-3 (42), allowing the C-terminus to leave the nucleus, thus linking its translocation to increased cell stress. Arimoclomol also reduced TDP-43 mislocalisation and TDP-43 expression in cultures exposed to inflammatory mediators, suggesting that the drug reduced levels of cell stress. Indeed, both β-APP overexpression and inflammatory mediators induced cell death, which was reduced by Arimoclomol.

Nuclear translocation of the NF-κB subunit p65, which was observed following exposure to IL1β and TNFα, was examined as an indication of NF-κB activation. β-APP overexpression also activated NF-κB as has been observed in cellular models of Alzheimer's disease in which Aβ42 peptide activates the inflammatory cascade (43), most likely as part of a non-specific stress response. Arimoclomol had an inhibitory effect on NF-κB activation in both the β-APP and the inflammatory cell models, which likely reflects upregulation of the heat shock response and downregulation of the NF-κB signaling cascade by heat shock proteins (44).

Arimoclomol also decreased the disruption in ER calcium ion homeostasis induced in both the β-APP and inflammatory cell models of sIBM and consequently reduced the ER stress response, a key mechanism triggered by aberrantly folded proteins. This was reflected by

restoration of cytosolic calcium ion concentrations. Indeed, in TNFα-treated rat myotube cultures, the reduction in cytosolic calcium ion concentration was prevented by Arimoclomol, as was the expression of the ER stress mediator CHOP. This protective effect of Arimoclomol may reflect, at least in part, the chaperone activity due to augmented expression of HSP70, which serves to improve the efficiency of handling and degradation of misfolded proteins.

Examination of the two major protein degradation pathways in the cell culture models revealed disruption in both proteasome function and increased autophagy. However, Arimoclomol had no effect on proteasome function, although formation of mature autophagosomes was reduced, suggesting a reduced misfolded protein load in the lysosomal pathway.

In vivo, treatment of mutant VCP mice with Arimoclomol improved the pathological features and functional deficits characteristic of the inclusion body myopathy associated with multisystem proteinopathy (MSP). Thus, in Arimoclomol-treated mutant VCP mice we observed an increase in muscle strength and a reduction in key pathological hallmarks of IBM, including decreased ubiquitin expression, a reduction in cytoplasmic mis-localisation of TDP-43, and a dramatic improvement in mitochondrial morphology accompanied by an increase in heat shock protein expression.

These findings provide evidence for the beneficial effects of Arimoclomol in experimental models of sIBM. This supports clinical assessment of the effects of Arimoclomol in sIBM patients. The primary endpoint of our randomised, double-blind, placebo-controlled, proof-of-concept trial in sIBM patients was met, with results showing that Arimoclomol was both safe and well tolerated by sIBM patients. Regarding the secondary endpoints (efficacy measures), there were no statistically significant differences between the treatment groups. However, this was not surprising since this first experimental study of a compound targeting the heat shock response in sIBM had several limitations. These included the small sample size, which had been advised by our ethics committees and the FDA because Arimoclomol had never been given to sIBM patients before and this study was intended as a proof-of-concept/safety study that was not powered for efficacy. The short duration of treatment (only 4 months) was also mandated by regulatory agencies. In a slowly progressing disease like sIBM, longer treatment periods are likely to be required in order to be able to detect changes in efficacy outcome measures. Whether Arimoclomol can ameliorate sIBM pathology will only be determined by performing adequately powered clinical trials of longer duration. A 12 month study powered for efficacy that will enroll 150 IBM patients has now been approved and fully funded and enrollment will commence in late 2016.

Finally, regarding the measurement of muscle HSP70 expression in sIBM patients, this read-out also had several limitations, namely the fact that muscle HSP70 expression is extremely sensitive to multiple factors, including disease stage, physical activity, age and gender (45-47) and the fact that muscles on opposite sides of the body were biopsied at baseline and post-treatment.

Arimoclomol has previously been shown to be safe in both animal models and humans (36, 39, 48) and has been indicated to be of potential therapeutic benefit in several neurological disorders including diabetic peripheral neuropathy and retinopathy (49). Arimoclomol has also been found to be beneficial in animal models of neurodegeneration, including models of acute injury-induced neuronal death (37) and has been shown to have therapeutic value in mouse models of motor neuron disease, including ALS (36, 50) and Kennedy's Disease (51). Arimoclomol has been through seven phase I clinical trials in healthy volunteers to assess its safety, tolerability and pharmacokinetic properties as well as a small-scale phase II trial in ALS patients (48). A phase IIa dose-ranging trial in ALS has shown Arimoclomol to be safe and well tolerated up to 100 mg three times daily (52) and a phase II/III randomised, double-blind, placebo-controlled trial is currently underway in familial SOD1-ALS patients (NCT00706147).

Our results show that Arimoclomol is safe and well tolerated in sIBM patients, and ameliorates key degenerative and inflammatory features of IBM pathology in experimental cellular and animal models. Together, these findings support further investigation of Arimoclomol for the treatment of sIBM.

Materials and Methods

Study Design

In this study, we took a 3-step translational approach to test whether augmentation of the heat shock response may be a potential therapy for sIBM, by examining the effects of Arimoclomol in rat muscle cells *in vitro*, in a preclinical study in mutant VCP mice, and in sIBM patients in a safety and tolerability trial. *In vitro*, the effects of Arimoclomol were examined in rat primary muscle cultures overexpressing β-APP or treated with inflammatory cytokines, by assessing the effects on IBM-relevant histopathological characteristics. All experiments were repeated in at least 3 independent cultures. In the preclinical efficacy study, mutant VCP mice were randomized to Arimoclomol or vehicle treatment arms and the effects on muscle force and histopathological characteristics were set as the study endpoints. The experimenter was blind to genotype and treatment group throughout. In sIBM patients, we undertook a randomized, double-blind, placebo-controlled, proof-of-concept trial of Arimoclomol versus placebo, to evaluate the safety and tolerability of Arimoclomol and to gather exploratory efficacy data, by testing muscle strength and HSP70 expression. This trial was an exploratory study conducted without prior knowledge of effect size of Arimoclomol in sIBM. The sample size was chosen based on feasibility. Randomization was performed centrally for both study sites.

In vitro model of IBM

Primary muscle cultures were used as an *in vitro* model of IBM by extracting satellite cells that lie under the basal lamina of myofibres. Muscle cells were induced to model either the degenerative features of sIBM, by over-expression of β-APP, or the inflammatory characteristics, by treatment with inflammatory mediators (see Supplementary Methods for details of muscle culture preparation and treatment).

In these *in vitro* models of sIBM, the following features of sIBM pathology were examined and the effects of treatment with 10 μM Arimoclomol investigated: i) inclusion body formation; ii) heat shock protein expression; iii) cytoplasmic translocation of TDP-43; iv) cell survival; v) NFκB activation; vi) ER Stress (for details see Supplementary Material).

Breeding and maintenance of mutant VCP mice

All experimental procedures were carried out under licence from the UK Home Office (Scientific Procedures Act 1986), and following approval by the UCL Institute of Neurology's Animal Welfare and Ethical Review Board. Mice overexpressing the wildtype or mutant (A232E) human VCP gene under the CMV-enhanced chicken β-actin promoter [see Custer *et al.* (40)] were bred and maintained at UCL Institute of Neurology. Transgenic females carrying the wildtype or mutant gene were mated with wild-type C57BL/6J males to generate transgenic and non-transgenic littermates. Only male offspring were used in this study to prevent gender differences. The mice were genotyped by PCR amplification of ear notches and analysed using agarose gel electrophoresis and visualized using GelRed™ stain (Sigma-Aldrich). All mice used in this study were housed in a controlled temperature and humidity environment with a 12-hour light/dark cycle and had access to drinking water and food *ad libitum*.

Treatment of mutant VCP mice with Arimoclomol

Following genotyping, male mice were randomly assigned to a treatment or vehicle arm of the study. From 4 months of age to the time of examination at 14 months, mutant VCP mice were treated with 120 mg/kg/day Arimoclomol dissolved in drinking water (n=10) or water alone (vehicle; n=10). The body weight of all mice was recorded fortnightly. Arimoclomol was obtained from Orphazyme ApS. All experiments were undertaken blinded to genotype and treatment.

Longitudinal assessment of grip strength and body weight

Grip strength was assessed fortnightly in all mice from 4-14 months of age, using a Bioseb™ force gauge according to the manufacturer's instructions. An average of four maximum readings was obtained. Body mass was recorded at the same time as grip strength. The ratio of Grip strength:body mass was determined for individual animals and were pooled by each genotype.

In vivo analysis of isometric muscle force

The mice were prepared for *in vivo* assessment of muscle function (see Kieran *et al.* (2004) (35)). Briefly, mice were deeply anaesthetized (inhalation of 1.5–2.0% isoflurane in oxygen delivered through a Fortec vaporizer (Vet Tech Solutions Ltd)). The distal tendons of the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles in both hindlimbs were dissected free and attached to isometric force transducers (Dynamometer UFI Devices). The sciatic nerve was exposed, sectioned and all branches cut except for the deep peroneal nerve that innervates the TA and EDL muscles. Muscle length was adjusted for maximum twitch tension. Isometric contractions were elicited by stimulating the nerve to the muscles using square-wave pulses of 0.02 ms duration at supra-maximal intensity using silver wire

electrodes. Contractions were elicited by trains of stimuli at frequencies of 40, 80 and 100 Hz for 450 ms and the maximum twitch and tetanic tension was measured using force transducers connected to a Picoscope 3423 oscilloscope (Pico Technology) and subsequently analysed using Picoscope Software v5.16.2 (PicoTechnology). Muscle histology, immunohistochemistry, western blot and electron microscopy protocols are described in the Supplementary material.

Clinical trial design and patient population

In this investigator-initiated, double-blind, placebo-controlled study, 24 patients meeting the Griggs diagnostic criteria for definite or probable sIBM (53, 54) were randomised to Arimoclomol (100 mg) or placebo (2:1 ratio), three times a day, over 4 months (treatment phase). This restricted 4-month treatment period, mandated by the US Food and Drug Administration (FDA), was followed by an 8-month blinded assessment phase. The study was conducted from August 2008 to May 2012, at two Centres in two countries (12 patients/Centre): University of Kansas Medical Centre (KUMC), Kansas, USA, and Medical Research Council (MRC) Centre for Neuromuscular Diseases, London, UK. Detailed inclusion and exclusion criteria can be found in the Supplementary Methods.

Randomization was performed centrally for both study sites at KUMC. This was done by a General Clinical Research Centre statistician, who sent the randomization codes, created using a random number generator table, to the respective research pharmacies. All personnel involved in the conduct of the trial were blind to the identity of the treatment assignments, except for the unblinded statistician and the pharmacist at each site who labelled the study medication using codes provided by the unblinded statistician. The appearance of the placebo was identical to that of Arimoclomol.

The study was conducted according to the ethical principles of the Declaration of Helsinki and approved by the Independent Ethics Committee or Institutional Review Board for each Centre. Informed consent was obtained from each patient before randomization. The study was registered with ClinicalTrials.gov (NCT00769860) and with International Standard Randomised Controlled Trial Number Register (ISRCTN80057573).

Clinical trial: Primary outcomes

The safety and tolerability of Arimoclomol compared to placebo was the primary outcome of the trial. Participants were seen for assessment of adverse events at every study visit. Unscheduled visits to evaluate potential adverse events could occur at any time. All serious adverse events were reported to the sponsor and regulatory authorities according to standard operating procedures. The trial was monitored by an Independent Safety Monitoring Committee.

During the first 4 months (treatment phase), all participants completed a study medication diary. Pill bottles were brought to each visit for a count by a research team member to check on whether participants were taking the study medication in the appropriate dosages. At screening and months 1, 2, 3, 4 and 12, the study participants had full safety laboratory analyses done which included: full blood count with differential, prothrombin time, activated partial thromboplastin time, urea, creatinine and electrolytes, glucose, phosphate and

calcium, alanine transaminase, aspartate transaminase, total bilirubin, albumin, full urine analysis and 24-hour urine protein content and creatinine clearance. At months 0.5, 1.5, 2.5, and 3.5, participants had partial safety laboratory analyses done including: serum creatinine, urea, electrolytes, glucose, phosphate, calcium and a full urine analysis. An electrocardiogram was performed at screening and months 1 and 3. An ophthalmic examination was performed at screening and month 10. The electrocardiogram and ophthalmic examination were introduced as safety measures following results from an animal model study which raised concerns of potentially accelerated cataract formation and apparent risk of sudden, unexplained death with Arimoclomol at very high doses and with Arimoclomol used in conjunction with riluzole (CytRx Corporation. Arimoclomol Investigator′s Brochure version 7.0, September 2010).

Clinical trial: Secondary outcomes

Physical function was measured using the IBM functional rating scale (IBMFRS) that is intended only for patients with sIBM. It includes 10 measures (swallowing, handwriting, cutting food and handling utensils, fine motor tasks, dressing, hygiene, turning in bed and adjusting covers, changing position from sitting to standing, walking, and climbing stairs), graded on a Likert scale from 0 (being unable to perform) to 4 (normal). The sum of the 10 items gives a value between 0 and 40, with a higher score representing less functional limitation. The IBMFRS is a sensitive and reliable tool for assessing activities of daily living in patients with sIBM and is quickly administered (see Supplementary Methods) (7, 30, 55-57).

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) (see Supplementary Methods). 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 sIBM (30, 57, 58). 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 disease studies, including sIBM (30, 57). Patient muscle HSP70 expression was determined as described in the Supplementary Methods.

Statistical methods

In the preclinical cellular model and mutant VCP mouse model, analysis of normally distributed results was performed using an unpaired t-test or, for comparison of greater than two groups, a one-way ANOVA. Otherwise, the non-parametric Mann-Whitney U-test was used.

Data management and statistical analysis were performed by General Clinical Research Centre (GCRC) informatics staff and a GCRC statistician. Data was entered blindly to a GCRC password-protected electronic database and analyses were performed after database lock. Descriptive statistics were used to summarize subject disposition and adverse events by

treatment group. In order to reduce measurement error, baselines scores were computed by calculating the average of visit 1 (screening visit) and visit 2 (baseline visit), which had to be less than 21 days apart. Continuous variables were compared between treatment groups using the Mann-Whitney U test. Categorical variables were compared between treatment groups using Chi-square or Fisher′s exact test, as appropriate. Treatment groups were compared at baseline as well as regarding changes in the several outcome measures at 4 months (IBMFRS, MMT, MVICT, DEXA and HSP70 content), 8 months (IBMFRS, MMT and MVICT) and 12 months (IBMFRS, MMT, MVICT and DEXA).

Statistical analyses were performed using STATA v10 and in all analyses statistical significance was set at the two-sided 5% level. Error bars represent standard error of the mean unless otherwise stated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We sincerely thank the participants who enrolled in this intensive study and the KIT and UK myositis support groups. The following clinicians, research nurses, coordinators and technicians contributed substantially to the trial: Maureen Walsh, Mimi Michaels, Faisal Raja, Arthur Dick, Kevin Latinis, Liz Dewar, Gisela Barreto, Iwona Skorupinska. We thank Orphazyme Aps and CytRx for providing drug and placebo.

Funding

This work was funded by Arthritis Research UK (Ref 19255), MRC Centre for Neuromuscular Diseases Grant (G0601943), Kansas University Neurology Department Ziegler Grant, Kansas University General Clinical Research Centre CReFF Grant and the Wellcome Trust (107116/Z/15/Z) (GS). LG is the Graham Watts Senior Research Fellow, supported by the Brain Research Trust, Rosetrees Trust, The Stoneygate Trust and funded by the European Community's Seventh Framework Programme (FP7/2007-2013). LC and AW are funded by Cancer Research UK, the MRC, BBSRC and EPSRC under grant award MR/K01580X/1 to LC and Peter O'Toole (York University). This project was also supported by an Institutional Clinical and Translational Science Award, NIH/NCATS Grant (UL1TR000001). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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Accessible Summary

Targeting protein dyshomeostasis in sporadic IBM

sIBM is a debilitating adult myopathy without treatment. Although both inflammation and protein dyshomeostasis are implicated in sIBM pathogenesis, previous trials have only targeted the inflammatory component, and all have failed. Here, we tested the effects of targeting protein dyshomeostasis, using Arimoclomol, a co-inducer of the heat shock response. In rat myoblast models, Arimoclomol reduced key pathological features of IBM. *In vivo*, in mutant valosin-containing protein (VCP) mice, which develop an inclusion body myopathy, treatment with Arimoclomol ameliorated disease pathology and improved muscle function. A safety and tolerability trial of Arimoclomol in sIBM patients showed that Arimoclomol was safe and well tolerated.

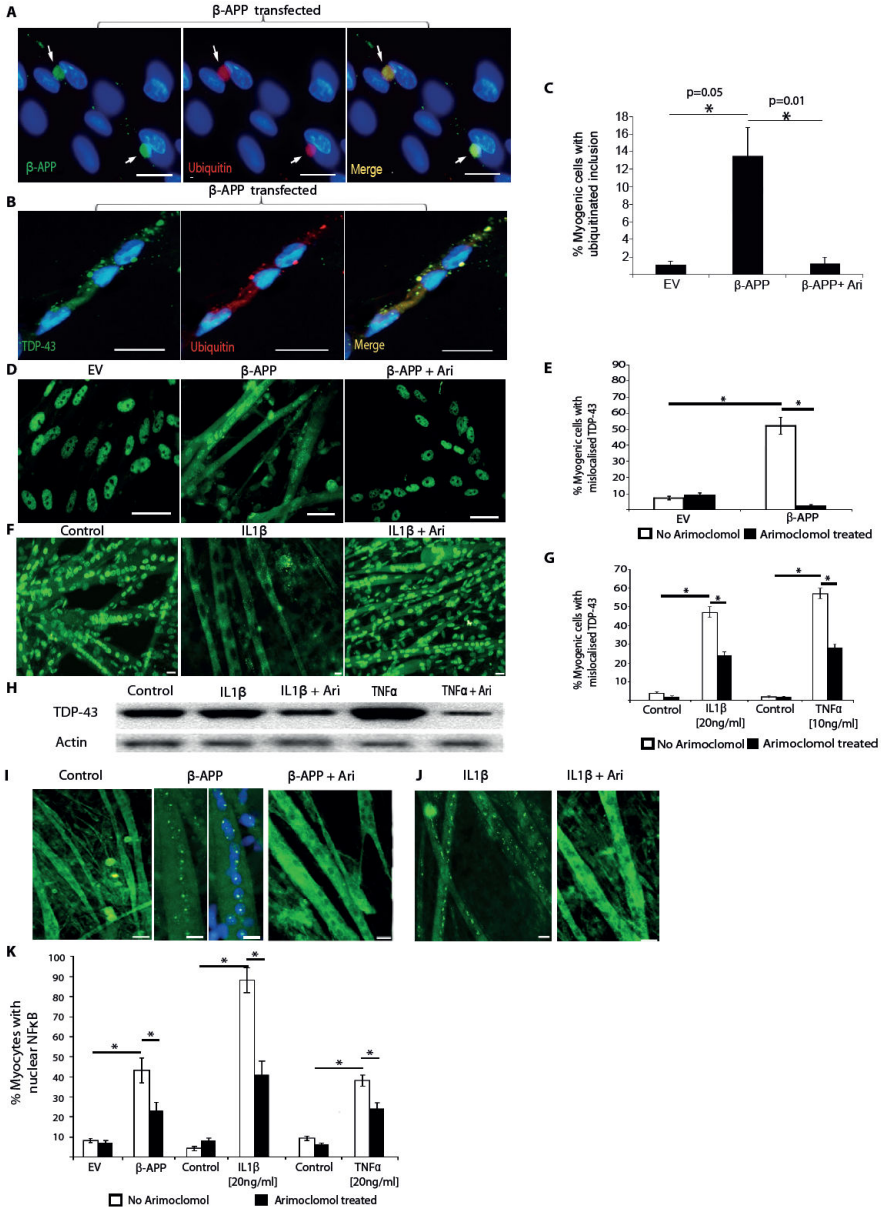

Fig. 1. β-APP overexpression or exposure to inflammatory mediators induces sIBM-like pathology in cultured rat myocytes that is abrogated by Arimoclomol

Shown is formation of cytoplasmic inclusion bodies (white arrows) in rat myocytes transfected with full-length human β-APP immunoreactive for (**A**) β-APP and ubiquitin and (**B**) TDP-43 and ubiquitin. (**C**) The number of myocytes containing ubiquitinated inclusion bodies as a percentage of the total number of myocytes present (n=3; $p<0.05$; one-way ANOVA). (**D**) Expression of TDP-43 (green) following empty vector (EV) or β-APP transfection and Arimoclomol treatment, and (**E**) the number of myocytes with cytoplasmic mislocalisation of TDP-43 (n=3, $p<0.001$; unpaired t-test). (**F**) TDP-43 expression (green) following exposure to inflammatory mediators and Arimoclomol, and (**G**) quantification of TDP-43 mislocalisation in cytokine-treated cultures (n=3, $p<0.05$; unpaired t-test). (**H**) Western blot analysis of TDP-43 expression in rat myocyte cultures exposed to cytokines in

the presence and absence of Arimoclomol. Images show the expression of NF-κB subunit p65 (green) in (**I**) β-APP transfected cultures (DAPI labelled nuclei in blue), and (**J**) cultures exposed to cytokines in the presence and absence of Arimoclomol. (**K**) The number of rat myocytes with nuclear NF-κB subunit p65 as a percentage of the total number of myocytes present (n=3, $p<0.05$; one-way ANOVA). Error bars = S.E.M; scale bars: **A**, **B** =10 μm, **D**, **I** and **J** =20 μm. * indicates statistical significance.

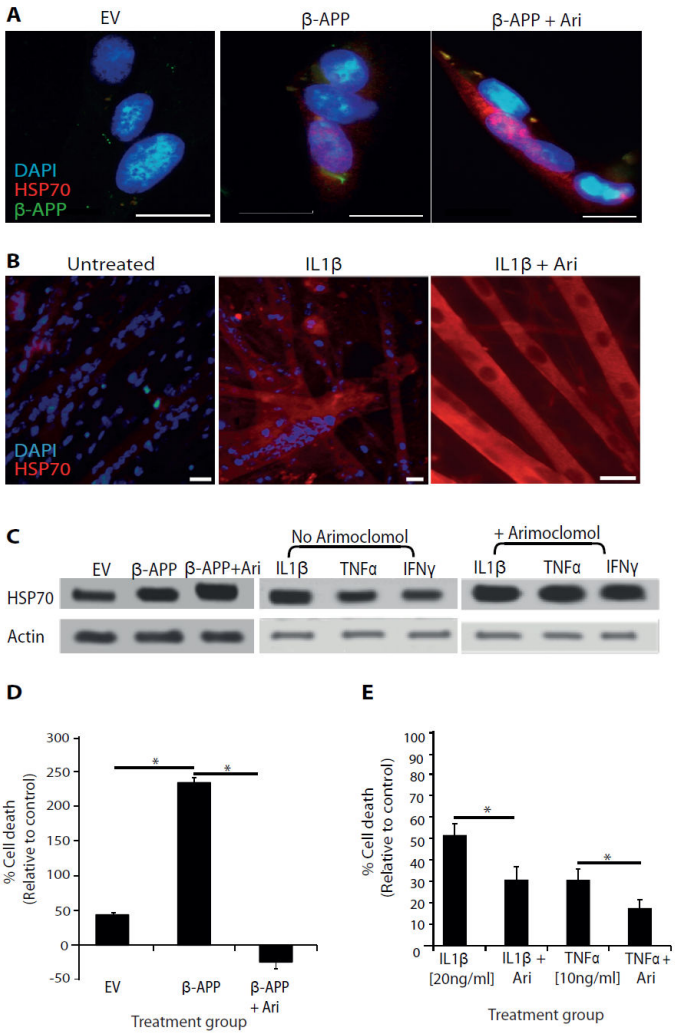

Fig. 2. Arimoclomol augments HSP70 expression and improves survival of cultured rat myocytes

(**A**) HSP70 expression (red) in cultured rat myocytes following empty vector (EV) transfection (left hand panel), β-APP overexpression (middle panel), or β-APP overexpression plus treatment with Arimoclomol (right hand panel). (**B**) HSP70 expression (red) in cultured rat myocytes that were untreated (control; left hand panel), or after exposure to IL-1β alone (middle panel) or IL-1β plus Arimoclomol (right hand panel). (**C**) Western blot analysis of HSP70 expression in rat myocyte cultures exposed to inflammatory mediators or after β-APP overexpression in the presence or absence of Arimoclomol. (**D**) Cytotoxicity in β-APP overexpressing rat myocyte cultures as a percentage of that in control cultures (n=3, $p<0.02$; one-way ANOVA), as assessed by a lactate dehydrogenase (LDH) assay. (**E**) Cytotoxicity following exposure to inflammatory mediators in the presence or absence of Arimoclomol (n=3, $p<0.05$; one-way ANOVA), as assessed by an MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. Error bars= S.E.M.; Scale bars=10 μm. * indicates statistical significance.

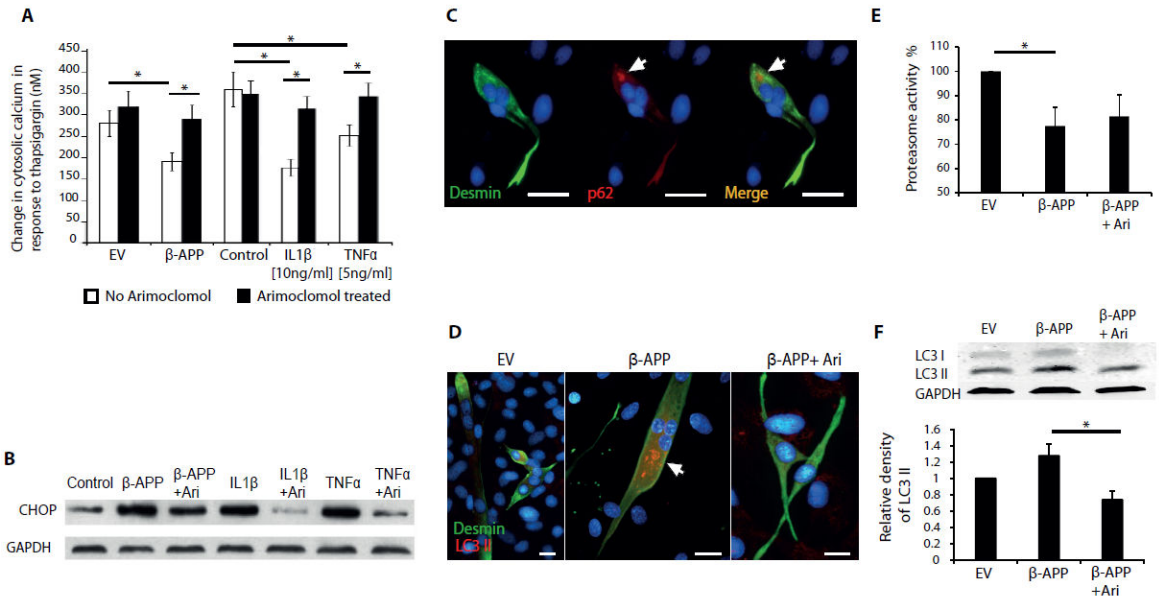

Fig. 3. Disruption of protein homeostasis in rat myocyte cultures is prevented by Arimoclomol

(**A**) The cytosolic calcium ion response induced by the ER stressor thapsigargin (an indicator of ER stress) in β-APP overexpressing rat myocyte cultures and in cell cultures exposed to inflammatory mediators (n=3, $p<0.05$; one-way ANOVA). (**B**) Expression of the ER stress mediator CHOP determined by Western blot analysis in rat myocyte cultures overexpressing β-APP or exposed to inflammatory mediators, in the presence or absence of Arimoclomol. (**C**) The images show the presence of a cytoplasmic aggregate immunoreactive for p62 (red; white arrow) in a desmin-positive rat myocyte following β-APP transfection. (**D**) The images show the expression of the autophagic protein LC3-II (red; white arrow) in β-APP transfected desmin-positive rat myocytes after Arimoclomol treatment. The white arrow indicates punctate LC3-II staining of autophagosomes. (**E**) The chymotrypsin-like proteasome activity assessed 48 hours after β-APP transfection in the presence or absence of Arimoclomol (n=3, $p<0.05$; one-way ANOVA). (**F**) The expression of the autophagosome marker LC3 II in β-APP transfected rat myocytes with or without Arimoclomol (n=3, $p<0.05$; one-way ANOVA). EV, empty vector. Error bars=S.E.M; Scale bars=10 μm. * indicates statistical significance.

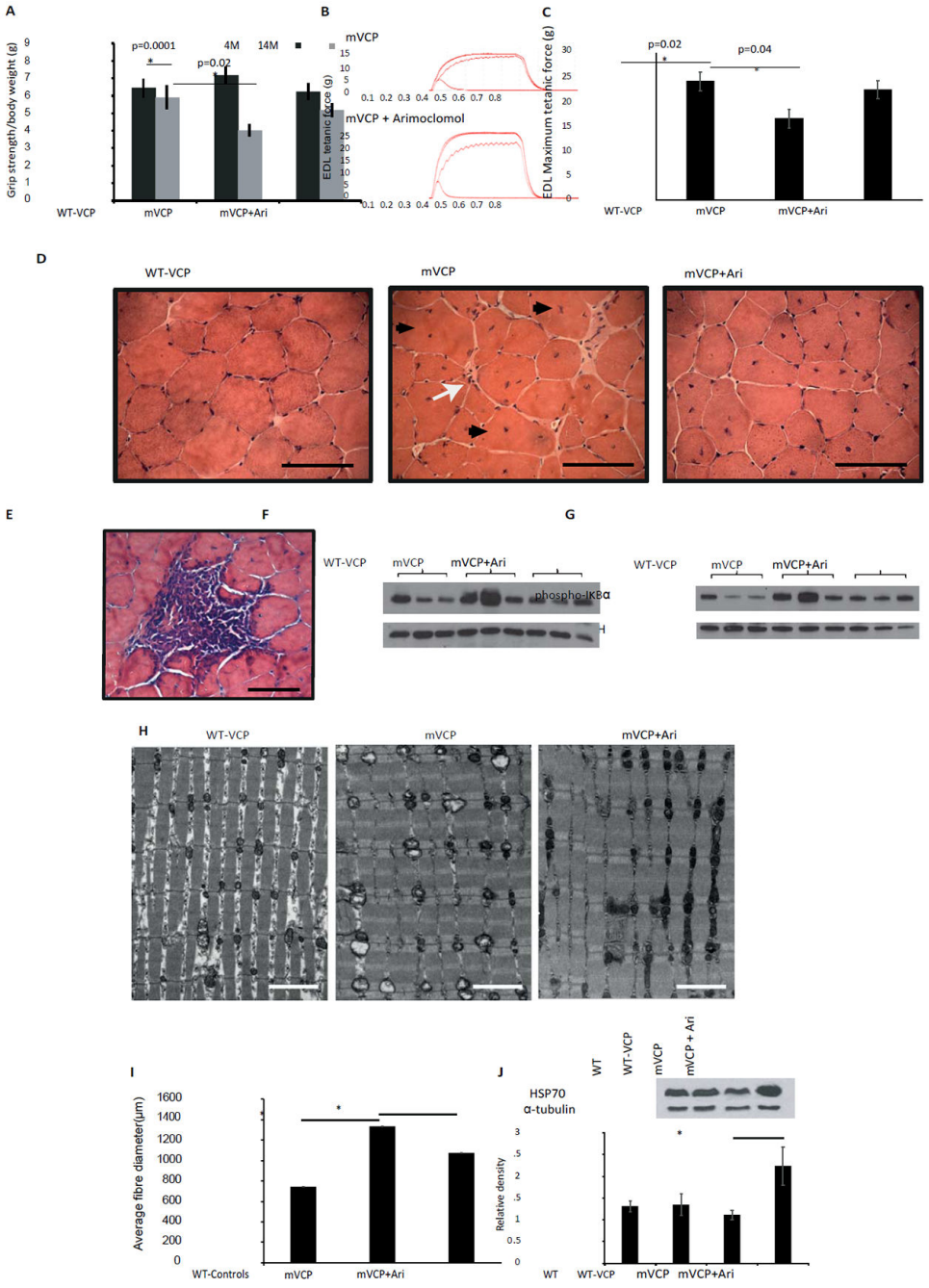

Fig. 4. Arimoclomol treatment improves muscle strength, muscle contractile characteristics and IBM-like pathology in mutant VCP mice

(**A**) The change in grip strength in wildtype VCP (WT-VCP), mutant VCP (mVCP) and Arimoclomol-treated mutant VCP (mVCP+Ari) mice between 4 and 14 months (n=10; p<0.0001; unpaired t-test). (**B**) Typical traces of muscle twitch and maximum tetanic force of extensor digitorum longus (EDL) muscles in untreated and Arimoclomol-treated mutant VCP mice. (**C**) Mean maximum force of extensor digitorum longus muscles of WT-VCP, mutant VCP and Arimoclomol-treated mutant VCP mice (n=10; p<0.04; one-way ANOVA). (**D**) H&E staining of tibialis anterior (TA) muscles of mice in each experimental group

(white arrow, atrophied fibre; black arrowheads, hypertrophic fibres). (**E**) H&E staining showing clear inflammatory cell infiltration in mutant VCP tibialis anterior muscle. Western blots show (**F**) MHC-1 and (**G**) phospho-IκBα expression in tibialis anterior muscles of mice in each experimental group. (**H**) Transmission electron microscopy of tibialis anterior muscles of mice in each experimental group. (**I**) Muscle fibre diameter in tibialis anterior muscles from untreated and Arimoclomol-treated mutant VCP mice compared to WT-VCP mice (n=3; $p<0.0001$; one-way ANOVA). (**J**) Western blot analysis of HSP70 expression in tibialis anterior muscles from mice in each experimental group. Bar chart shows mean relative optical density (n=3; $p=0.01$; unpaired t-test). Error bars = S.E.M, Scale bars: **D, E** =50 μm; **H**=2μm. * indicates statistical significance.

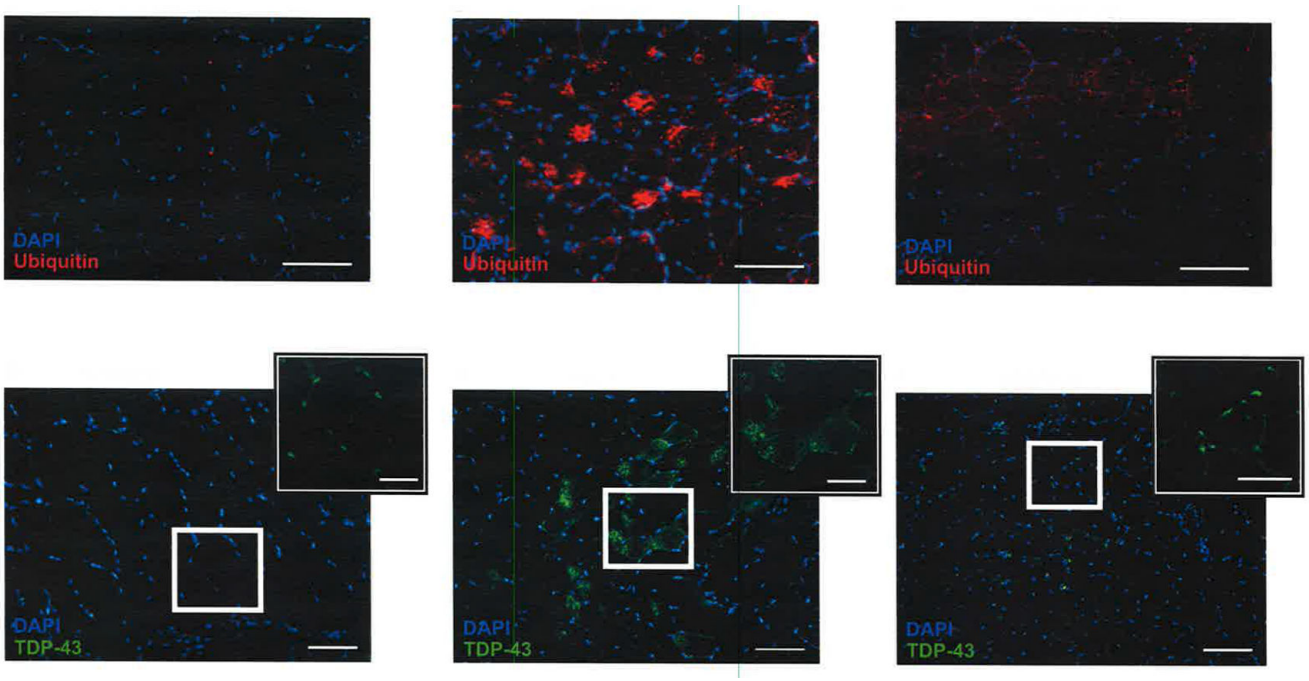

Fig. 5. Arimoclomol abrogates IBM-like pathology in mutant VCP mouse muscle

Cross sections of tibialis anterior muscles from wildtype VCP (WT-VCP), mutant VCP (mVCP) and Arimoclomol-treated mutant VCP (mVCP+Ari) mice, immunostained for ubiquitin (red; **A**-**C**) and TDP-43 (green; **D**-**F**); nuclei stained with DAPI (blue). Scale bar = 50 μm; inserts = 25 μm.

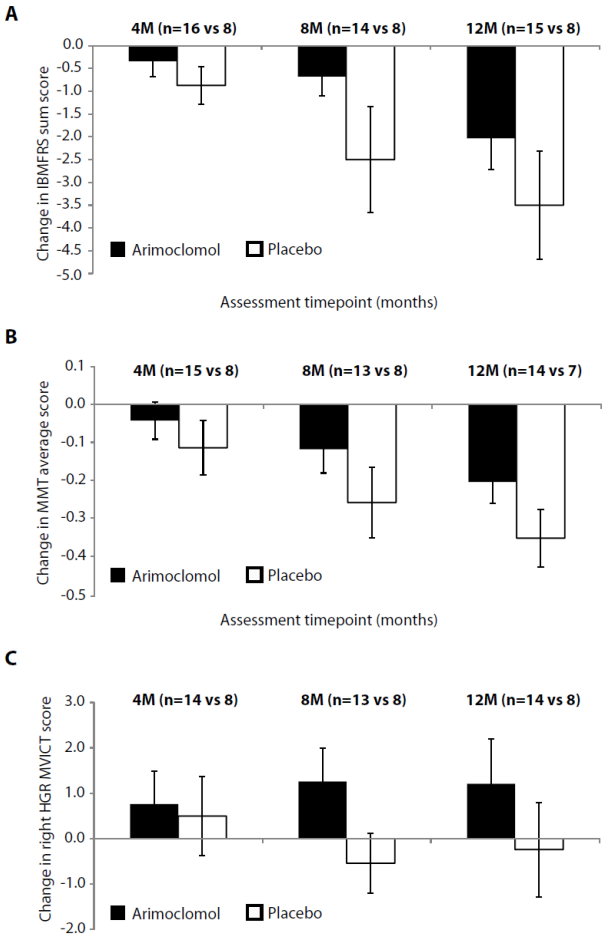

Fig. 6. Clinical trial secondary outcomes (efficacy measures)

The change from baseline to endpoint on 3 different clinical scales assessed at 4, 8 and 12 months (M) (mean ± S.E.M) in sIBM patients treated with Arimoclomol for 4 months. (**A**) IBMFRS score, (**B**) MMT average score, and (**C**) right hand grip MVICT score. The IBMFRS is a disease-specific functional questionnaire for patients with sIBM and measures physical function/disability. MMT is a measure of muscle strength scored by the physician based on the clinical assessment. MVICT is a measure of muscle strength performed using a quantitative muscle assessment system that uses an adjustable cuff to attach the patient's arm or leg to an inelastic strap that is connected to a force transducer. Error bars = S.E.M. No statistically significant clinical efficacy measures were observed.