Preventing muscle wasting by osteoporosis drug alendronate in vitro and in myopathy models via sirtuin-3 down-regulation

Hsien-Chun Chiu1†, Chen-Yuan Chiu1,2†, Rong-Sen Yang3†, Ding-Cheng Chan4, Shing-Hwa Liu1,5,6* & Chih-Kang Chiang1,7*

1Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, 2Institute of Food Safety and Health, College of Public Health, National Taiwan University, Taipei, Taiwan, 3Departments of Orthopaedics, College of Medicine, National Taiwan University, Taipei, Taiwan, 4Department of Geriatrics and Gerontology, College of Medicine, National Taiwan University, Taipei, Taiwan, 5Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan, 6Department of Pediatrics, College of Medicine, National Taiwan University, Taipei, Taiwan, 7Department of Internal Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan

Abstract

Background  A global consensus on the loss of skeletal muscle mass and function in humans refers to sarcopenia and cachexia including diabetes, obesity, renal failure, and osteoporosis. Despite a current improvement of sarcopenia or cachexia with exercise training and supportive therapies, alternative and specific managements are needed to discover for whom are unable or unwilling to embark on these treatments. Alendronate is a widely used drug for osteoporosis in the elderly and post-menopausal women. Osteopenic menopausal women with 6 months of alendronate therapy have been observed to improve not only lumbar bone mineral density but also handgrip strength. However, the effect and mechanism of alendronate on muscle strength still remain unclear. Here, we investigated the therapeutic potential and the molecular mechanism of alendronate on the loss of muscle mass and strength in vitro and in vivo.

Methods  Mouse myoblasts and primary human skeletal muscle-derived progenitor cells were used to assess the effects of low-dose alendronate (0.1–1 μM) combined with or without dexamethasone on myotube hypertrophy and myogenic differentiation. Moreover, we also evaluated the effects of low-dose alendronate (0.5 and 1 mg/kg) by oral administration on the limb muscle function and morphology of mice with denervation-induced muscle atrophy and glycerol-induced muscle injury.

Results  Alendronate inhibited dexamethasone-induced myotube atrophy and myogenic differentiation inhibition in mouse myoblasts and primary human skeletal muscle-derived progenitor cells. Alendronate significantly abrogated dexamethasone-up-regulated sirtuin-3 (SIRT3), but not SIRT1, protein expression in myotubes. Both SIRT3 inhibitor AKG7 and SIRT3-siRNA transfection could also reverse dexamethasone-up-regulated atrogin-1 and SIRT3 protein expressions. Animal studies showed that low-dose alendronate by oral administration ameliorated the muscular malfunction in mouse models of denervation-induced muscle atrophy and glycerol-induced muscle injury with a negative regulation of SIRT3 expression.

Conclusions  The putative mechanism by which muscle atrophy was improved with alendronate might be through the SIRT3 down-regulation. These findings suggest that alendronate can be a promising therapeutic strategy for management of muscle wasting-related diseases and sarcopenia.

Keywords  Alendronate; Skeletal muscle; Atrophy; Dysfunction; Sirtuin3
Introduction

There is a growing global health awareness of the risk factors of skeletal muscle in many physiological and disease processes, including skeletal muscle mass declines with ageing (sarcopenia), obesity, other chronic diseases, and muscle growth retardation. Loss of skeletal muscle is related to the frailty of skeletal muscle function by weakness, fatigue, disability, and glucose intolerance. Moreover, muscle fibre atrophy has been reported to be an obvious histological phenomenon of people with sarcopenia and muscle wasting diseases. Muscle growth retardation related to low birth weight of foetus has also been shown to be associated with myogenic inhibition by several risk factors, including polycyclic aromatic hydrocarbons and arsenic. An appropriate remedy for skeletal muscle atrophy and dysfunction needs to be discovered. To date, pharmacological treatments of sarcopenia and disease-related muscle atrophy and dysfunction are focused on elevation of the exercise and physical activity, nutritional supplement, hormone therapy, and vitamin D. However, some people with sarcopenia or disease-induced muscle atrophy and dysfunction are unable or unwilling to embark on exercise training or other supportive therapies after diagnosis. Therefore, alternative management are needed to find out for the clinical efficacy of sarcopenia and disease-related muscle atrophy and dysfunction.

Alendronate (ALN), the most popular first-line bisphosphonate drug, has been widely used for the treatment of osteoporosis by increasing bone density and micro-architecture and prohibiting bone resorption in the elderly and postmenopausal women. Due to the hazardous duet of the decline in bone mineral density (osteoporosis) and muscle mass (sarcopenia) leading to frailty, recent reports on the treatment of the osteoporosis with ALN or other bisphosphonates have also indicated that these anti-osteoporosis drugs provided a positive effect on muscle mass and strength in osteoporotic patients. However, Widrick et al. have found that ALN can improve bone loss but has no independent effect on muscle function in ovariecotomized rats, an animal model of post-menopausal osteoporosis. Therefore, the effect and mechanism of ALN on skeletal muscle function still remain to be clarified and in need of further investigation. In addition, potential therapeutic interventions for cachexia, ageing or other muscle wasting-related diseases have been extensively discovered. Trimetazidine, a metabolic modulator, elicited a significant increase in muscle strength in sarcopenic mice. A proteasome inhibitor, bortezomib, has been suggested to prevent skeletal muscle from wasting in cancer cachexia-induced muscle loss rats. Enoki et al. have suggested that indoxyl sulfate is an aetiology of chronic kidney disease-associated sarcopenia and indoxyl sulfate-targeted intervention, AST-120, could prevent from chronic kidney disease-induced muscle atrophy and lower exercise endurance. Wendowski et al. have also indicated that dihydrotestosterone could rescue an age-dependent reduction in protein synthesis of skeletal muscle fibres in sarcopenic mice. A natural product, salidroside, sourced from Rhodiola rosea L., has been found to exhibit potential therapy against cancer-associated cachexia-induced limb muscle loss in tumour-borne BALB/c mice, including gastrocnemius (Gascn) muscles. Moreover, anamorelin, a ghrelin-analog, could show an increase in muscle mass in cancer anorexia-cachexia syndrome. It is critical to discover the potential interventions against muscle wasting from sarcopenia, cachexia, and other diseases.

In the present study, the aim was to characterize the action and molecular mechanism of ALN-inhibited skeletal muscle atrophy and regeneration impairment in cultured cell models [mouse myoblasts and human skeletal muscle-derived progenitor cells (HSMPCs)] and mouse models of denervation-induced skeletal muscle atrophy and glycerol-induced muscle injury (a model for muscle regeneration in response to injury).

Materials and methods

Animals

Five to 6-week-old male ICR mice were purchased from the animal centre of the College of Medicine, National Taiwan University, Taipei, Taiwan, and housed in the controlled vivarium (22 ± 2°C and 40–60% relative humidity with a cycle of 12 h light and 12 h dark) with free access to food and water. All animal protocols of this study were conformed to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources) and approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University, Taipei, Taiwan. All animals were handled with humane treatment and alleviated the suffering while experimental challenges. The 48 mice were divided into eight groups (n = 6/group) including (a) sham control mice for both denervation and glycerol injection, (b) mice with or without ALN (0.5 and 1 mg/kg) (Merck Millipore, Temecula, CA, USA) administration for 1 month until 7 days after denervation prior to sacrifice, and (c) mice with or without ALN (0.5 and 1 mg/kg) administration for 1 month until 5 days after glycerol-induced muscle injury prior to sacrifice.

Denervation model

The mice were anaesthetized with inhalational application of a mixture gas of isoflurane (3%) (Baxter Healthcare of Puerto Rico, Guayama, PR, USA) and oxygen (97%), and denervation was achieved by surgical transection of the sciatic nerve from right hind limb as previously described by MacDonald et al.
Control mice were sham operated without removal of the sciatic nerve.

**Induction of muscle regeneration**

The mice were intramuscularly injected 100 μL glycerol (50% vol/vol) (Avantor Performance Materials, Center Valley, PA, USA) into monolateral soleus muscles under inhalational anaesthesia with a mixture of isoflurane (3%) (Baxter Healthcare of Puerto Rico, Guayama, PR, USA) and oxygen (97%) as described by Chiu et al.25

**Muscle fatigue task**

Muscular endurance was measured by the muscle fatigue task as previously described by Chiu et al.25 Briefly, the mice were accommodated to training prior to the fatigue task by using an accelerating rota-rod (Ugo Basile, Varese, Italy). The mice were given the training trial with the rota-rod at a constant speed of 13 r.p.m. for 15 min. After 15 min of rest, the mice were replaced on the rota-rod adjusted to accelerate from 13 to 25 r.p.m. in 180 s for 15 min. The next day, each mouse received the muscle fatigue task on the rota-rod operated at speeds ramping from 13 to 25 r.p.m. in 180 s and maintained at 25 r.p.m. for 30 min intertrial interval. Latency to fall off the rota-rod of each individual mouse was measured. The fatigued mouse was identified while the mouse fell off 4 times within 1 min and the task terminated.

**Sampling**

The mice were anaesthetised and euthanized after the fatigue task. Soleus and Gascn muscles were dissected, harvested, weighed, and fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) at room temperature overnight for following histological analysis.

**Assessment of muscle tension**

Isometric contraction of isolated soleus muscles was assessed as described by Liu et al.26 with modification. Briefly, harvested soleus muscles were suspended in the modified Krebs solution (130.6 mM NaCl, 4.8 mM potassium chloride (KCl), 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 12.5 mM NaHCO$_3$, and 11.1 mM glucose, pH = 7.4) constantly gassed with 5% CO$_2$ and 95% O$_2$ at 37 ± 0.5°C in a 10 mL organ bath. The soleus muscle was stimulated with a Grass S88 (Astro-Med Inc., West Warwick, RI, USA) constant voltage stimulator (rectangular pulses of 0.5 ms width at 0.2 Hz) or 30 mM KCl to evoke single twitch responses and the contracture. The muscle was loaded with a resting tension of 1 g, and the changes of tension were recorded with an isometric transducer (Mantracourt Electronics Limited, Devon, UK) and a high-resolution laboratory data recorder (e-corder) (eDAQ Pty Ltd, Denistone East, NSW, Australia). In some experiments, the KCl-induced tonic contracture was detected in isolated soleus muscles. The entry of external calcium has been suggested to play an essential role in the potassium-induced tonic contracture.27

**Histological assessment**

Fixed skeletal muscles imbedded in paraffin were sectioned into 4 μm sections. Histological examination was assessed by using haematoxylin and eosin staining. For assessment of the collagen deposition during muscle regeneration, the paraffin-embedded soleus muscle sections were stained with Masson’s trichrome as described previously.25 Immunohistochemistry was performed on fixed soleus muscle sections for Atrogin-1 and SIRT-3 staining with anti-mouse Atrogin-1 rabbit polyclonal antibody and anti-mouse SIRT-3 rabbit polyclonal antibody (Abcam, Cambridge, UK). A SuperPicture horseradish peroxidase polymer conjugate kit (Invitrogen, Carlsbad, CA, USA) was used to signal visualization according to the manufacturer’s instructions, and sections were counterstained with haematoxylin. Histology images were collected with an optical Nikon Eclipse TS100 microscope equipped with a Nikon D5100 digital camera. Cross-sectional area (CSA) and myofibres with central nuclei of individual myofibres were counted and calculated by using the ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA) in five random fields of each section. The regenerative capacity of injured skeletal muscles was determined by the ratio of the numbers of myofibres with a centrally located nucleus over the total numbers of myofibres.

**C2C12 mouse myoblasts**

C2C12 mouse myoblasts were acquired from American Type Culture Collection (Manassas, VA, USA) and grown in proliferation medium composed of Dulbecco’s modified Eagle’s medium (Gibco, Bethesda, MD, USA) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin/amphotericin B (Biological Industries, Cromwell, CT, USA) in 5% CO$_2$ at 37°C.

**Isolation and culture of primary human skeletal muscle-derived progenitor cells**

Human tissue sampling of the present study was reviewed and approved by the National Taiwan University Hospital Institutional Review Board for clinical investigation and
collected after written informed consent from all participating subjects. The process of isolation and culture of primary HSMPCs was described previously. Briefly, human skeletal muscle biopsies (~0.2 g) were collected from 10 rectus muscles of patients under orthopaedic surgery (mean age, 64 years; range, 34 to 81 years, both male and female). The minced segregate muscle was processed for HSMPC isolation within a few hours by serial enzymatic dissociation (0.5% type XI collagenase for 1 h, Sigma; 2.4 IU/mL dispase for 45 min, Invitrogen; 0.2% trypsin–EDTA for 15 min, Biological Industries). The cell suspension was passed through a 70 μm mesh filter and seeded in a collagen-coated dish in proliferative growth medium (20% foetal bovine serum (Gibco), 1% PSA (Biological Industrial), and 5 ng/mL basic fibroblast growth factor (ProSpec, East Brunswick, NJ, USA) in Ham’s F-10 (Gibco)). After serial transfers and pre-plates based on different adhesion abilities between fibroblasts and muscle-derived progenitor cells as described previously, the isolated HSMPCs with desmin-positive (as a myogenic progenitor marker) staining were used for myogenic differentiation.

**Myogenic differentiation and differentiated myotube treatment**

C2C12 myoblasts and HSMPCs were seeded on six-well plates in growth media and induced to differentiate by replacement of nutrient mixture F-12K Ham solution (Gibco)/MCDB201 (Sigma) (1:1) with 2% horse serum (Gibco). The differentiated medium was freshly exchanged every 24 h with or without dexamethasone (Dexa) (Sigma) (1 μM) or ALN (0.1–1 μM) treatment during the 4 days of differentiation. To investigate muscle anti-atrophic effects of ALN in vitro, C2C12 and HSMPC differentiated myotubes on the fourth day of differentiation were treated with Dexa (1 μM) or ALN (0.1–1 μM) for 2 days. Effects of ALN on the cellular signalling pathway of Dexa-induced myogenic inhibition and atrophic myotube formation were determined by a pharmacological inhibitor of SIRT3 (AGK7) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and small interfering RNA (siRNA) of SIRT3 (Invitrogen) transfection. AKG7 was added for 1 h followed by treatment with Dexa and ALN in C2C12 and HSMPC myogenic differentiation and myotubes. Transfection was performed a day before treatment with Dexa and ALN in C2C12 and HSMPC myogenic differentiation and myotubes by using the lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. A non-targeting siRNA was used as a negative control (Invitrogen).

**Morphological myotube analysis**

To analyse myotube formation and atrophic myotubes, five visual fields of haematoxylin and eosin staining were chosen for calculation of multinucleated myotube formation and diameters under an optical Nikon Eclipse TS100 microscope equipped with a Nikon DS100 digital camera. The percentage of myotubes that contained the indicated number of nuclei and myotube diameters were calculated by using the ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA).

**Immunoblotting**

Immunoblotting was performed as described previously. Briefly, cell protein concentration was quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Following electrophoresis, proteins were transferred on to polyvinylidene difluoride membranes blocked with 5% non-fat powdered milk and subsequently incubated overnight at 4°C with anti-Atrogin-1 (Abcam), anti-SIRT1, anti-SIRT3 (Cell Signalling Technology, Danvers, MA, USA), anti-myosin heavy chain (MHC), and anti-β actin (Santa Cruz Biotechnology). The signals were detected by using horseradish peroxidase-conjugated secondary antibodies (Cell Signalling Technology) and revealed with an enhanced chemiluminescence reagent (BioRad Laboratories, Redmond, WA, USA). The gel band quantitative densitometric analysis was quantified by the ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA).

**Statistics**

All data are presented as mean ± standard errors of the means of at least three independent experiments. The statistical significance of differences in the means of experimental groups was analysed by one-way analysis of variance and unpaired two-tailed Student’s t-test with a significance threshold of 0.05 via GraphPad Prism software.

**Results**

**Alendronate promotes hypertrophy and ameliorate dexamethasone dexa-induced atrophy in C2C12 and human skeletal muscle-derived progenitor cell-derived myotubes**

As a generally applied model of skeletal muscle atrophy in vitro, the C2C12 murine myoblasts and primary HSMPCs were induced to differentiate into myotubes. The myotube atrophy and protein degradation were induced by Dexa. As shown in Figure 1A and B, ALN (0.1–1 μM) dose-dependently promoted the enlargement in diameters of myotubes, which were over 20 and 40 μm of myotubes differentiated from C2C12 myoblast and HSMPCs, respectively. Moreover, treatment with 1 μM Dexa for 24 h caused...
morphological alteration and reduced diameter in myotubes differentiated from both C2C12 myoblasts (Figure 2A) and HSMPCs (Figure 2B), which could be significantly inhibited by ALN (1 μM) treatment. The total numbers of myotubes were not changed by Dexa or ALN treatment (total number of myotubes, control: 56.0 ± 2.2; Dexa: 51.4 ± 1.8; Dexa + ALN: 59.3 ± 2.2, *P < 0.05). Alendronate prevents Dexa-induced atrophy in C2C12-derived and human skeletal muscle-derived progenitor cell-derived myotubes via SIRT3 mediation

Muscle atrophy in vitro is ordinarily determined by both the reduction of myotube diameters and the induction of ubiquitin ligase muscle atrophy F-box or Atrogin-1 expressions, which drive skeletal muscle specific protein degradation. As shown in Figure 3A, myotubes were treated with 1 μM Dexa in the presence or absence of 0.1–1 μM ALN for 24 h. Dexamethasone significantly elevated the Atrogin-1 protein expressions in both C2C12-derived [Figure 3A(a)] and HSMPC-derived [Figure 3A(b)] myotubes, which could be significantly reversed by the treatment of ALN in a dose-dependent manner.

Sirtuins (SIRT), a family of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases with seven members (SIRT1–SIRT7), have been performed to play an important role in skeletal muscle metabolism in which both SIRT1 and SIRT3 were the most extensively studied. As shown in Figure 3B(a) and 3B(b), Dexa significantly up-regulated the SIRT3 protein expression in both C2C12-derived and HSMPC-derived myotubes, which could be abrogated by the treatment of ALN in a dose-dependent manner. Dexamethasone could also up-regulate the SIRT1 protein expression in C2C12-derived myotubes, but ALN did not affect this Dexa-up-regulated SIRT1 expression [Figure 3C(a)]. Dexamethasone did not induce the SIRT1 protein expression in HSMPC-derived myotubes [Figure 3C(b)]. In addition, treatment with 1 μM Dexa for 24 h caused morphological alteration and reduced diameter in myotubes differentiated from C2C12 myoblasts, which could be significantly inhibited by ALN treatment at higher concentrations (5 and 10 μM) (Figure 4A). Alendronate treatment at higher concentrations (5 and 10 μM) could also ameliorate Dexa-induced Atrogin-1 [Figure 4B(a)] and SIRT3 [Figure 4B(b)] protein expressions in C2C12-derived myotubes, while higher concentrations of ALN did not affect Dexa-up-regulated SIRT1 protein expression [Figure 4B(c)]. Moreover, we used the pharmacological SIRT3 inhibitor (AKG7) and small interfering RNA of SIRT3 (SIRT3 siRNA) to determine whether the down-regulation of SIRT3 could reverse the Dexa-induced myotube atrophy. As shown in Figure 5, both AKG7 [Figure 5A(a) and B(a)] and SIRT3 siRNA [Figure 5A(b) and B(b)] significantly reversed the atrophic
effect of Dexa on the C2C12-derived myotubes by down-regulating protein expressions of Atrogin-1 (Figure 5A) and SIRT3 (Figure 5B), whereas there were no observed effects of both AGK7 and SIRT3 siRNA on the protein expressions of SIRT1 in the Dexa-induced atrophic myotubes (Figure 5C). In addition, resveratrol, an SIRT3 activator, could blockade the inhibitory effect of ALN on the Dexa-induced atrophic myotubes by up-regulating protein expressions of Atrogin-1 [Figure 5D(a)] and SIRT3 [Figure 5D(b)] but not SIRT1 [Figure 5D(c)]. These results suggest that the anti-atrophic activity of ALN on Dexa-treated C2C12-derived and HSMPC-derived myotubes may be through the regulation of SIRT3 signalling.

Figure 2. Alendronate (ALN) protected myotube from atrophy induced by dexamethasone in C2C12 myoblasts and primary human skeletal muscle-derived progenitor cells (HSMPCs). C2C12 myoblasts (A) and primary HSMPCs (B) were differentiated for 4 days under differentiation medium, followed by an additional 24 h with ALN (1 μM) in the presence or absence of dexamethasone (1 μM). The representative haematoxylin and eosin stained myotubes and the frequency distribution of myotube diameter were shown. All data are presented as means ± standard errors of the means for at least three independent experiments. The solid arrows indicated the multi-nuclei myotubes. Scale bar = 100 μm. *P < 0.05 as compared with control group; #P < 0.05 as compared with dexamethasone group.
Alendronate ameliorate Dextra-diminished myogenesis of C2C12 myoblasts and primary human skeletal muscle-derived progenitor cells

In response to skeletal muscle damage, quiescent mononucleated myogenic cells, called satellite or progenitor cells, are activated and initiated to proceed myogenic differentiation as revealing the skeletal muscle regeneration and functional recovery. Besides, the signalling regulation has been shown the similar correspondence between skeletal muscle atrophy and retarded regeneratio. Therefore, we next investigated whether ALN reversed Dextra-induced myogenic

Figure 3 Alendronate (ALN) inhibited dexamethasone (Dextra)-induced Atrogin-1 and SIRT3 protein expressions in myotubes. C2C12 myoblasts and primary human skeletal muscle-derived progenitor cells (HSMPCs) were differentiated for 4 days under differentiation medium, followed by an additional 24 h with ALN (0.1–1 μM) in the presence or absence of Dextra (1 μM). Western blot analysis of protein expressions of Atrogin-1 (A), SIRT3 (B), and SIRT1 (C) in myotubes from C2C12 myoblasts (a) and primary HSMPCs (b) was shown. All data are presented as means ± standard errors of the means for at least three independent experiments. *P < 0.05 as compared with control group; #P < 0.05 as compared with Dextra group.
Figure 4  Alendronate (ALN) at a higher concentrations protected myotube from atrophy induced by dexamethasone (Dexa) in C2C12 myoblasts. C2C12 myoblasts were differentiated for 4 days under differentiation medium, followed by an additional 24 h with Dexa (1 μM) in the presence or absence of ALN (1, 5, and 10 μM). (A) The representative haematoxylin and eosin stained myotubes and the frequency distribution of myotube diameter were shown. (B) Western blot analysis of protein expressions of (a) Atrogin-1, (b) SIRT3, and (c) SIRT1 in myotubes from C2C12 myoblasts was shown. All data are presented as means ± standard errors of the means for at least three independent experiments. The solid arrows indicated the multi-nuclei myotubes. Scale bar = 100 μm. *P < 0.05 as compared with control group; #P < 0.05 as compared with Dexa group.
Both SIRT3 inhibitor AKG7 treatment and SIRT3-siRNA transfection suppressed dexamethasone (Dexa)-induced Atrogin-1 and SIRT3 protein expressions in myotubes. Fully differentiated C2C12 myotubes (day 4) were pre-treated with AKG7 (1 μM) or transfected with scramble or SIRT3 siRNA (50 nM) or resveratrol (RSV, 100 μM) and subsequently treated with Dexa (1 μM) for 24 h in the presence or absence of alendronate (1 μM). Western blot analysis of protein expressions of Atrogin-1 (A), SIRT3 (B), and SIRT1 (C) in myotubes with AKG pre-treatment (a) or SIRT3 siRNA transfection (b) was shown. (D) Western blot analysis of protein expressions of Atrogin-1 (a), SIRT3 (b), and SIRT1 (c) in myotubes with RSV pre-treatment was shown. All data are presented as means ± standard errors of the means for at least three independent experiments. *P < 0.05 as compared with control group; #P < 0.05 as compared with Dexa group; &P < 0.05 as compared with Dexa + ALN group.
Aldronate ameliorate muscle wasting/weakness and lower regenerative capacity in the mouse models of denervation and glycerol-induced skeletal muscle injury

The present study demonstrated for the first time that low-dose ALN inhibited muscle atrophy and promoted muscle regenerative capacity via SIRT3 down-regulation in vitro and in vivo. We also postulated that the reversibility of ALN in muscle atrophy might be linked to the stimulation of hypertrophic myotube formation. We used a well-established murine model of skeletal muscle atrophy-denervation-induced muscle atrophy to investigate the effect of ALN on muscle wasting. We hypothesized that this anti-osteoporosis drug could effectively not only enhance skeletal muscle mass and strength in patients with osteoporosis but also improve skeletal muscle loss and dysfunction from cachexia, sarcopenia, and other muscle wasting-related diseases. These findings suggest that ALN can be a promising therapeutic strategy for management of muscle wasting-related diseases and sarcopenia.

The effects of bisphosphonates osteoporosis drugs on muscle growth and function are controversial. Yoon et al. have
Figure 6 Alendronate (ALN) suppressed dexamethasone (Dexa)-induced myogenic differentiation inhibition and SIRT3 expression in C2C12 myoblasts and primary human skeletal muscle-derived progenitor cells (HSMPCs). C2C12 myoblasts and primary HSMPCs were cultured in growth medium or differentiation medium for 4 days with ALN (0.1–1 μM) in the presence or absence of Dexa (1 μM). (A and B) Representative haematoxylin and eosin E stained myotube formation during myogenic differentiation of C2C12 myoblasts (A) and primary HSMPCs (B). (C) Western blot analysis of the protein expressions of myosin heavy chain (MHC) and SIRT3 in C2C12 myoblasts (a) and primary HSMPCs (b) during myogenic differentiation was shown. (D) Western blot analysis of the protein expressions of MHC and SIRT3 in C2C12 myoblasts with or without scramble or SIRT3 siRNA (50 nM) transfection during myogenic differentiation was shown. All data are presented as means ± standard errors of the means for at least three independent experiments. The solid arrows indicated the multi-nuclei myotubes. Scale bar = 100 μm. *P < 0.05 as compared with control group; #P < 0.05 as compared with Dexa group.
Figure 7  Alendronate (ALN) treatment protected skeletal muscle from denervation-induced atrophy in mice. Alendronate (0.5 and 1 mg/kg) or vehicle was provided 4 weeks in mice, and mice were kept administration of ALN until 7 days after sham operation or sciatic nerve resection (DN) from right hind limb. (A) Muscle endurance was determined as latency to fall in the muscle fatigue after denervation. (B) The average muscle weights of soleus muscles (a) and gastrocnemius (Gascn) muscles (b) were measured. (C) The isometric contractions of soleus muscles evoked by a voltage stimulator and potassium chloride-induced tonic contracture were shown. (D) The representative haematoxylin and eosin stained muscle sections (i), the average cross-sectional area (CSA) of myofibres (ii), and the frequency distribution of myofibres (iii) in soleus muscles (a) and Gascn (b) were shown. The average CSA of myofibre was evaluated from random five visual fields of each group. The frequency distribution of myofibre CSA from each group was presented as percent fibres/size of fibres. Cross-sectional area was calculated by the ImageJ analysis software. Data are presented as means ± standard errors of the means, n = 6 of each group. Scale bar = 100 μm. *P < 0.05 as compared with sham control group; #P < 0.05 as compared with DN group.
indicated that pamidronate, a member of bisphosphonates, provided positive effects on both bone (an increase in bone growing and strength) and skeletal muscle function (an increase in grip strength) in Mdx mice, an animal model used as a surrogate for Duchenne muscular dystrophy. Børsheim et al. have suggested that pamidronate attenuates muscle loss as indicated by a positive net balance of muscle protein synthesis and breakdown and preserve muscle strength in post-burned paediatric patients. However, the considerable controversy also exists concerning the positive effects of bisphosphonates on skeletal muscle preservation. Uchiyama et al. have indicated that the skeletal muscle CSA around femur in 32 osteoporotic patients with 3 years of bisphosphonate (ALN, risedronate, or minodronate) was smaller than that of the bone mineral density-matched control. Widrick et al. have also shown that ALN, at a dosage shown to improve bone loss, did not affect the skeletal muscle function in ovariectomized rats. Moreover, the results from Shiomori et al. found that ALN (at a higher concentration of 100 μM) disrupted proliferation and myogenic differentiation in primary cultured human myogenic cells. These negative results still remain some concerns for dosage and sample size. In the present study, our results exhibited a beneficial effect on myotube atrophy and myogenic differentiation in C2C12 myoblasts and HSMPCs at relative lower concentrations (1 μM) of ALN. We also found that ALN monotherapy could attenuate skeletal muscle atrophy in the denervated mice and improve the skeletal muscle regenerative capacity in mice with glycerol-induced skeletal muscle injury. These results are consistent with the positive observations of ALN on muscle function in the previous studies. Furthermore, we also suggested that the possible mechanism of the fact may be through SIRT3 inhibition.

Figure 8  Alendronate (ALN) treatment down-regulated the expressions of Atrogin-1 and SIRT3 in the soleus muscles of denervated mice. Alendronate (0.5 and 1 mg/kg) or vehicle was provided 4 weeks in mice, and mice were kept administration of ALN until 7 days after sham operation or sciatic nerve resection from right hind limb. The representative immunohistochemical images for the expressions of Atrogin-1 and SIRT3 in the soleus muscles isolated from each group were shown (n = 4 of each group).
Alendronate treatment protects skeletal muscle from glycerol-impaired regenerative capacity in mice. Alendronate (ALN, 0.5 and 1 mg/kg) or vehicle was provided 4 weeks in mice, and mice were kept administration of ALN until 5 days after sham operation or glycerol (Gly; 50% vol/vol)-induced muscle injury from right hind limb. (A) Muscle endurance was determined as latency to fall in the muscle fatigue after 5 days of Gly injection. (B) The average muscle weight of soleus muscles was measured. (C) Representative haematoxylin and eosin stained sections of soleus muscles with Gly-induced muscle injury (a), quantification of the percent of soleus myofibres containing central nuclei (b), the average cross-sectional area (CSA) of myofibres (c), and the frequency distribution of myofibres of each group (d) were shown. Data are presented as means ± standard errors of the means, n = 6 of each group. Scale bar = 100 μm. *P < 0.05 as compared with sham control group; #P < 0.05 as compared with Gly group. (D) The representative immunohistochemical images for the expressions of SIRT3 in the soleus muscles isolated from each group were shown (n = 4 of each group). (E) The representative Masson’s trichrome-stained sections of soleus muscles isolated from each group were shown (n = 4 of each group).
Glucocorticoids such as Dexamethasone (Dexa) are steroid hormones widely used against inflammatory and immunosuppressive activities. However, high doses and prolonged administration may induce adverse effects including hyperglycaemia, insulin resistance, osteoporosis, and skeletal muscle weakness/wasting, as well called ‘steroid myopathy’. Its characteristics represent lower skeletal muscle endurance, poor skeletal muscle regeneration, and skeletal muscle atrophy mainly in the proximal skeletal muscles of upper or lower limbs and neck flexors. The mechanisms of glucocorticoids on myopathy have been suggested to affect the IGF/Pi3K/Akt-dependent mTOR pathway, resulting in increased atrogen-1 and MURF1 protein expressions for muscle degradation/myotube atrophy, and to modulate myogenic regulatory factors, interfering with myogenic differentiation process for suppression of muscle myogenesis/myotube formation. In the clinical presentation of steroid myopathy, muscle weakness has been found in 2.4% to 21% of patients with exogenous glucocorticoid administration. Miller has suggested that rapid onset of clinically significant muscle weakness can be observed within 2 weeks after the initiation of over 10 mg/day prednisone administration. To date, reducing steroid dose and endurance exercise training have been applied as the treatment of glucocorticoid excess state-induced muscle weakness. Moreover, many factors have contributed to ageing—such as chronic kidney disease-related loss of muscle mass and strength, including physical inactivity, a disruption of several positive regulators of muscle hypertrophy (e.g. the Akt pathway), and hormonal changes (e.g. testosterone, vitamin D, and parathyroid hormone).

However, in the present study, we found that an anti-osteoporotic drug, ALN at low doses, could effectively reverse the impaired effects of Dexamethasone on the muscle hypertrophy and the regenerative capacity via SIRT3 down-regulation in vitro and muscle degeneration therapy in vivo, suggesting that ALN may be provided as a novel potential remedy for steroid myopathy in the clinic in the future.

Sirtuin proteins are mammalian homologues of yeast silent information regulator 2 encoding NAD+-dependent class III histone deacetylases. The mammalian silent information regulator 2 family comprised seven orthologues (SIRT1–SIRT7), which all have a conserved catalytic core NAD+-binding domain but have diverse substrates, subcellular locations, and functions, which have gained growing attention for their connections with many biological processes including cellular metabolism regulation, neuroprotection, apoptosis, inflammation, and cancer progression. In skeletal muscle metabolism, both SIRT1 and SIRT3 are the most studied members of SIRT family serving as an important role in skeletal muscle energy homeostasis. Fulco et al. have reported that SIRT1 is shown as a negative regulator to skeletal muscle differentiation by associating with the complex PCAF/GCN5. Although SIRT1 may inhibit myogenic differentiation, Lee and Goldberg have suggested that SIRT1 activation can block muscle atrophy by suppressing FoxO expressions and activating PGC-1α to combat muscle wasting and restore muscle mass from muscle-wasting related diseases. On the other hand, Jing et al. reported that deletion of SIRT3 contributes to impair glucose oxidation in skeletal muscle, which is correlated with a decrease in pyruvate dehydrogenase activity and accumulation of pyruvate metabolites to inhibit fatty acid oxidation. Despite a positive modulator of SIRT3 in skeletal muscle oxidative capacity, Lin et al. have indicated that SIRT3 activation may improve muscle metabolic function under caloric restriction condition but it may unfortunately contribute to reduce muscle mass through up-regulation of FOXO1/MuRF-1 atrophic signalling pathway. In the present study, our results showed that ALN could ameliorate muscle atrophy and promote skeletal muscle regenerative capacity due to SIRT3 regulation but not SIRT1 regulation. We found that SIRT3 protein expressions were activated after muscle injury (denervation or glycerol-induced muscle injury) in vitro and in vivo and ALN could effectively ameliorate muscle atrophy and reduce regenerative capacity through suppressing SIRT3 protein expressions.

In the current study, SIRT3 regulation was performed in both skeletal muscle atrophy and myogenic differentiation. This observation postulated that muscle fibre atrophy may be associated with retardation of skeletal muscle regeneration, which results from lower satellite cell number in myofibres. Van der Velden et al. have suggested that muscle regrowth from muscle atrophy facilitates myogenic differentiation dependently through the inactivation of muscle-specific glycogen synthase kinase-3β. Our previous study has been shown that advanced glycation end products performed similar regulative mechanism between muscle atrophy and impairment of muscle regeneration through receptor for advanced glycation end product-mediated AMPK-down-regulated Akt signalling pathway in diabetic animals and cultured skeletal muscle cells. These findings support our postulation that muscle fibre atrophy correlates with retardation of skeletal muscle regeneration through the similar regulative mechanism. In the present study, inhibition of SIRT3 protein expressions by ALN could be applied to ameliorate both skeletal muscle atrophy and impairment of skeletal muscle regeneration. However, the detailed mechanism participated in the positive regulation of muscle satellite cell/stem cell homeostasis by ALN remains to be further elucidated.

Sirtuin-1 and SIRT3 have also been reported to exert several organ protection from fibrosis, including kidney, lung, liver, and heart. Yang et al. have reported that resveratrol, a well-known SIRT1 activator, could ameliorate inflammation and fibrosis in rats with unilateral ureteral obstruction-induced renal failure. Huang et al. have provided the evidence that overexpression of SIRT1 could attenuate extra-cellular matrix expression through the down-regulation of TGF-β1/Smad3 pathway in cultured mesangial cells.
regard to regulation of SIRT3 involved in fibrosis, Sundaresan et al. have reported that SIRT3-knockout mice were shown tissue fibrosis of several organs including lungs, kidney, liver, and heart, whereas there was not shown tissue fibrosis of these organs in SIRT3 overexpressing mice.64 Controversially, in the present study, we found the fibrotic feature shown in glycerol-injured soleus muscles accompanying with SIRT3 activation, which ALN could reverse the collagen infiltration with down-regulation of SIRT3 protein expression. This different regulatory effect of SIRT3 on fibrosis from the study of Sundaresan et al. may be due to different target organs and different injured models. However, further investigation is required to evaluate the detail molecular mechanism between ALN and collagen production under SIRT3 regulation in injured skeletal muscles.

In conclusion, the present study demonstrates for the first time that osteoporosis drug ALN can significantly prevent muscle wasting in vitro and in denervation-induced and glycerol-induced myopathy animal models through a SIRT3 down-regulation. However, there are some limitations for our animal models. As a result, the denervation-induced and glycerol-induced myopathy animal models used in this study seem not to mimic the muscle wasting-related diseases in human. Further investigation for the action and mechanism of ALN on osteoporosis-induced or ageing-induced skeletal muscle wasting animal models needs to be considered.

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Author contributions

All authors approved the final version to be published. Study conception and design: S.-H.L., R.-S.Y., and C.-K.C. Acquisition of data: C.-Y.C. and H.-C.C. Analysis and interpretation of data: C.-Y.C. and H.-C.C. Provide reagents and technical support: D.-C.C. and R.-S.Y. Wrote the paper: C.-Y.C., C.-K.C., and S.-H.L.

Conflict of interest

Hisen-Chun Chiu, Chen-Yuan Chiu, Rong-Sen Yang, Ding-Cheng Chan, Shing-Hwa Liu, and Chih-Kang Chiang stated that they have no conflict of interest.

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