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Research Paper

Synergistic action of statins and nitrogen-containing bisphosphonates in the development of rhabdomyolysis in L6 rat skeletal myoblasts

Sumio Matzno[a,b], Tomoko Nishiguchi[a], Takeshi Akiyoshi[c], Setsuko Anami[d], Toshikatsu Nakabayashi[a] and Kenji Matsuyama[c]

[a]School of Pharmaceutical Sciences and [b]Institute for Biosciences, Mukogawa Women's University, Hyogo; [c]Department of Clinical Pharmacy, Keio University Faculty of Pharmacy, Tokyo and [d]Pharmacy Department, Sakai Municipal Hospital, Osaka, Japan

Abstract

Objectives Nitrogen-containing bisphosphonates, which are widely used to treat osteoporosis, act as inhibitors of farnesyl pyrophosphate synthase, one of the key enzymes of the mevalonate pathway, and thus may have the potential to enhance the effect of statins (inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase). In this study, we evaluated the synergistic effect of two nitrogen-containing bisphosphonates, alendronate and risedronate, in statin-induced apoptosis in rat skeletal L6 myoblasts.

Methods L6 rat myoblasts were differentiated with drugs. DNA fragmentation was measured and small GTPase was detected by immunoblotting.

Key findings Alendronate and risedronate caused dose-dependent apoptosis of L6 myoblasts. Risedronate induced detachment of rho GTPase from the cell membrane, followed by activation of the caspase-8-related cascade. Risedronate-induced apoptosis was synergistically enhanced with atorvastatin and significantly reduced by addition of geranylgeraniol. By contrast, alendronate did not reduce membrane GTPases and the apoptosis was caspase independent.

Conclusions These results suggest that risedronate-induced apoptosis is related to geranylgeranyl pyrophosphate depletion followed by rho detachment, whereas alendronate affects are independent of rho. Our results suggest a risk of synergistic action between nitrogen-containing bisphosphonates and statins in the development of rhabdomyolysis when treating osteoporosis in women with hyperlipidaemia.

Keywords apoptosis; N-containing bisphosphonates; protein prenylation; statins

Introduction

Bisphosphonates are widely used for the management of bone metabolism disorders such as Paget's disease and osteoporosis.[1,2] Oral bisphosphonates are the most widely used drugs in the treatment of osteoporosis; they reduce the risk of fractures in women with postmenopausal osteoporosis. Bisphosphonates have a common P-C-P chemical structure; the presence of two phosphonate groups allows the molecule to act as a 'bone hook', which is essential for targeting the bone and for the molecular mechanism of action of the compounds.[2,3]

The main action of bisphosphonates in the treatment of metabolic bone diseases is to inhibit bone resorption by inducing osteoclast apoptosis. The first-generation bisphosphonates (such as clodronate and etidronate) are internalised by osteoclasts, followed by conversion into methylene-containing (AppCp)-ATP analogues.[4,5] These AppCp-ATP analogues accumulate in high concentrations in the osteoclast cytosol and consequently induce osteoclast apoptosis. By contrast, the nitrogen-containing bisphosphonates, which contain a nitrogen side chain at the R_2 position and are more potent inhibitors of bone resorption, are not metabolised to AppCp. They act as inhibitors of farnesyl pyrophosphate (FPP) synthase, one of the key enzymes of the mevalonate pathway.[6,7] Both FPP and geranylgeranyl pyrophosphate (GGPP) are covalently added to members of the small GTP-binding protein superfamily (small GTPases: ras, rho and rab families).[8] Protein prenylation is crucial for the targeting and activity of GTPases involved in cell activity and

Correspondence: Sumio Matzno PhD, School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68, Kyuban-cho, Koshien, Nishinomiya, Hyogo 663-8179, Japan.
E-mail: smatzno@mukogawa-u.ac.jp

survival. Nitrogen-containing bisphosphonates inhibit the prenylation of GTPases, resulting in osteoclast dysfunction and apoptotic cell death.

Statins, which are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme located upstream of the mevalonate pathway, are widely used to treat hypercholesterolaemia.[9] Rhabdomyolysis is a severe potential adverse effect of statin administration.[10] Our earlier study revealed that statin-induced rhabdomyolysis results from apoptosis in muscular tissue, initiated by depletion of farnesyl-anchored ras protein from the cell membrane.[11] As nitrogen-containing bisphosphonates also act on the mevalonate pathway, co-administration of these agents with statins might exacerbate the risk of rhabdomyolysis. As postmenopausal women commonly develop both osteoporosis and hypercholesterolaemia, combined treatment of statins and bisphosphonates is likely.[12]

The purpose of this study was to clarify the synergism between statins and nitrogen-containing bisphosphonates on the onset of apoptosis using L6 rat skeletal myoblasts in the in-vitro myopathy evaluation model that we established in previous studies.[13,14] Our findings revealed that statins synergistically enhance apoptosis induced by nitrogen-containing bisphosphonates in skeletal myoblasts, thus indicating the need for care in the use of these agents with statins.

Materials and Methods

Drugs and solutions

Transferrin was purchased from Gibco BRL (Carlsbad, CA, USA). Hoechst 33342, bovine serum albumin and insulin were from Sigma-Aldrich, Inc. (St Louis, MO, USA). Proteinase K was from Wako Pure Chemicals (Osaka, Japan). Caspase-specific substrates (acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) for caspase-3; Ac-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC) for caspase-8; Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC) for caspase-9), and the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OCH_3)-fluoromethylketone (z-VAD-FMK) were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Anti-rho A and anti-ras primary antibodies were from Lab Vision Corp. (Fremont, CA, USA). Peroxidase-conjugated anti-mouse IgG was from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). All other materials were from Nacalai Tesque Ltd (Kyoto, Japan).

Cell culture

The growth medium used for routine maintenance of L6 rat skeletal myoblasts was the alpha-modification of Eagle's medium (α-MEM) containing 50 IU/ml penicillin G, 50 μg/ml streptomycin and 10% fetal bovine serum. Fewer than 20 passages were used in the experiments. Generally, myoblasts (3×10^4 cells/cm^2) were seeded into tissue culture plates for subsequent experiments. After 24 h' cultivation, the standard growth medium was changed to the differentiation medium (α-MEM supplemented with 1 mg/ml bovine serum albumin, 10 μg/ml insulin, 5 μg/ml transferrin and 10 nM sodium selenite) to induce myotube formation, and treated with the drugs as described below.

Evaluation of cell viability by intracellular esterase activity

Cell counting kit-F (Dojindo Laboratories, Kumamoto, Japan) was used to assess cell viability.[15] Briefly, cells were seeded onto culture plates and incubated for 24 h with growth medium. The medium was then changed to differentiation medium and the cells were treated with 100 μM bisphosphonates for 12, 24, 36, 48 and 72 h. (The bisphosphonates were dissolved in DMSO. The DMSO concentration did not exceed 0.1% in the medium, which did not affect cell viability.) At the end of the treatment period, cells were washed with phosphate-buffered saline (PBS) and allowed to react with calcein-AM for 30 min. The released fluorescent calcein was measured in a CytoFluor plate reader (PerSeptive Biosystems, Foster City, CA, USA) at an excitation wavelength of 490 nm/emission 515 nm.

Evaluation of apoptosis and nuclear DNA fragmentation

Cells were seeded onto cover slips and treated with the drugs as described above. After 20 h' differentiation, they were fixed with 1% glutaraldehyde for 5 min, washed twice with PBS and stained with 0.5 mM Hoechst 33342 solution (dissolved in PBS) for 5 min. After staining, the solution was removed, and the cells were washed three times with PBS and then observed using a bright-field fluorescent microscope under UV excitation. Apoptotic cells appeared blue, peripherally clumped or had fragmented chromatin.

For the DNA fragmentation assay, cells were plated onto 100 mm tissue culture dishes and treated with drugs for 20 h. They were then harvested with 100 μl lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100, pH 7.4) for 10 min at 4°C. After centrifugation (10 000g at 4°C for 10 min), the supernatant was treated with 20 μg RNase A for 60 min at 37°C and subsequently with 20 μg proteinase K for 30 min at 50°C. The fragmentated DNA was precipitated with 0.4 M NaCl/50% isopropanol for 17 h at –20°C, and then electrophoresed on 2% agarose gel.

Determination of caspase activities

Caspase activities were assessed by cleavage of their specific substrates, as described previously.[16] Briefly, 24 h after drug treatment, the cells were washed twice with PBS and scraped. They were then harvested with 0.1% Triton X-100 and centrifuged at 4000g at 4°C for 10 min. Three aliquots of supernatant were then incubated at 37°C with an equal volume of reaction buffer (100 mM NaCl, 50 mM HEPES, 1 mM EDTA and 10% glycerol; pH 7.4 adjusted with NaOH) and 50 μM Ac-DEVD-AFC for caspase-3, Ac-IETD-AMC for caspase-8 and Ac-LEHD-AFC for caspase-9. After a 2 h reaction, the production of the fluorescent dyes was detected using an excitation wavelength of 360 nm/emission 530 nm for AFC and excitation 360 nm/emission 460 nm for AMC.

Western blotting

Membrane-bound small GTPase was detected using immunoblot analysis, as described previously.[11] The cells were treated with bisphosphonates for 2, 4 and 6 h and then harvested, sonicated for a few seconds and centrifuged at 50 000*g* at 4°C for 30 min. Membrane (precipitate) and cytosolic (supernatant) fractions were then collected and their protein contents determined using a bicinchoninate protein assay (Pierce, Rockford, IL, USA).

Electrophoresis was performed onto 12.5% T gel and the separated proteins were electrotransferred to the membrane at 150 mA for 1 h. After washing twice with TBST (0.1% Tween 20, 150 mM NaCl and 10 mM Tris-HCl, pH 7.4), the blots were blocked in 5% skimmed milk for 1 h. Next, each primary antibody against ras or rho A (1 : 500 dilution) was applied for 17 h at 4°C. After washing, blots were treated with peroxidase-conjugated anti-mouse IgG (1 : 5000 dilution) for 1 h, followed by enhanced chemiluminescence detection of antigens.

Data analysis

Tukey's or Dunnett's multiple comparison tests was used to evaluate the effects of bisphosphonates *per se*. To assess synergistic action between bisphosphonates and statins, the data were analysed using two-way analysis of variance (ANOVA). Results are expressed as mean ± SD. A *P* value of less than 0.05 was considered statistically significant.

Results

Effects of bisphosphonates on DNA fragmentation in L6 myoblasts

The cytotoxic effects of bisphosphonates in L6 myoblasts are illustrated in Figure 1. Typical photographs of apoptotic cells are shown in Figure 1a. L6 myoblasts showed abnormal nuclear condensation after treatment with 100 μM alendronate or risedronate for 20 h, whereas 100 μM etidronate did not cause nuclear cleavage. The cytotoxic effect of bisphosphonates on L6 myoblasts was quantified by the loss of cytosolic esterase activity (Figure 1b). Alendronate and risedronate at 100 μM significantly diminished the esterase activity in a time-dependent manner, whereas 100 μM etidronate did not. In accordance with loss of cytosolic esterase activity, DNA fragmentation was observed in myoblasts treated with alendronate or risedronate using agarose gel electrophoresis. As shown in Figure 1c, alendronate and risedronate dose-dependently induced 180 bp interval chromosomal DNA fragmentation in L6 myoblasts, indicating that these nitrogen-containing bisphosphonates caused apoptotic cell death, which would result in muscular dysfunction.

Caspase activation in myoblasts

We also tried to clarify the mechanism of action of muscular apoptosis induced by nitrogen-containing bisphosphonates by examining the role of various caspases during the apoptosis using specific substrates. The activities of caspase-3, -8 and -9 increased significantly in myoblasts treated with 100 μM risedronate (Figure 2a). Caspase-8 activity was initiated at 6 h, followed by activation of caspase-3 and -9 at 8 h. This finding indicates that risedronate induces apoptosis through the caspase-8-related pathway, followed by activation of the effector caspase (caspase-3), whereas caspase-9 might be secondarily activated. In contrast, alendronate did not induce either apical (caspases-8 and -9) or executioner (caspase-3) activation; instead, these enzyme activities time-dependently declined in alendronate-treated L6 myoblasts (Figure 2b). This result suggests that alendronate induces apoptosis through caspase-independent pathway(s), in contrast to risedronate. To complement this assumption, the fluoromethylketone variant was used as a caspase inhibitor.[17] When L6 myoblasts were treated with each nitrogen-containing bisphosphonate for 24 h, cytosolic esterase activity was significantly reduced. The broad-spectrum caspase inhibitor z-VAD-FMK (1 μM) prevented only risedronate-induced apoptotic cell death (Figure 3). It did not inhibit alendronate-related apoptosis, indicating that risedronate-induced muscular apoptosis is related to the caspase-dependent cascade, whereas alendronate causes apoptosis through a caspase-independent mechanism.

Synergism in the development of cell damage with statins

The findings described above revealed differences between risedronate and alendronate in the apoptotic mechanism. Our earlier studies indicated that statin-induced apoptosis in muscular tissue was directly initiated by depletion of farnesyl-anchored Ras protein from the cell membrane and the consequent activation of caspase-8.[11,14] In the current study we show that risedronate also causes apoptosis of L6 myoblasts through a caspase-8-related cascade, revealing that this agent can also inhibit the prenylation of GTPase and thereby cause myoblast death.

We next examined the relationship between prenylation and apoptosis (Figure 4). In L6 myoblasts treated with risedronate (300 μM), detachment of the rho protein from the cell membrane occurred at 6 and 9 h, whereas ras GTPase detachment did not occur. No detachment of either rho or ras was observed in alendronate-treated myoblasts (Figure 4a).

We also examined the effect of downstream mevalonate metabolites. Risedronate-induced apoptosis was significantly recovered by addition of 20 μM geranylgeraniol (GGOH) but was not affected by farnesol (FOH) (Figure 4b). Neither isoprenoid affected alendronate-treated myoblasts.

Finally, we evaluated the synergistic action between bisphosphonates and statins on myoblast apoptosis. As shown in Figure 5, risedronate-induced L6 cell death was synergistically potentiated by the addition of statins. The multiplier action was strongly evident in the presence of 1 μM atorvastatin with risedronate. By contrast, only an additive effect was observed in myoblasts treated with alendronate and statins. Etidronate-treated cells did not undergo myopathy even with statin addition. These results suggest that risedronate inhibits the geranylgeranylation of rho GTPase in skeletal muscle cells and the consequent signal loss, resulting in apoptosis. Statins also inhibit the upstream mevalonate pathway, thus suggesting that addition of a statin exacerbates only risedronate-induced apoptosis.

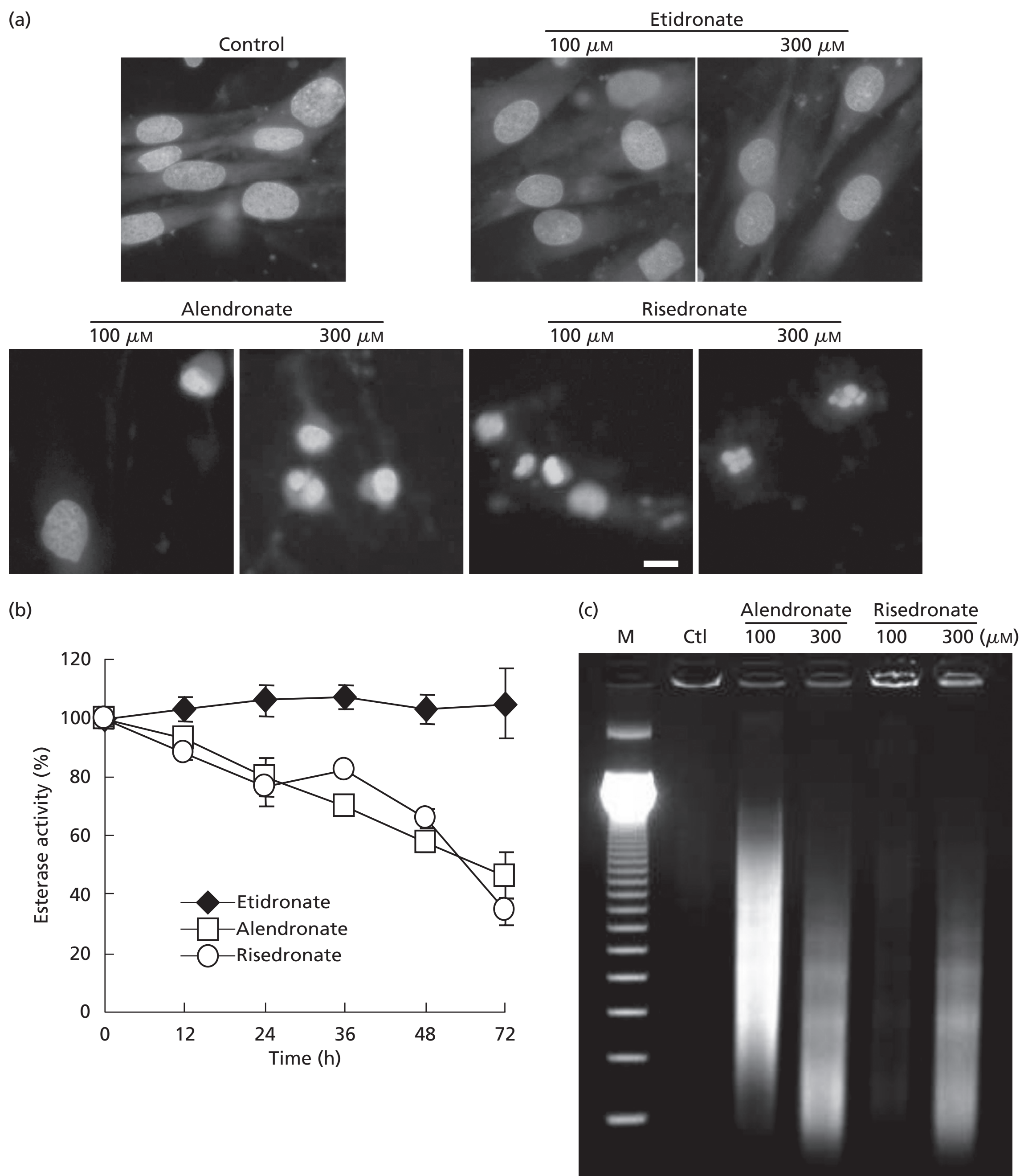

Figure 1 Induction of apoptosis in L6 rat skeletal myoblasts by bisphosphonates. (a) Typical fluorescence micrographs showing nuclear condensation and cleavage by the nitrogen-containing bisphosphonates. Cell were treated with 100 or 300 μM drug for 24 h, fixed with glutarardehyde and stained with Hoechst 33342. Bar: 10 μm. (b) Cytosolic esterase activities in L6 myoblasts treated with alendronate, risedronate and etridonate. Relative intensity of calcein fluorescence was expressed as a viability index (n = 5). (c) DNA fragmentation in myoblasts treated with nitrogen-containing bisphosphonates. M, 100-bp DNA ladder marker; Ctl, negative control without drug.

Discussion

Bisphosphonates have a common P-C-P chemical structure, which is essential for biological activity.[3] This P-C-P component does not exist in natural products and is metabolically stable. Hence we initially examined the characteristics of these 'artificial' stable compounds from the viewpoint of biological safety. This study revealed the mechanism of action of rhabdomyolysis induced by nitrogen-containing bisphosphonates, which induced cell apoptosis with nuclear condensation and DNA fragmentation in L6 myoblasts (Figures 1 and 2). In contrast with non-nitrogen-containing bisphosphonates, these agents inhibit FPP synthase, a key enzyme in the mevalonate pathway, and consequently reduce the prenylation of small GTPases that are essential for cell function and survival. Our results show

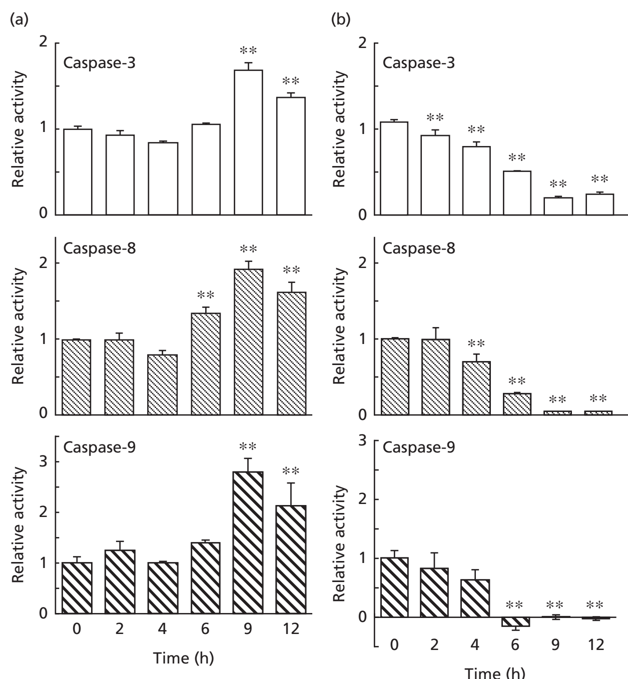

Figure 2 Caspase activation during incubation of L6 myoblasts with nitrogen-containing bisphosphonates. Cells were treated with 100 μM risedronate (a) or alendronate (b) and were allowed to react with caspase-specific substrates: Ac-DEVD-AFC (top), Ac-IETD-AMC (middle) and Ac-LEHD-AFC (bottom) for 2 h (n = 4). **P < 0.01 vs 0 h.

that risedronate-induced apoptosis was prevented by GGOH, which is metabolised to GGPP, followed by geranylgeranylation of the rho protein (Figure 4b). This apoptosis could not be prevented by FOH (Figure 4b). Furthermore, immunoblot analysis (Figure 4a) clearly revealed that risedronate selectively inhibits the anchoring of rho GTPase to the cell membrane. These results strongly suggest that geranylgeranylation of rho GTPase is essential to the survival of skeletal muscle and that risedronate causes direct inhibition at this point and a consequent onset of apoptosis.

Unlike with skeletal muscle cells, Benford and colleagues reported that the cytotoxic action of nitrogen-containing bisphosphonates in the mouse macrophage-like J774.1 cell line could be prevented by addition of both FOH and GGOH.[18] A recent structural study revealed that nitrogen-containing bisphosphonates also have an inhibitory effect on GGPP synthase as well as FPP synthase in humans.[19] These findings suggest that although nitrogen-containing bisphosphonates can bind to both GGPP synthase and FPP synthase, there are possibilities for selective inhibition in skeletal muscle tissues. Further investigation is needed to clarify the mechanism of tissue selectivity.

We also treated L6 myoblasts with both nitrogen-containing bisphosphonates in combination with statins (Figure 5). Risedronate-induced apoptosis was synergistically enhanced by 1 μM atorvastatin, whereas other statins caused only slight enhancement. The difference might be attributed to the selectivity of statins in inhibiting GTPase prenylation. Several investigators have demonstrated selective rho prenylation by atorvastatin. Sabbatini and colleagues reported that atorvastatin depresses the progress of acute renal failure by reducing membrane-bound rho A, whereas the ras system was not affected.[20] An in-vitro study showed that atorvastatin decreased prenylation of rho B and consequently caused apoptosis in vascular smooth muscle cells.[21] In contrast, our recent study indicated that cerivastatin caused apoptosis by inhibiting farnesylation of p21-ras, not rho GTPases, resulting in the lack of the membrane-associated active ras proteins.[11] The reasons for this discrepancy of prenylation action by statins are still unclear, but these studies show the benefits of the concomitant use of bisphosphonates with statins.

What remains to be clarified is the mechanism of alendronate-induced muscular cell death. Alendronate also caused apoptosis in L6 myoblasts with DNA fragmentation, but did not activate any caspases, and apoptosis was not blocked by z-VAD-FMK. It has been reported that alendronate also inhibits protein prenylation[22] and apoptosis in osteoclasts is blocked by GGOH.[23] On the other hand, our findings showed that alendronate-induced muscle cell apoptosis was not reduced by addition of GGOH or FOH (Figure 4). This suggests that the mechanism of alendronate-induced apoptosis differs in osteoclasts and myoblasts.

Regarding the interaction of nitrogen-containing bisphosphonates and statins, the P-C-P component is responsible for the strong affinity of the bisphosphonates for the bone mineral; the hydroxyl group at the R_1 position markedly increases this binding affinity.[3,24] The gastrointestinal absorption of bisphosphonates is poor.[25] Thus, the accumulation and low absorption of nitrogen-containing bisphosphonates with oral administration may decrease the likelihood of synergistic action and the risk of rhabdomyolysis during therapy of osteoporosis. On the other hand, recent reports indicate that the risk of serious atrial fibrillation events is increased in patients treated with alendronate[26] and zoledronic acid.[27] These clinical studies suggest that long-term treatment with nitrogen-containing bisphosphonates results in drug accumulation in plasma. In addition, epidemiological studies reveal that a certain number of alendronate users are also exposed to statins, estrogen and antihypertensive agents, and the risk of atrial fibrillation is higher in patients also taking statins.[28] Taking this into consideration, we propose that long-term use of nitrogen-containing bisphosphonate in combination with statins may increase serum levels of the bisphosphonate and consequently increase the risk of bisphosphonate-related muscular dysfunction in cardiac muscle and, most likely, in skeletal muscle.

It is particularly interesting that alendronate induces apoptosis through a caspase-independent pathway in L6 myoblasts (Figures 2 and 3). Although further studies are needed to elucidate the mechanism of action, our current findings suggest that, with appropriate care, bisphosphonates can be prescribed in combination with statins for the treatment of osteoporosis in postmenopausal women with hyperlipidaemia.[12]

Because protein prenylation is required for cell survival, nitrogen-containing bisphosphonates also affect the viability of tumour cells, and intravenous infusion of nitrogen-containing bisphosphonates has been used for patients with bone metastases from breast and prostate cancer.[29,30] In chemotherapeutic use, it is important to be aware of the

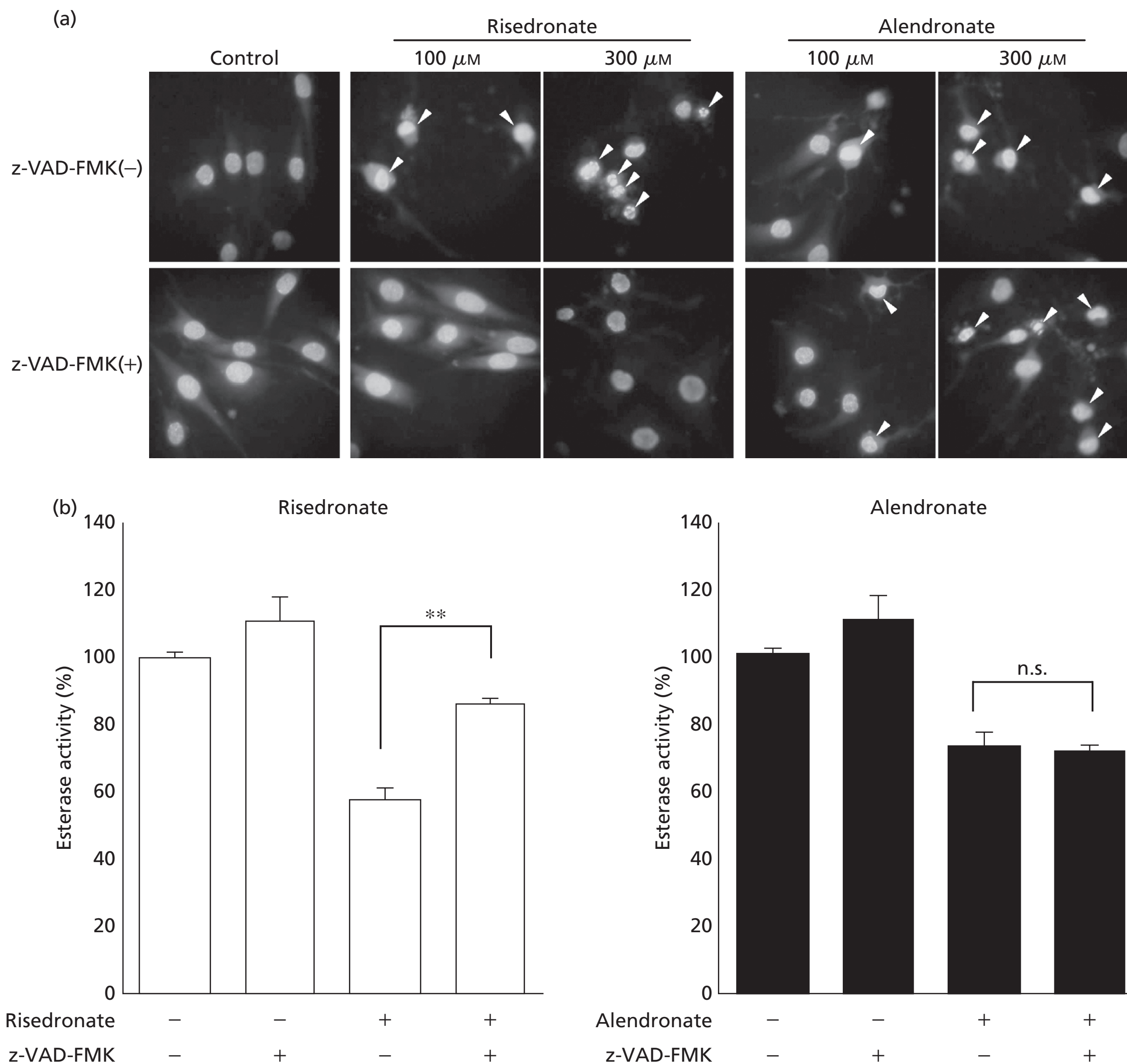

Figure 3 The caspase inhibitor z-VAD-FMK prevents apoptosis induced by nitrogen-containing bisphosphonates in L6 myoblasts. (a) Fluorescent micrographs. Cells were treated with 100 or 300 μM bisphosphonate for 24 h in the presence or absence of 1 μM z-VAD-FMK. Nuclear DNA was stained with 0.5 mM Hoechst 33342. (b) Cytosolic esterase activity in cells incubated with 100 μM bisphosphonates. Values are means ± SD (n = 5). $^{**}P < 0.01$; n.s., not significant.

possibility of synergistic action between nitrogen-containing bisphosphonates and statins in the development of rhabdomyolysis. Our findings indicate that the combination of risedronate and atorvastatin is likely to synergistically elevate the risk of rhabdomyolysis.

Conclusions

Although the P-C-P structure of bisphosphonates leads to their accumulation in bone tissue, awareness is needed of the possible onset of rhabdomyolysis in patients given these agents in combination with statins. In particular, the risk of cardiac and skeletal muscle failure may be increased in patients taking a nitrogen-containing bisphosphonate and a statin together for an extended period.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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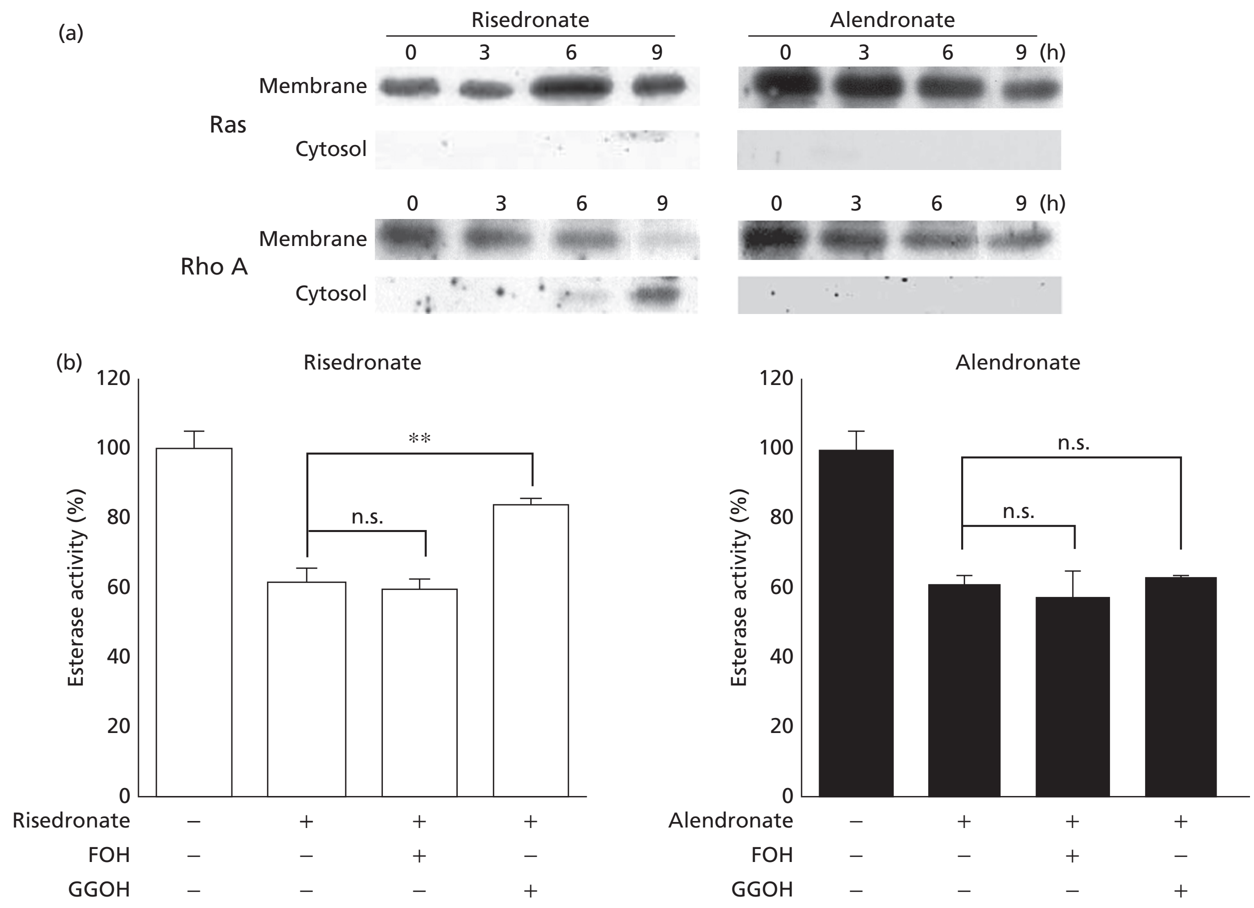

Figure 4 Effect of nitrogen-containing bisphosphonates on prenylation of small GTPases and related cell survival in L6 myoblasts. (a) Immunoblot analysis of rho A and ras small GTPases. (b) Effects of geranylgeraniol (GGOH) and farnesol (FOH) on apoptosis induced by the bisphosphonates ($n = 5$). $^{**}P < 0.01$; n.s., not significant.

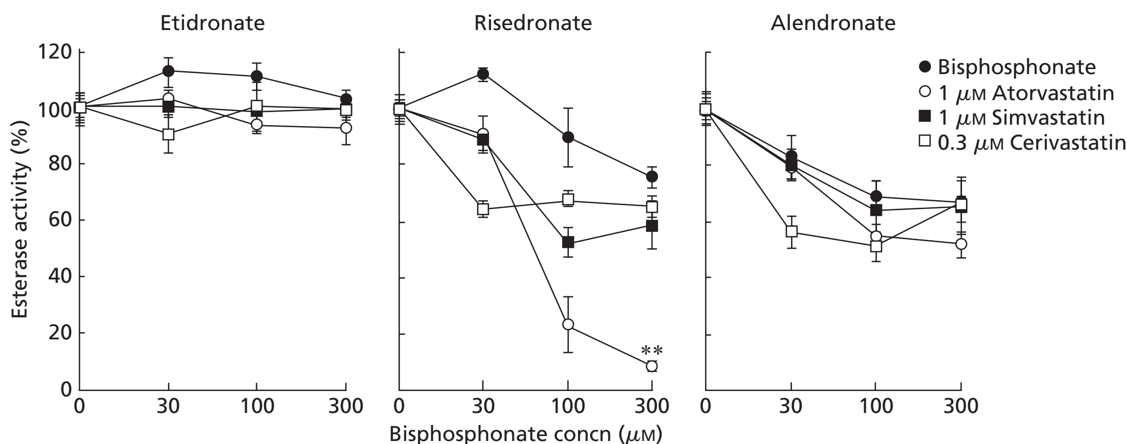

Figure 5 Evaluation of the synergistic action on muscular apoptosis of nitrogen-containing bisphosphonates and statins. Cells were treated with each bisphosphonate alone or in the presence of 1 μM atorvastatin, 1 μM simvastatin or 0.3 μM cerivastatin ($n = 5$). Synergism was evaluated using two-way analysis of variance; $^{**}P < 0.01$.

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