

Original Article

Bisphosphonates and statins inhibit expression and secretion of MIP-1 α via suppression of Ras/MEK/ERK/AML-1A and Ras/PI3K/Akt/AML-1A pathways

Masanobu Tsubaki¹, Tomoya Takeda¹, Kotaro Sakamoto¹, Hiroataka Shimaoka¹, Arisa Fujita¹, Tatsuki Itoh², Motohiro Imano³, Kenji Mashimo^{1,4}, Daiichiro Fujiwara^{1,4}, Katsuhiko Sakaguchi⁴, Takao Satou⁵, Shozo Nishida¹

¹Division of Pharmacotherapy, Kinki University School of Pharmacy, Kowakae, Higashi-Osaka, Japan; ²Department of Food Science and Nutrition, Kinki University School of Agriculture, Nara, Nara, Japan; ³Department of Surgery, Kinki University School of Medicine, Osakasayama, Osaka, Japan; ⁴Department of Pharmacy, Japanese Red Cross Society Wakayama Medical Center, Wakayama, Japan; ⁵Department of Pathology, Kinki University School of Medicine, Osakasayama, Osaka, Japan

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Abstract: Osteolytic bone disease in multiple myeloma (MM) is associated with upregulated osteoclast activity. Macrophage inflammatory protein-1 α (MIP-1 α) is crucially involved in the development of osteolytic bone lesions in MM. We previously reported that minodronate inhibited lipopolysaccharide-induced MIP-1 α secretion in mouse myeloma cells. However, it remains unknown whether bisphosphonates and statins inhibit MIP-1 α secretion by human MM cells. In present study, we investigated whether bisphosphonates and statins had any inhibitory effect on MIP-1 α secretion by human myeloma cells and the mechanism underlying this effect. In this study, we found that bisphosphonates and statins inhibited MIP-1 α mRNA and MIP-1 α secretion and suppressed extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt phosphorylation by inhibiting Ras prenylation. Moreover, bisphosphonates and statins suppressed the expression of acute myeloid leukemia-1A (AML-1A) mRNA, a MIP-1 α transcription factor. These results indicate that bisphosphonates and statins suppress the Ras/mitogen-activated protein kinase kinase/ERK/AML-1A and Ras/phosphatidylinositol-3 kinase/Akt/AML-1A pathways, thereby inhibiting MIP-1 α secretion by MM cells. Therefore, use of MIP-1 α expression inhibitors such as bisphosphonates and statins may provide a new therapeutic approach to inhibiting tumour progression and bone destruction in MM patients.

Keywords: Bisphosphonate, statin, MIP-1 α , AML-1A, myeloma, Ras, ERK, Akt

Introduction

Multiple myeloma (MM) is an incurable plasma cell neoplasm that accounts for 13% of all the haematological malignancies [1, 2]. MM patients are at an increased risk of infection, anaemia, thrombocytopenia, renal failure, and bone disease. Osteolytic bone disease occurs in 70-80% of MM patients and results in severe bone pain, pathological fractures, paralysis through nerve compression, hypercalcemia, and death [2]. Bone destruction results from highly localised osteoclastic bone resorption induced by the osteoclastic stimulatory factors secreted by MM cells and bone marrow stromal cells that are adjacent to the MM cells [3].

Studies have implicated several cytokines as potential factors responsible for MM-related bone destruction, including interleukin-1 β (IL-1 β), IL-6, and tumour necrosis factor- α . However, none of these cytokines has been found to be consistently elevated in the peripheral blood or bone marrow plasma of MM patients [4, 5].

Macrophage inflammatory protein-1 α (MIP-1 α) is expressed and secreted by the myeloma cells freshly isolated from patients and by human myeloma cell lines [6-9]. MIP-1 α is elevated in the bone marrow plasma of patients with MM than in the bone marrow plasma of patients with other lymphoid hematologic malignancies and healthy donors [6, 8]. It has been indicated

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that MIP-1 α stimulates chemotaxis of human osteoclast precursors, promotes the expression of the receptor activator of NF- κ B ligand by osteoblastic and interstitial cells, and acts directly on the osteoclasts to promote osteoclastogenesis and osteoclastic bone resorption [10-14]. Moreover, MIP-1 α accelerates cell growth in MM cell lines [15, 16]. It is further possible that osteoclastic activity will be inhibited by the use of a neutralising antibody to control MIP-1 α . It has been confirmed that bone resorption is inhibited in severe combined immunodeficient mice through the inhibition of MIP-1 α by using an antisense strand [17]. Accordingly, it is possible that MIP-1 α inhibition has the potential to serve as a therapeutic method for inhibiting tumour cell growth or preventing bone destruction.

Bisphosphonates are structural analogues of pyrophosphoric acid that are used as therapeutic agents for osteoporosis and hypercalcemia through the apoptotic induction of osteoclasts [18]. Nitrogen-containing bisphosphonates have an inhibitory effect on farnesyl pyrophosphate (FPP) synthase and geranylgeranyl pyrophosphate (GGPP) synthase in the mevalonate pathway; such inhibition of the intermediates in this pathway results in disruption of protein prenylation that is vital to the function of essential signaling proteins such as small GTPases (Ras and Rho) [19-21]. Statins, which are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (the rate-limiting enzyme in cholesterol synthesis), suppress cell proliferation, and induce apoptosis in various cell types such as osteoclasts and various tumour cells, by inhibiting GGPP biosynthesis and Ras prenylation [21-24].

If the MIP-1 α released from the myeloma cells can be inhibited by bisphosphonates and statins that act not only on osteoclasts but also on tumour cells, it would be possible to inhibit the growth of myeloma cells or prevent osteolysis. We previously reported that minodronate inhibited lipopolysaccharide-induced MIP-1 α secretion in mouse myeloma [16]. However, it remains unknown whether bisphosphonates and statins can inhibit MIP-1 α secretion by human MM cells. Moreover, the mechanism underlying this suppression is yet to be elucidated. Therefore, the use of bisphosphonates and statins may possibly prolong lives, alleviate

osteolytic pain, or contribute to improved quality of life for patients. Thus, in this study, we investigated whether bisphosphonates and statins can inhibit MIP-1 α secretion by the human myeloma cells and the mechanism possibly underlying this inhibition.

Materials and methods

Materials

Minodronate was supplied from Astellas Pharmaceutical Co., Ltd. (Tokyo, Japan). Alendronate was purchased from Sigma (St. Louis, MO, USA). Fluvastatin and simvastatin were purchased from Calbiochem (San Diego, CA, USA), and dimethyl sulfoxide (DMSO). This reagent was dissolved in phosphate buffer saline (PBS; 0.05 M, pH 7.4, Wako, Osaka, Japan), filtrated through syringe filters (0.45 μ m, IWAKI GLASS, Tokyo, Japan) and used for various assays described below.

Mevalonic acid lactone (MVA), FPP, GGPP, squalene, isopentenyladenine, ubiquinone, and dolichol were purchased from Sigma. MVA, FPP and GGPP were dissolved in dry ethanol. U0126 and LY294002 were purchased from Promega (Madison, WI, USA) and dissolved in DMSO. Rapamycin and Y27632 were purchased from Wako (Tokyo, Japan) and dissolved in DMSO. And then, these dissolved reagents were resuspended in PBS (0.05 M, pH 7.4), filtrated through syringe filters (0.45 μ m, IWAKI GLASS) before use.

Cell culture

IM9 cells, RPMI8226 cells, and HS-sultan cells, were obtained from Health Science Research Resources Bank (Osaka, Japan). ARH77 cells were obtained from DS Pharma biomedical (Osaka, Japan). These human myeloma cell lines were cultured in RPMI1640 medium (Sigma) supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA, USA), 100 μ g/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), and 25 mM HEPES (pH 7.4; Wako) in an atmosphere containing 5% CO₂.

Enzyme-linked immunosorbent assay (ELISA)

Cells were cultured at 1×10^4 cells/mL and conditioned media were harvested. The MIP-1 α levels were measured using Quantikine MIP-1 α

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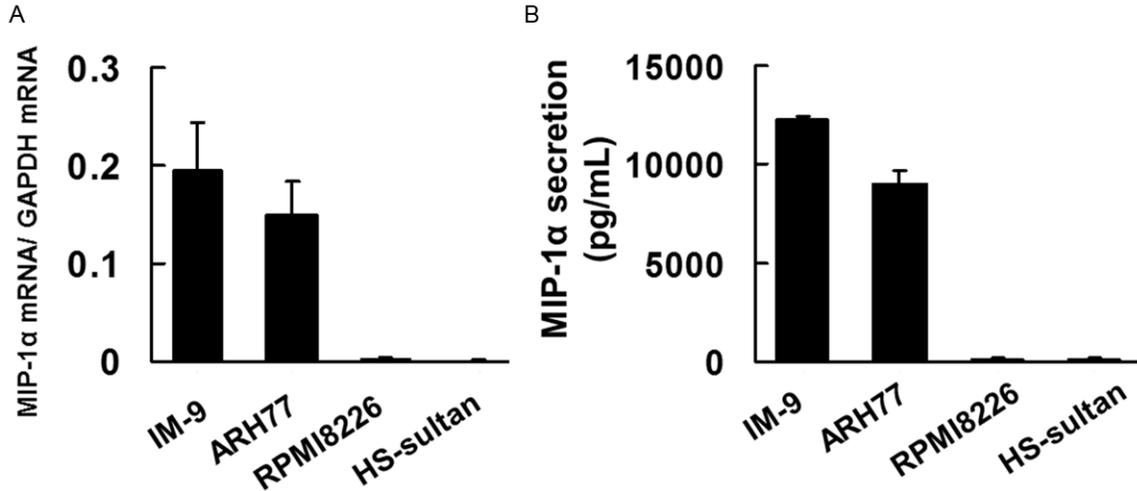


Figure 1. Multiple cell lines myeloma express MIP-1 α . A. A total RNA was extracted, and MIP-1 α mRNA levels were determined by real-time PCR. The results are expressed as treated over control ratio after correction to GAPDH mRNA levels. B. Cells (1×10^4 cells per well) were incubated for 48 h. Culture supernatant was collected and analyzed for MIP-1 α by enzyme-linked immunosorbent assay (ELISA). The results are representative of five independent experiments.

enzyme immunoassay kits (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIZOL (Invitrogen; Carlsbad, CA). One microgram of purified total RNA was used for the real-time PCR analysis with the SuperScript First-Strand Synthesis System (Invitrogen). cDNA was subjected to quantitative real-time PCR by using SYBR Premix Ex *Taq* (Takara Biomedical; Siga, Japan) and the Thermal Cycler Dice Real Time system (Takara Biomedical) in a 96-well plate according to the manufacturer's instructions. The PCR conditions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MIP-1 α , AML-1A, CREB, NF-AT, and C/ERB β were 94°C for 2 min; followed by 40 cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 0.5 min. The following primers were used: for MIP-1 α , 5'-CAG CGA GTA CCA GTC CCT TTT-3' (5'-primer); 5'-CCT CGC TGC CTC CAA GA-3' (3'-primer), for AML-1A, 5'-CTG GTC ACT GTG ATG GCT GG-3' (5'-primer); 5'-CTG CCT TAA CAT CTC CAG GG-3' (3'-primer), for CREB, 5'-GGC CTG CAA ACA TTA ACC AT-3' (5'-primer); 5'-ACG ACA CTC TCG AGC TGC TT-3' (3'-primer), for NF-AT, 5'-TCC TCG CAT CGA GAT AAC CT-3' (5'-primer); 5'-ACG ACA CTC TCG AGC TGC TT-3' (3'-primer), for C/ERB β , 5'-ACA GCG

ACG AGT ACA AGA TCC-3' (5'-primer); 5'-GCA GCT GCT TGA ACA AGT TCC-3' (3'-primer), and for GAPDH, 5'-ACT TTG TCA AGC TCA TTT-3' (5'-primer), 5'-TGC AGC GAA CTT TAT TG-3' (3'-primer). As an internal control for each sample, the GAPDH gene was used for standardization. Cycle threshold (Ct) values were established, and the relative difference in expression from GAPDH expression was determined according to the $2^{-\Delta\Delta Ct}$ method of analysis and compared to the expression in control cells.

Cell viability

Cells (2×10^3 cells/well) were plated in 96-well plates and incubated with various concentration of minodronate, alendronate, fluvastatin, and simvastatin. After incubation for 5 days, cells were stained with trypan blue dye, and the number of stained cells was counted.

Western blotting

Cells treated under various conditions were lysed with a lysis buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP40, 1 μ g/mL leupeptin, 1 μ g/mL anti-pain, and 1 mM phenylmethylsulfonyl fluoride), and the protein concentrations of the resulting cell lysates were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The membrane fraction of cells was extracted with

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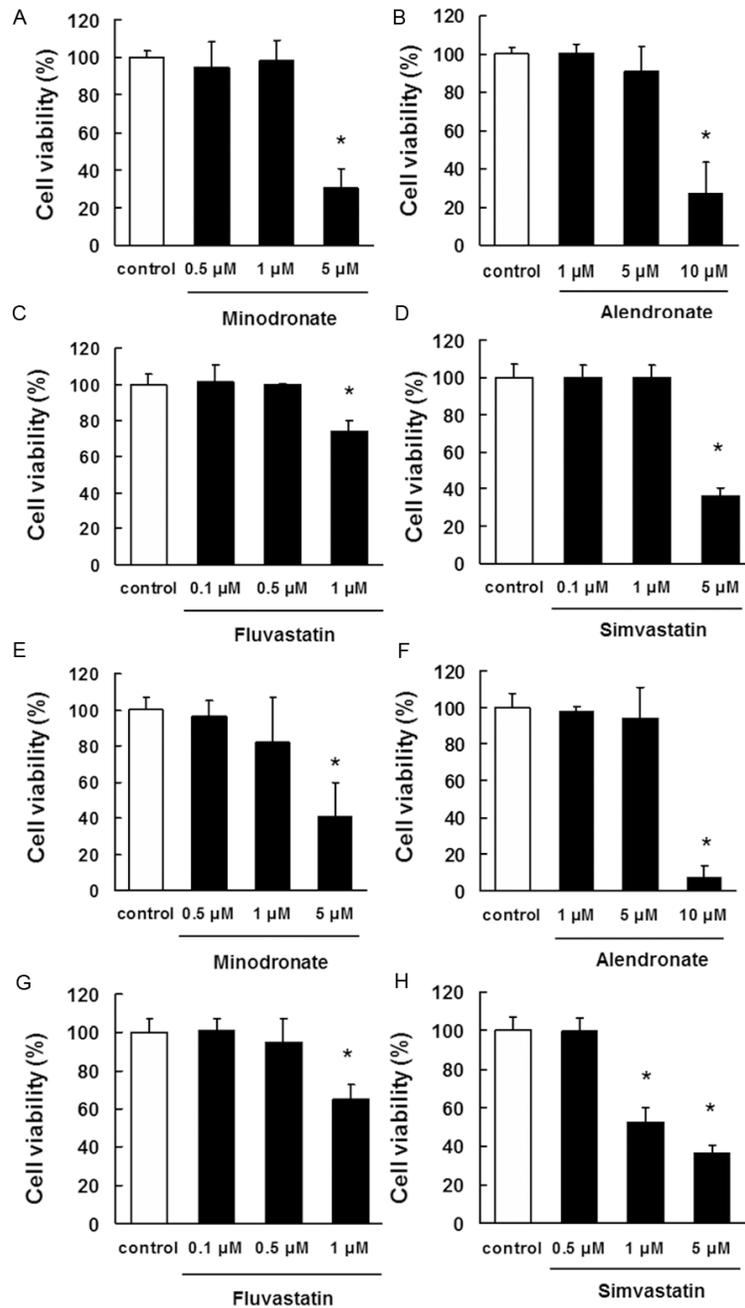


Figure 2. Determination of the appropriate concentrations of bisphosphonates and statins that are not cytotoxic to IM9 cells and ARH77 cells. IM9 cells (A-D) and ARH77 cells (E-H) were incubated in 96-well plates for 24 h and then treated with various concentrations of (A, E) minodronate, (B, F) alendronate, (C, G) fluvastatin, or (D, H) simvastatin. After 5 days, cell viability was quantified by trypan blue dye assays. The results are representative of 5 independent experiments. * $p < 0.01$ vs. the controls (ANOVA with Dunnet's test).

the ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem, San Diego, CA, USA). An aliquot of each extract (containing 40 μ g protein) was fractionated by electrophoresis in sodium dodecyl sulfate -polyacrylamide gel

and transferred to a polyvinyl difluoride membrane (Amersham, Arlington Heights, IL, USA). Membranes were blocked with a solution containing 3% skim milk and then incubated overnight at 4°C with the following antibodies: anti-phospho-p44/42 mitogen-activated protein kinase (MAPK; ERK1/2) (Thr²⁰²/Tyr²⁰⁴) antibody, anti-phospho-Akt (Ser⁴⁷³) antibody, anti-phospho-p38MAPK (Thr¹⁸⁰/Tyr¹⁸²) antibody, anti-phospho-mTOR (Ser²⁴⁴⁸) antibody, anti-p44/42 MAPK (ERK1/2) antibody, anti-Akt antibody, anti-p38MAPK antibody, anti-mTOR antibody, I- κ B antibody (Cell Signaling Technology, Beverly, MA, USA), anti-Ras antibody (clone RAS-10), anti-Rho antibody (clone 55) (Upstate Biology, Charlottesville, VA, USA), Na/K-ATPase (Santa Cruz Biotechnologies, CA, USA), and anti- β -actin antibody (Sigma). Subsequently, the membranes were incubated for 1 h at room temperature with anti-rabbit IgG sheep antibody or anti-mouse IgG sheep antibody coupled to horseradish peroxidase (Amersham). Reactive proteins were visualized using a chemiluminescence (ECL-plus) kit (Amersham) according to the manufacturer's instructions.

Statistical analysis

All results are expressed as means and S.D. of several independent experiments. Multiple comparisons of the data were done by ANOVA with Dunnet's test. P values less than 5% were regarded as significant.

Results

Expression and secretion of MIP-1 α in MM cell lines

To assess whether different MM cell lines express MIP-1 α mRNA, we analysed MIP-1 α

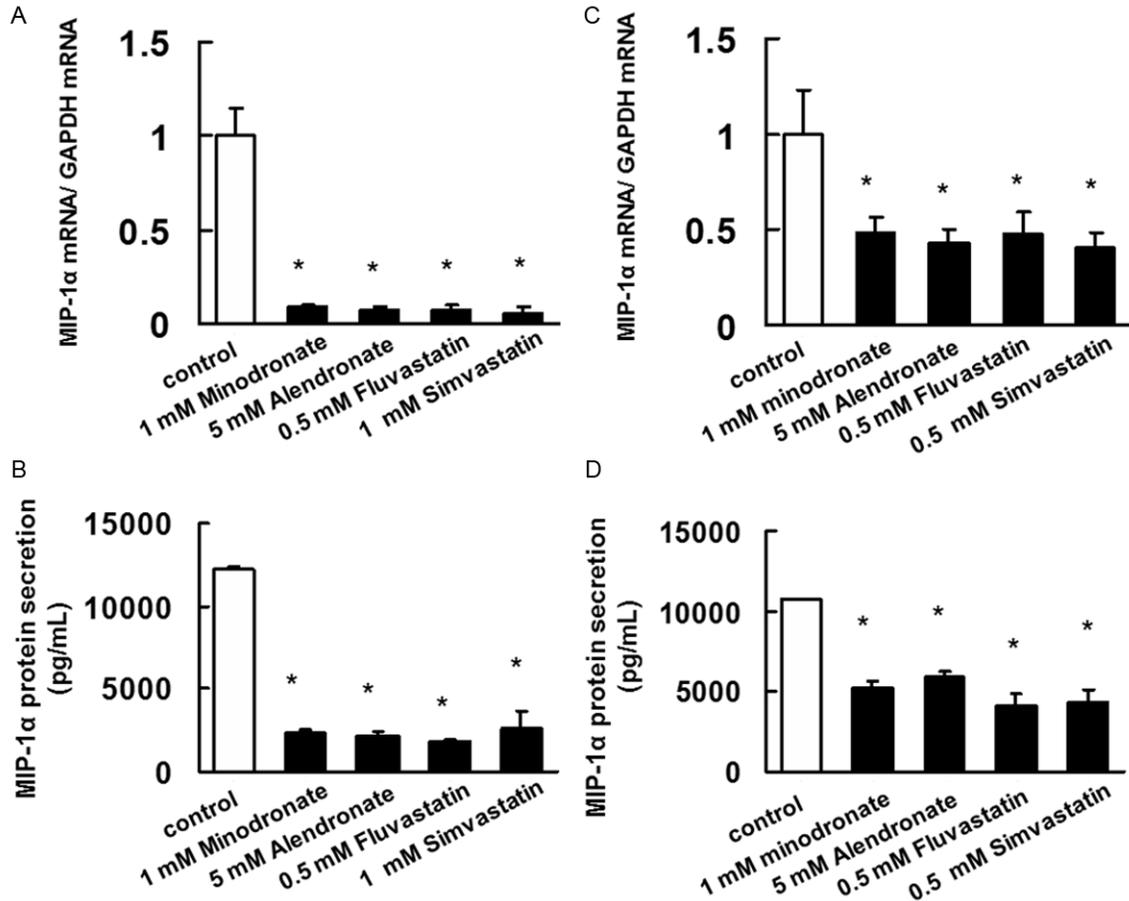


Figure 3. Inhibitory effect of bisphosphonates and statins on the mRNA expression and protein secretion of MIP-1 α . (A, C) A total RNA was extracted, and MIP-1 α mRNA levels were determined by real-time PCR. (A) IM-9 cells and (C) ARH77 cells treated with minodronate, alendronate, fluvastatin, or simvastatin for 3 days. The results are representative of 5 independent experiments. *P < 0.01, as compared to controls (ANOVA with Dunnett's test). (B) IM-9 cells and (D) ARH77 cells were treated with minodronate, alendronate, fluvastatin, or simvastatin for 3 days. MIP-1 α concentration of the cell culture supernatants was assayed using ELISA. minodronate, alendronate, fluvastatin, or simvastatin suppressed MIP-1 α secretion in IM-9 cells and ARH77 cells. The results are representative of five independent experiments. *p < 0.01 vs. the controls (ANOVA with Dunnett's test).

mRNA expression by real time PCR. MIP-1 α mRNA expression was detected on IM9 cells and ARH77 cells (Figure 1A). To confirm that IM9 cells, ARH77cells, RPMI8226 cells, and HS-Sultan cells secrete MIP-1 α , cells were seeded at a concentration of 1×10^4 cells/mL in conditioned media. MIP-1 α secretion was measured in 48-h culture supernatants by ELISA. MIP-1 α secretion was detectable in IM9 cells and ARH77 cells (Figure 1B).

Cytotoxicity toward IM9 cells and ARH77 cells

The cytotoxic effects of minodronate, alendronate, fluvastatin, and simvastatin on IM9 cells were measured by Trypan blue dye exclusion

assay. The results showed that minodronate and simvastatin did not affect cell viability at a concentration of 0.5 μ M to 1 μ M for 5 days (Figure 2A, 2D). We also found that alendronate did not affected cell viability at a concentration of 1 μ M to 5 μ M for 5 days (Figure 2B). Fluvastatin did not affect cell viability at a concentration of 0.1 μ M to 0.5 μ M for 5 days (Figure 2C). On the basis of these results, we selected 1 μ M as the effective concentration of minodronate; 5 μ M, of alendronate; 0.5 μ M, of fluvastatin; and 1 μ M, of simvastatin that did not show cytotoxicity toward IM9 cells.

Next, we examine the cytotoxic effects of minodronate, alendronate, fluvastatin, and simvas-

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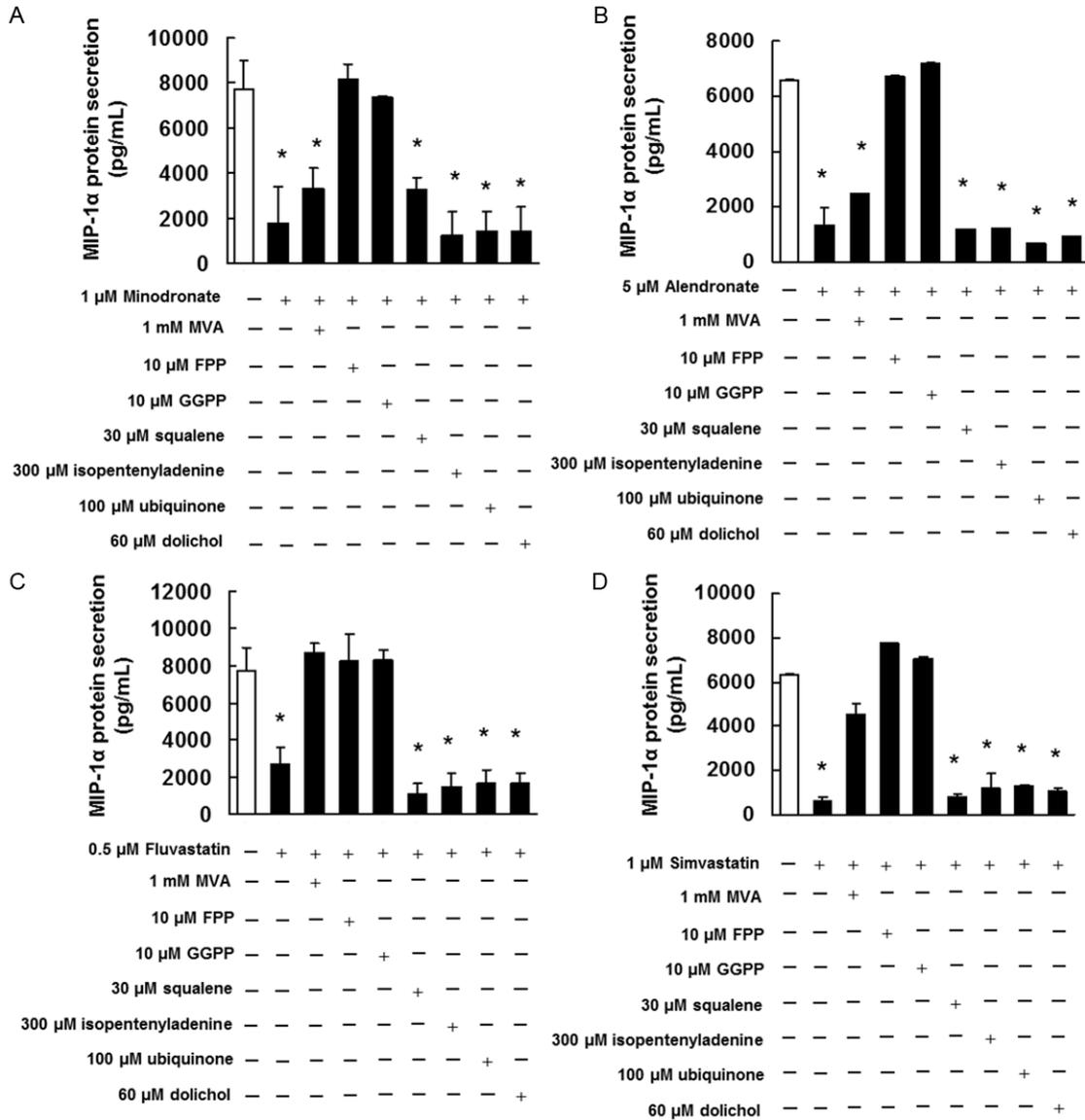


Figure 4. Effect of MIP-1 α secretion in IM-9 cells by intermediate of the mevalonate pathway. IM9 cells were pre-treated with 1 mM MVA, 10 μ M FPP, 10 μ M GGPP, 30 μ M squalene, 300 μ M isopentenyladenine, 100 μ M ubiquinone, or 60 μ M dolichol for 4 h and then treated with (A) 1 μ M minodronate, (B) 5 μ M alendronate, (C) 0.5 μ M fluvastatin, or (D) 1 μ M simvastatin for 72 h. Culture supernatant was collected and analyzed for MIP-1 α by ELISA. These results are representative of 5 independent experiments. *p < 0.01 vs. the controls (ANOVA with Dunnet's test).

tatin on ARH77 cells. The results showed that minodronate did not affect cell viability at a concentration of 0.5 μ M to 1 μ M for 5 days (**Figure 2E**). We also found that alendronate did not affected cell viability at a concentration of 1 μ M to 5 μ M for 5 days (**Figure 2F**). Fluvastatin and simvastatin did not affect cell viability at a concentration of 0.5 μ M for 5 days (**Figure 2G, 2H**). On the basis of these results, we selected 1 μ M as the effective concentration of minodronate; 5 μ M, of alendronate; 0.5 μ M, of fluvas-

tatin; and 0.5 μ M, of simvastatin that did not show cytotoxicity toward ARH77 cells.

Inhibitory effects of bisphosphonates and statins on MIP-1 α mRNA expression and secretion by IM9 cells and ARH77 cells

To determine whether bisphosphonates and statins inhibited MIP-1 α mRNA expression by IM9 cells and ARH77 cells, we treated IM9 cells or ARH77 cells with bisphosphonates and

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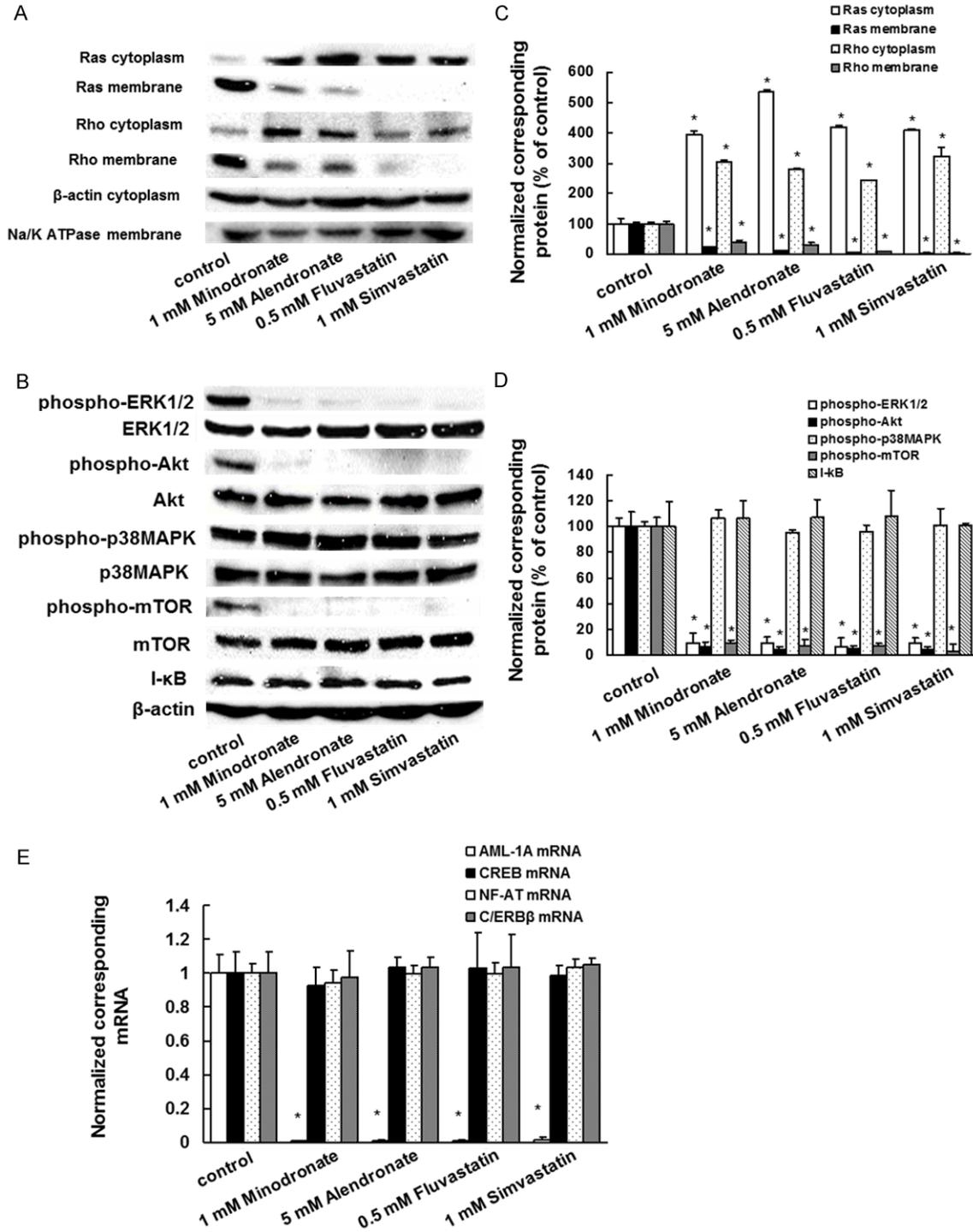


Figure 5. Effect of bisphosphonates and statins on Ras/Rho membrane localization, activation of signaling molecules, and expression of AML-1A mRNA in IM9 cells. The cells were incubated with 1 μ M minodronate, 5 μ M alendronate, 0.5 μ M fluvastatin, or 1 μ M simvastatin for 3 days. A. The cytoplasmic fractions and membrane fractions were extracted and then subjected to SDS-PAGE/immunoblotting with anti-Ras and anti-Rho antibodies. Anti-Na/K-ATPase antibody or Anti- β -actin antibody was used as the membrane or cytoplasm internal standard, as the primary antibody to detect Na/K-ATPase or β -actin protein. B. The cytoplasmic fractions were extracted and then subjected to SDS-PAGE/immunoblotting with antibodies against phosphorylated ERK1/2 (phospho-ERK1/2), phosphorylated Akt (phospho-Akt), phosphorylated p38MAPK (phospho-p38MAPK), I- κ B, phosphorylated mTOR (phospho-mTOR), ERK, Akt, p38MAPK, mTOR, and β -actin. C. Quantification of the amount of Ras or Rho, normalized to the amounts of the corresponding proteins, respectively. The results are representative of 5 independent experiments. *P <

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0.01, as compared to controls (ANOVA with Dunnett's test). D. Quantification of the amount of phospho-ERK1/2, phospho-Akt, phospho-p38MAPK, phospho-mTOR, or I- κ B, normalized to the amounts of the corresponding proteins, respectively. The results are representative of 5 independent experiments. *P < 0.01, as compared to controls (ANOVA with Dunnett's test). E. A total RNA was extracted, and AML-1A, CREB, NF-AT, and C/ERB β mRNA levels were determined by real-time PCR. IM-9 cells treated with minodronate, alendronate, fluvastatin, or simvastatin for 3 days. The results are representative of 5 independent experiments. *P < 0.01, as compared to controls (ANOVA with Dunnett's test).

statins. As shown in **Figure 3A** and **3C**, bisphosphonates and statin downregulated MIP-1 α mRNA expression. Bisphosphonates and statins also suppressed the secretion of MIP-1 α in IM9 cells and ARH77 cells (**Figure 3B** and **3D**).

Confirmation of the inhibitory effect of bisphosphonates and statins plus mevalonate pathway intermediates on MIP-1 α secretion by IM9 cells

We administered bisphosphonates and statins in combination with mevalonate pathway intermediates to investigate whether the reduced MIP-1 α secretion observed in IM9 cells was due to bisphosphonate- or statin-induced inhibition of the intermediates of the mevalonate biosynthesis pathway.

Bisphosphonates inhibited MIP-1 α secretion, but when used in combination with FPP or GGPP, MIP-1 α secretion was restored to the degree observed in control (0.1% DMSO-treated) cells (**Figure 4A** and **4B**). This finding suggests that the inhibition of MIP-1 α secretion by IM9 cells treated with bisphosphonates was due to the inhibition of FPP or GGPP biosynthesis. Statins inhibited MIP-1 α secretion, whereas statins in combination with MVA, FPP, or GGPP led to the restoration of MIP-1 α secretion to the degree observed in control cells (**Figure 4C** and **4D**). This finding suggests that the inhibition of MIP-1 α secretion in IM9 cells treated with statins was due to the inhibition of FPP or GGPP biosynthesis.

Bisphosphonates and statins suppress Ras and Rho prenylation in IM9 cells

To demonstrate whether bisphosphonates and statins inhibit the functions of Ras and Rho by suppressing their prenylation, the protein samples were subjected to a standard western blot assay to detect the presence of small GTPases in both the membrane and the cytoplasm lysates of IM9 cells incubated with or without bisphosphonates and statins. Membrane local-

ization of Ras or Rho proteins significantly decreased in the bisphosphonate- and statin-treated cells compared to the control cells. In contrast, the cytoplasmic localization of Ras or Rho proteins increased in the reagent-treated cells compared to the control cells (**Figure 5A** and **5C**).

Bisphosphonates and statins suppress ERK1/2, Akt, and mTOR activation in IM9 cells

We further investigated whether bisphosphonates and statins inhibit signal transduction factors that are downstream of Ras and found that they suppressed ERK1/2, Akt, and mTOR activation. There was no substantial change in the level of phosphorylated p38MAPK or expression of I- κ B in the bisphosphonate- and statin-treated cells compared to that in the control cells (**Figure 5B** and **5D**).

Bisphosphonates and statins inhibit AML-1A mRNA expression

Next, we investigated whether bisphosphonates and statins suppress the transcription factor for MIP-1 α expression. Real time PCR analyses revealed that bisphosphonates and statins induced markedly decreased expression of AML-1A mRNA. There were no substantial changes in the cyclic adenosine monophosphate response element-binding (CREB), NF-AT, or C/ERB β mRNA expression levels in the bisphosphonate- and statin-treated cells compared to those in the control cells (**Figure 5E**).

Inhibitory effect of U0126 and LY294002 on MIP-1 α and AML-1A mRNA expression and MIP-1 α secretion

Our results indicated that the inhibitory effects of bisphosphonates and statins on MIP-1 α expression are exerted via the inhibition of Ras/MEK/ERK, Ras/PI3K/Akt, Ras/mTOR, and Rho/ROCK signalling pathways. Therefore, we treated IM9 cells with U0126 (MEK inhibitor), LY294002 (PI3K inhibitor), Y27632 (ROCK

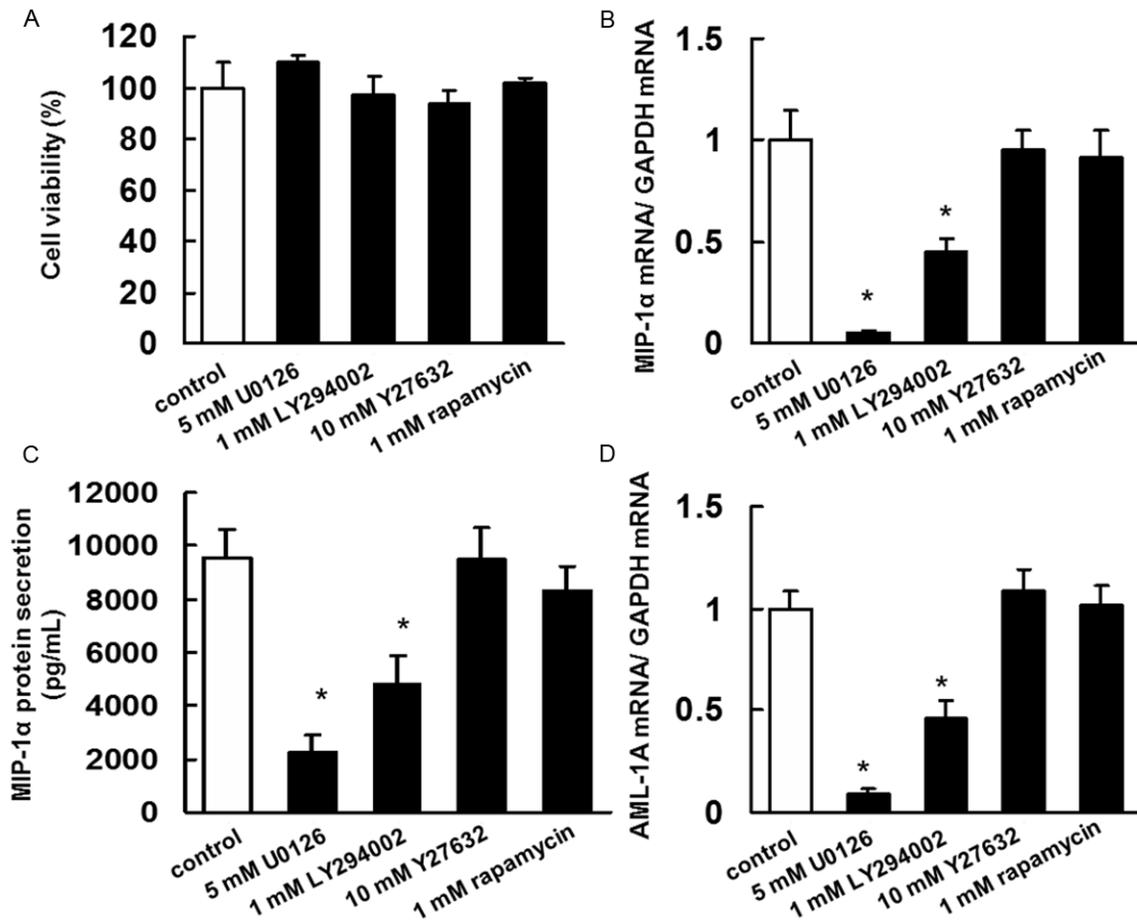


Figure 6. Inhibitory effect of U0126, LY294002, Y27632, or rapamycin on the secretion of MIP-1 α and AML-1A mRNA expression in IM9 cells. A. Determination of the appropriate concentrations of U0126, LY294002, Y27632, or rapamycin that are not cytotoxic to IM9 cells. IM9 cells were incubated in 96-well plates for 24 h and then treated with 5 μ M U0126, 1 μ M LY294002, 10 μ M Y27632, or 1 μ M rapamycin. After 5 days, cell viability was quantified by trypan blue dye assays. The results are representative of 5 independent experiments. B. A total RNA was extracted, and MIP-1 α mRNA levels were determined by real-time PCR. IM-9 cells treated with U0126, LY294002, Y27632, or rapamycin for 3 days. The results are representative of 5 independent experiments. *P < 0.01, as compared to controls (ANOVA with Dunnett's test). C. IM-9 cells were treated with U0126, LY294002, Y27632, or rapamycin for 3 days. MIP-1 α concentration of the cell culture supernatants was assayed using ELISA. The results are representative of five independent experiments. *p < 0.01 vs. the controls (ANOVA with Dunnett's test). D. A total RNA was extracted, and AML-1A mRNA levels were determined by real-time PCR. IM-9 cells treated with U0126, LY294002, Y27632, or rapamycin for 3 days. The results are representative of 5 independent experiments. *P < 0.01, as compared to controls (ANOVA with Dunnett's test).

inhibitor), and rapamycin (mTOR inhibitor) to determine whether the suppression of ERK, Akt, ROCK, or mTOR would result in MIP-1 α expression inhibition. Administration of U0126 or LY294002 inhibited MIP-1 α and AML-1A mRNA expression and MIP-1 α secretion even at concentrations of these reagents that did not show anti-proliferative effects on IM9 cells (Figure 6). However, neither Y27632 nor rapamycin affected MIP-1 α and AML-1A mRNA expression or MIP-1 α secretion (Figure 6).

Discussion

In the present study, we demonstrated that bisphosphonates and statins inhibit MIP-1 α expression and secretion by suppressing FPP and GGPP biosynthesis in human MM cells. We also found that bisphosphonates and statins suppressed Ras and Rho prenylation in our experimental model. It has been reported that statins decrease Ras and Rho prenylation by suppressing FPP and GGPP biosynthesis in myeloma cells [25]. We also previously indicat-

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ed that minodronate and mevastatin induced apoptosis by inhibiting GGPP biosynthesis and Ras prenylation [20, 21, 24, 26]. Collectively, these findings suggest that bisphosphonate- and statin-induced inhibition of small GTPase prenylation through FPP and GGPP biosynthesis plays an important role in the suppression of MIP-1 α secretion by MM cells.

Activation of Ras can lead to the activation of MAPKs, the PI3K/Akt pathway, and the PI3K/mTOR pathway [27]. MAPKs are serine/threonine kinases that are present in most cell types and can activate MIP-1 α gene expression [28]. The PI3K/Akt pathway has also been reported to regulate MIP-1 α expression [28]. Our results clearly demonstrate that bisphosphonates and statins induce decreases in ERK1/2, Akt, and mTOR phosphorylation. Moreover, we observed that U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor) inhibited MIP-1 α mRNA expression in IM9 cells. However, rapamycin (mTOR inhibitor) and Y27632 (ROCK inhibitor) had no effect on MIP-1 α and AML-1A mRNA expression or MIP-1 α secretion. MIP-1 α expression can be induced by various growth factors and cytokines, including acidic fibroblast growth factor and IL-6 [29, 30]. These inductions require activation of MAPKs or the PI3K/Akt pathway [31]. Therefore, our present findings indicate that bisphosphonates and statins inhibit MIP-1 α expression by suppressing Ras/MEK/ERK and Ras/PI3K/Akt pathways.

MIP-1 α expression is related to AML-1A, a transcription factor located downstream of ERK1/2 [32, 33]. It has been reported that AML-1A regulates the upregulation of MIP-1 α gene promoter activity. We found that bisphosphonates and statins inhibited AML-1A mRNA expression. Moreover, U0126 and LY294002 also inhibited the expression of AML-1A mRNA. These findings suggest that bisphosphonates and statin suppressed MIP-1 α expression by inhibiting AML-1A mRNA expression.

In the previous study, we have shown that minodronate inhibited LPS-induced MIP-1 α secretion by inhibiting Ras/ERK and Ras/Akt pathways. However, the detailed mechanism by which bisphosphonates and statins suppress MIP-1 α secretion by myeloma cells, though, has not been clearly elucidated. The findings of this study suggest that bisphosphonates and statin suppress MIP-1 α expression by inhibiting

Ras/MEK/ERK/AML-1A and Ras/PI3K/Akt/AML-1A pathways. Our results also indicate that AML-1A expression possibly is regulated by ERK1/2 and Akt activation.

In conclusion, our data showed that bisphosphonates and statins inhibited MIP-1 α expression by suppressing Ras/MEK/ERK/AML-1A and Ras/PI3K/Akt/AML-1A pathways in human MM cells. Therefore, the use of MIP-1 α expression inhibitors such as bisphosphonates and statins may provide a new therapeutic approach for inhibiting tumour progression and bone destruction in MM patients.

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Disclosure of conflict of interest

The authors have no competing interest.

Address correspondence to: Dr. Shozo Nishida, Division of Pharmacotherapy, Kinki University School of Pharmacy, Kowakae, Higashi-Osaka 577-8502, Japan. Tel: +81-6-6721-2332 Ext. 3851; Fax: +81-6-6730-1394; E-mail: nishida@phar.kindai.ac.jp

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