Citrus limonoid nomilin inhibits osteoclastogenesis in vitro by suppression of NFATc1 and MAPK signaling pathways

Yoshifumi Kimira*, Yuri Taniuchi, Sachie Nakatani, Yuusuke Sekiguchi, Hyoun Ju Kim, Jun Shimizu, Midori Ebata, Masahiro Wada, Akiyo Matsumoto, Hiroshi Mano

Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

*Corresponding author:

Yoshifumi Kimira, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

Tel: +81-49-271-7208; fax: +81-49-271-7984

E-mail address: kimira@josai.ac.jp (Y.K.).

Abstract

Background

Animal experiment studies have revealed a positive association between intake of citrus fruits and bone health. Nomilin, a limonoid present in citrus fruits, is reported to have many biological activities in mammalian systems, but the mechanism of nomilin on bone metabolism regulation is currently unclear.

Purpose

To reveal the mechanism of nomilin on osteoclastic differentiation of mouse primary bone marrow-derived macrophages (BMMs) and the mouse RAW 264.7 macrophage cell line into osteoclasts.

Study design

Controlled laboratory study. Effects of nomilin on osteoclastic differentiation were studied in *in vitro* cell cultures.

Methods

Cell viability of RAW 264.7 cells and BMMs was measured with the Cell Counting Kit.

TRAP-positive multinucleated cells were counted as osteoclast cell numbers. The number and area

of resorption pits were measured as bone-resorbing activity. Osteoclast-specific genes expression was evaluated by quantitative real-time PCR; and proteins expression was evaluated by western blot.

Results

Nomilin significantly decreased TRAP-positive multinucleated cell numbers compared with the control, and exhibited no cytotoxicity. Nomilin decreased bone resorption activity. Nomilin downregulated osteoclast-specific genes, *NFATc1* and *TRAP* mRNA levels. Furthermore, nomilin suppressed MAPK signaling pathways.

Conclusion

This study demonstrates clearly that nomilin has inhibitory effects on osteoclastic differentiation *in vitro*. These findings indicate that nomilin-containing herbal preparations have potential utility for the prevention of bone metabolic diseases.

Keywords: Limonoid; nomilin; osteoclastic differentiation

Abbreviations:
α-MEM, alpha-modified Eagle's medium; BMMs, bone marrow-derived macrophages; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; JNK, c-Jun- N-terminal kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony stimulating factor; PCR, polymerase chain reaction; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase

Introduction

Bone metabolic balance is maintained by osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Bone metabolic diseases, such as osteoporosis, are caused by an imbalance in bone metabolism, so that osteoclastic bone resorption exceeds osteoblastic bone formation (Boyle et al., 2003). Osteoclasts are multinucleated cells that differentiated from cells of the monocyte/macrophage lineage. Macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are essential cytokines for osteoclast differentiation. Ligation of RANKL to the receptor activator of NF-κB (RANK) activates mitogen-activated protein kinases (MAPKs) pathway, including extracellular signal-regulated kinase (ERK), c-Jun- N-terminal kinase (JNK), and p38. These signaling pathways, together with other signaling pathways, including M-CSF, initiate the expression of *NFATc1*, a master transcription factor that regulates the differentiation of osteoclasts. NFATc1 regulates the expression of osteoclast-specific genes, such as tartrate-resistant acid phosphatase (*TRAP)* (Wada et al., 2006, Takayanagi et al., 2002 and Asagiri and Takayanagi 2007).

Epidemiology studies have revealed a positive association between increased consumption of fruit and vegetables and bone health (Liu 2003 and Liu 2000). Furthermore, previous rodent studies have shown that citrus crude extract, juice or pulp significantly improves bone density in rats

(Deyhim et al., 2006, Deyhim et al., 2008 and Mandadi et al., 2009). These studies suggest that the bone protective property of herbal preparations from citrus may be because of its bioactive compounds, such as flavonoids or limonoids. Limonoids are a group of triterpene derivatives found in the *Rutaceae* and *Meliaceae* families (Manners 2007). Recently, it has been reported that 7-oxo-7-deacetoxygedunin, a gedunin-type limonoid, inhibits RANKL-induced osteoclastogenesis by suppressing activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways (Wisutsitthiwong et al., 2011). Therefore, citrus limonoids might play an important role in bone metabolism. Nomilin is one of the limonoids present in citrus fruits, such as yuzu (*Citrus junos*) or grapefruit, which is reported to have anti-proliferative effects on several types of cancer cells, an anti-hyperglycemic activity and anti-obesity properties (Minamisawa et al., 2014, Tian et al., 2001, Kim et al., 2013 and Ono et al., 2011). However, the effect of nomilin on bone metabolism is currently unclear. In this study, we investigated that nomilin would help to osteoclastic bone resorption. This study evaluated the effect of nomilin on the differentiation of mouse RAW 264.7 macrophage cells and mouse primary bone marrow-derived macrophages (BMMs) into mature osteoclasts.

Materials and methods

Reagents

Nomilin was purchased from LKT Laboratories (St. Paul, MN, USA). The purity of nomilin is 98%. The chemical structure of nomilin is shown as Fig. 1A. Recombinant human M-CSF was purchased from Kyowa Hakko Kogyo (Leukoprol; Tokyo, Japan). Recombinant mouse M-CSF and recombinant mouse RANKL were purchased from R&D Systems, Inc. (Minneapolis, MA, USA). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St. Louis, MN, USA). Alpha-modified Eagle's medium (α-MEM) was purchased from GIBCO (Grand Island, NY, USA). Antibodies for western blot analysis were purchased from Cell Signaling Technology (Beverly, MA, USA)

Cell culture

Mouse RAW 264.7 macrophage cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Mouse primary BMMs were obtained from the tibiae and femora of 7 week old ddY male mice and were cultured for 3 days in α-MEM containing 10% FBS and recombinant human M-CSF (1000 U/ml) in 100-mm tissue culture dishes. After 3 days incubation,

adherent cells were removed using trypsin and the isolated cells were used as BMMs (Okayasu et al., 2012). All cells were cultured in α-MEM supplemented with 10% FBS and 100 U/mL penicillin. Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Animal care and experiments were approved by the Animal Committee of Josai University.

Cell viability

RAW 264.7 cells were treated with various concentrations of nomilin (0.1–50 μM) or a dimethyl sulfoxide (DMSO) vehicle control, and incubated for 1 or 3 days. BMMs were treated with various concentrations of nomilin (0.1–50 μM) or DMSO vehicle control, in the presence of M-CSF (20 ng/ml), and incubated for 1 or 3 days. The effect of nomilin on cell viability of RAW 264.7 cells and BMMs was measured with the Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Plates were read using a microplate reader (Perkin Elmer, Inc, Waltham, MA, USA) at a wavelength of 450 nm.

TRAP staining

RAW 264.7 cells were seeded into 96-well plates at a density of 3×10^3 cells/well. Cells were treated with nomilin at the indicated concentrations or DMSO vehicle control, and cultured in α-MEM containing RANKL (10 ng/ml) for 3 days. Meanwhile, BMMs were seeded into 96-well

plates at a density of 1×10^4 cells/well. Cells were treated with various concentrations of nomilin (0.1, 1 or 10 μM) or DMSO vehicle control, and cultured in α-MEM containing M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 4 days. After culturing, cells were fixed and stained using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich, St. Louis, MO, USA). Multinucleated cells were visualized by TRAP staining and numbers of TRAP-positive multinucleated cells (three or more nuclei per cell) were counted to determine osteoclast cell number.

Bone resorption assay

To determine the bone-resorbing activity of osteoclasts, we measured the number and area of resorption pits formed by BMMs on dentine slices. Briefly, BMMs were seeded onto dentine slices in 96-well plates at a density of 1×10^4 cells/well. Cells were treated with various concentrations of nomilin (0, 1 or 10 μM) and cultured in α-MEM containing M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 14 days. After culturing, the dentine slices were brushed to remove the cells, and were stained with acid hematoxylin for 2 min. Pit formation areas on dentine slices were scanned and analyzed qualitatively using Image J software (Kameda et al., 1997).

Quantitative real-time RT-PCR

Total RNA was extracted from the cells using a NucleoSpin II Column Kit (Takara Bio Japan, Osaka, Japan). First-strand cDNA was converted with the PrimeScript™ Reagent Kit (Takara Bio Japan, Osaka, Japan). Quantitative real-time PCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems, Carlsbad, CA, USA). TaqMan probes were as follows: *NFATc1* (Mm00479445_m1) and *TRAP* (Mm00475698_m1). *β-actin* (Mm00607939_s1) was used as the internal control for normalization of target gene expression.

Western blot analysis

RAW 264.7 cells were incubated with or without 10μM nomilin for 1h and then stimulated with or without RANKL (20 ng/ml) for the indicated times. Cells were washed twice with ice-cold PBS and then lysed with RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail. Cell lysates were centrifuged at 15,000 rpm for 30 min, and the supernatants were collected as the protein samples. The protein concentration of each sample was measured with BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). Proteins (5 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 2% BSA in TBS-T (10 mM Tris-HCl, pH 7.4

containing 1.37 M NaCl and 0.1% Tween 20) for 30 min at room temperature. The membranes were probed with antibodies against phosphor-ERK, ERK, phospho-p38, p38, phospho-JNK, JNK and β-actin for 1 h at room temperature or overnight at 4°C. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was applied as the secondary antibodies for 1 h at room temperature. Finally, labeled proteins were detected with EZ west Lumi plus (ATTO, Tokyo, Japan). Protein bands were analyzed using EZ capture MG (ATTO, Tokyo, Japan).

Statistical analysis

Results are presented as means ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's post hoc test was used for statistical analysis. Values of $p < 0.05$ were considered significant.

Results

Effect of nomilin on osteoclastic differentiation of RAW 264.7 cells and BMMs

To examine the effect of nomilin on osteoclastic differentiation, RAW 264.7 cells and BMMs were treated with various concentrations of nomilin in the presence of RANKL. Osteoclastic differentiation was determined by counting TRAP-positive multinucleated cell numbers. TRAP staining analysis is the most popular method to confirm osteoclastic differentiation. Furthermore, we examined the effect of nomilin on the viability of RAW 264.7 cells and BMMs.

Treatment with a nomilin concentration greater than 1 μM markedly decreased the formation of TRAP-positive multinucleated osteoclasts from RAW 264.7 cells (IC50 = 12 μM) (Fig. 1B, C). Nomilin had no cytotoxic effect on RAW 264.7 cells at concentrations of less than 50 μM compared with the control treatment (Fig. 1D). Furthermore, treatment with nomilin inhibited the formation of TRAP-positive multi nucleated osteoclasts from BMMs, which were subjected to RANKL-induced osteoclastogenesis, in a dose-dependent manner (IC50 = 0.6 μM) (Fig. 2A, B). Nomilin had no cytotoxic effects on BMMs at concentrations of less than 50 μM compared with the control treatment (Fig. 2C).

Effect of nomilin on osteoclastic bone resorption

We evaluated whether nomilin treatment could inhibit the bone resorbing activity of osteoclasts derived from BMMs. To examine the effect of nomilin on osteoclastic bone resorption, BMMs were cultured with various concentrations of nomilin in the presence of M-CSF and RANKL for 14 days. Pit formation areas were narrower after nomilin treatment. In addition, treatment with nomilin at a concentration of higher than 1 μM decreased the resorption activity and pit formation area of osteoclasts (Fig. 3A,B).

Effect of nomilin on the gene expression of *NFATc1* and *TRAP*

To examine the effect of nomilin on osteoclastic gene expression in BMMs, the BMMs were treated with various concentrations of nomilin (1 or 10 μM) in the presence of M-CSF and RANKL. Osteoclastic gene expression in BMMs was determined by quantitative real-time RT-PCR. The expression of osteoclastic genes was induced during osteoclastogenesis, including *NFATc1* and *TRAP*. Treatment with nomilin (10 μM) significantly suppressed the induction of *NFATc1* mRNA (Fig. 3C). In addition, *TRAP* mRNA levels were decreased by nomilin in a dose-dependent manner (Fig. 3D). These results indicated that nomilin could inhibit the osteoclastic differentiation by suppression osteoclastic gene expression.

Effect of nomilin on MAPK signaling pathways

We examined the effect of nomilin on RANKL-induced signaling pathways. We evaluated whether nomilin treatment affected the RANKL-induced phosphorylation of ERK, p38 and JNK. These signaling pathways are essential for the differentiation of osteoclasts. Treatment with nomilin inhibited the phosphorylation of ERK p38 and JNK (Fig. 4). These results indicated that nomilin could inhibit the RANKL-induced phosphorylation of MAPKs (ERK, p38 and JNK) in osteoclasts.

Discussion

Bioactive citrus compounds have been shown to enhance bone mineral density in rats (Deyhim et al., 2006, Deyhim et al., 2008; Mandadi et al., 2009). Moreover, limonoid, which is present in citrus seeds, is also reported to exhibit an anti-osteoclastogenic activity *in vitro* (Wisutsitthiwong et al., 2011). In this study, we investigated the effect of nomilin, a citrus limonoid, on osteoclastic differentiation from both mouse RAW 264.7 cells and BMMs.

A previous report has shown that a gedunin-type limonoid has a strong anti-osteoclastogenic activity in RANKL-treated RAW 264.7 cells as a model of osteoclastogenesis (Wisutsitthiwong et al., 2011). We used the same cell line and found that nomilin inhibited osteoclastic differentiation from RAW 264.7 cells without cytotoxicity. This result suggests that the citrus limonoid nomilin has an anti-osteoclastogenic activity.

In vivo, osteoclasts differentiate from BMM precursors. Hence, we investigated the effect of nomilin on osteoclastic differentiation from primary BMMs. We demonstrated that nomilin reduced RANKL-induced osteoclastogenesis in a dose-dependent manner from primary BMMs. Bone resorption occurs in the bone matrix and is mediated by mature osteoclasts (Boyle et al., 2003). Nomilin suppressed osteoclastic differentiation from both RAW 264.7 cells and BMMs. In addition, we evaluated whether nomilin treatment could inhibit the bone-resorbing activity of osteoclasts

derived from BMMs. Treatment with nomilin decreased bone-resorbed areas on dentine slices compared with the control. Thus, nomilin has an anti-bone resorption activity. This result indicates that nomilin can suppress physiological bone resorption.

In addition, we examined the effect of nomilin on the regulation of osteoclastic genes. NFATc1 acts as a master regulator of osteoclastogenesis and regulates the expression of TRAP, which is marker gene in the early stage of osteoclastic differentiation (Asagiri and Takayanagi 2007, Takayanagi et al., 2002). We found that treatment of nomilin suppressed *NFATc1* and *TRAP* mRNA levels in osteoclasts compared with the control. These results indicate that nomilin can suppress osteoclastogenesis at an early stage.

Previous reports have shown that treatment with a gedunin-type limonoid decreases the expression of *NFATc1* by suppressing activation of the NF-κB and MAPK pathways in RAW 264.7 cells (Wisutsitthiwong et al., 2011). It has also been reported that nomilin reduces tumor necrosis factor-alpha-induced p38 MAPK activity in human aortic smooth muscle cells (Kim et al., 2011). It has reported that RANKL-activated MAPKs (ERK, p38 and JNK) signaling has been associated with osteoclastogenesis (Boyle et al., 2003). The RANKL-mediated phosphorylation of these MAPKs upregulate NFATc1 activity (Monje et al., 2005, Matsumoto et al., 2000 and Ikeda et al 2004). In this study, we evaluated the effects of nomilin on MAPK signaling pathways. Nomilin inhibited the

phosphorylation of ERK, p38 and JNK signaling. These results indicated that nomilin suppress osteoclastogenesis via suppression of RANKL-mediated phosphorylation of MAPKs (ERK, p38 and JNK).

Conclusion

This study demonstrates for the first time that nomilin has an anti-osteoclastogenic activity by suppressing the expression of *NFATc1* gene and the RANKL-mediated phosphorylation of MAPKs (ERK, p38 and JNK). These findings indicate that nomilin-containing herbal preparations have potential utility for the prevention of bone metabolic diseases.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

This work was supported by a President's Special Research Fellowship at Josai University.

References

Asagiri, M, Takayanagi., H, 2007. The molecular understanding of osteoclast differentiation. Bone 40, 251–264.

Boyle, W.J, Simonet, W.S., Lacey, D.L., 2003. Osteoclast differentiation and activation. Nature 423, 337–342.

Deyhim, F., Garica, K., Lopez, E., Gonzalez, J., Ino, S., Garcia, M., Patil, BS., 2006. Citrus juice modulates bone strength in male senescent rat model of osteoporosis. Nutrition 22, 559–563.

Deyhim, F., Mandadi, K., Patil, B.S., Faraji, B., 2008. Grapefruit pulp increases antioxidant status and improves bone quality in orchidectomized rats. Nutrition 24, 1039–1044.

Ikeda, F., Nishimura, R., Matsubara, T., Tanaka, S, Inoue, J., Reddy, S.V., Hata, K., Yamashita, K., Hiraga, T., Watanabe, T., Kukita, T., Yoshioka, K., Rao, A., Yoneda, T., 2004. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. J. Clin. Invest. 114, 475–484.

Kameda, T., Mano, H., Yuasa, T., 1997. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. J. Exp. Med. 186, 489–495.

Kim, J., Jayaprakasha, G.K., Muthuchamy, M., Patil, B.S., 2011. Structure-function relationships of citrus limonoids on p38 MAP kinase activity in human aortic smooth muscle cells. Eur. J. Pharmacol. 670, 44–49.

Kim, J., Jayaprakasha, G.K., Patil, B.S., 2013. Limonoids and their anti-proliferative and anti-aromatase properties in human breast cancer cells. Food Funct. 4, 258–265.

Liu, R.H., 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am. J. Clin. Nutr. 78, 517S–520S.

Liu, S., Manson, J.E., Lee, I.M., Cole, S.R., Hennekens, C.H., Willett, W.C., Buring, J.E., 2000. Fruit and vegetable intake and risk of cardiovascular disease: the Women's Health Study. Am. J. Clin. Nutr. 72, 922–928.

Mandadi, K., Ramirez, M., Jayaprakasha, G.K., Faraji, B., Lihono, M., Deyhim, F., 2009. Citrus bioactive compounds improve bone quality and plasma antioxidant activity in orchidectomized rats. Phytomedicine 16, 513–520.

Manners, G.D., 2007. Citrus limonoids: analysis, bioactivity, and biomedical prospects, J. Agric. Food. Chem. 55, 8285–8294.

Matsumoto, M. Sudo, T., Saito, T., Osada, H., Tsujimoto, M., 2000. Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). J. Biol. Chem. 275, 31155–31161.

Minamisawa, M., Yoshida, S., Uzawa, A., 2014. The functional evaluation of waste yuzu (Citrus junos) seeds. Food Funct. 5, 330–336.

Monje, P., Losa, J.H., Lyons, R.J., Castellone, M.D., Gutkind, J.S., 2005. Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. J. Biol. Chem. 280, 35081–35084.

Okayasu, M., Nakayachi, M., Hayashida, C., Ito, J., Kaneda, T., Masuhara, M., Suda, N., Sato, T., Hakeda, Y., 2012. Low-density lipoprotein receptor deficiency causes impaired osteoclastogenesis and increased bone mass in mice because of defect in osteoclastic cell-cell fusion. J. Biol. Chem. 287, 19229–19241.

Ono, E., Inoue, J., Hashidume, T., Shimizu, M., Sato, R., 2011. Anti-obesity and anti-hyperglycemic effects of the dietary citrus limonoid nomilin in mice fed a high-fat diet. Biochem. Biophys. Res. Commun. 410, 677–681.

Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E.F., Mak, T.W., Kodama, T., Taniguchi, T., 2002. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev. Cell 3, 889–901.

Tian, Q., Miller, E.G., Ahmad, H., Tang, L., Patil, B.S., 2001. Differential inhibition of human cancer cell proliferation by citrus limonoids, Nutr. Cancer 40, 180–184.

Wada, T., Nakashima, T., Hiroshi, N., Penninger, J.M., 2006. RANKL-RANK signaling in osteoclastogenesis and bone disease. Trends. Mol. Med. 12, 17–25.

Wisutsitthiwong, C., Buranaruk, C., Pudhom, K., Palaga, T., 2011. The plant limonoid 7-oxo-deacetoxygedunin inhibits RANKL-induced osteoclastogenesis by suppressing activation of the NF-κB and MAPK pathways. Biochem. Biophys. Res. Commun. 415, 361–366.

Figure legends

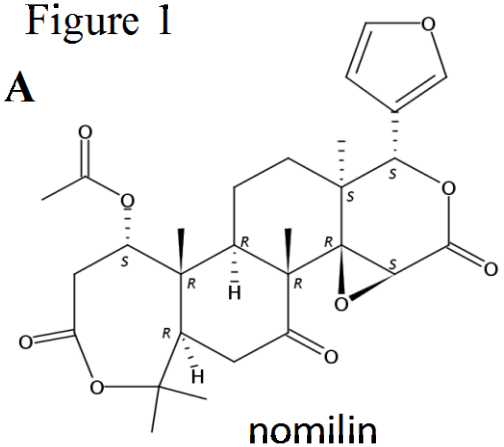
Fig. 1. Effect of nomilin on osteoclastic differentiation of RANKL-stimulated RAW 264.7 cells. (A) Structure of nomilin. (B) RAW 264.7 cells were treated with nomilin at the indicated concentrations or DMSO vehicle control, with culture in α-MEM containing RANKL (10 ng/ml) for 3 days. Multinucleated cells were visualized by TRAP staining. Scale bars represent 200 μm. (C) TRAP-positive multinucleated cells were counted to determine osteoclast numbers. (D) RAW 264.7cells were treated with nomilin at the indicated concentrations for 1 or 3 days. Cell viability was measured with the Cell Counting Kit. Data are presented as means ± SD (n = 3), $*p < 0.05$.

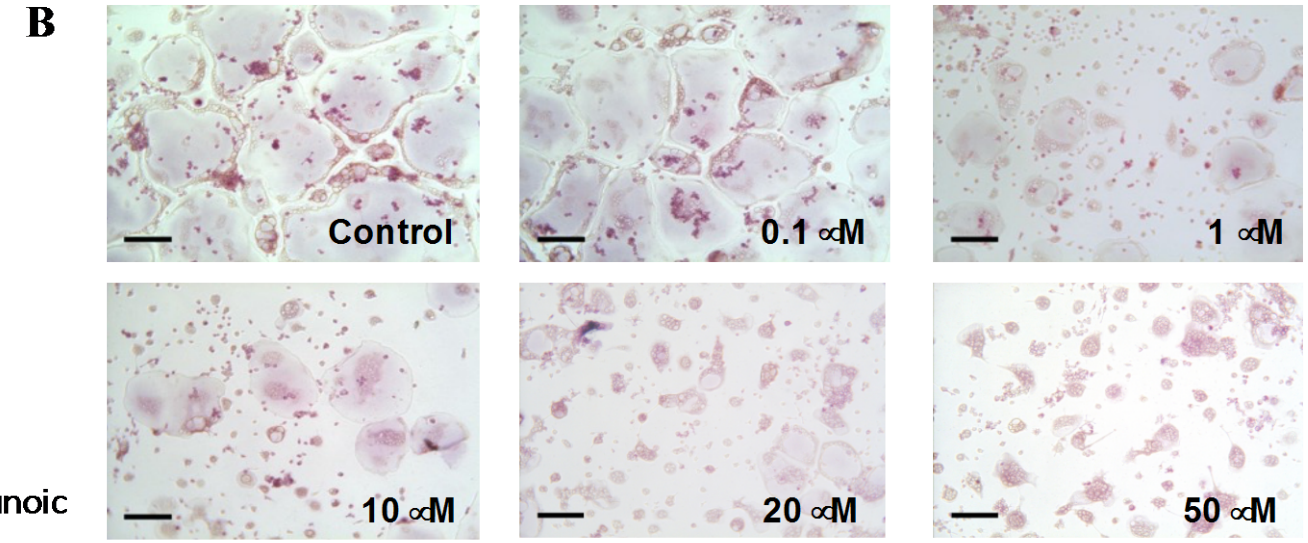
Fig. 2. Effect of nomilin on osteoclastic differentiation of RANKL-stimulated BMMs. (A) BMMs were treated with various concentrations of nomilin (0.1, 1 or 10 μM) or DMSO vehicle control, with culture in α-MEM containing M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 4 days. Multinucleated cells were visualized by TRAP staining. Scale bars represent 200 μm. (B) TRAP-positive multinucleated cells were counted to determine osteoclast cell numbers. (C) BMMs were treated with nomilin at the indicated concentrations in the presence of M-CSF (20 ng/ml) and incubated for 1 or 3 days. Cell viability was measured with the Cell Counting Kit. Data are presented as means ± SD (n = 3), $*p < 0.05$.

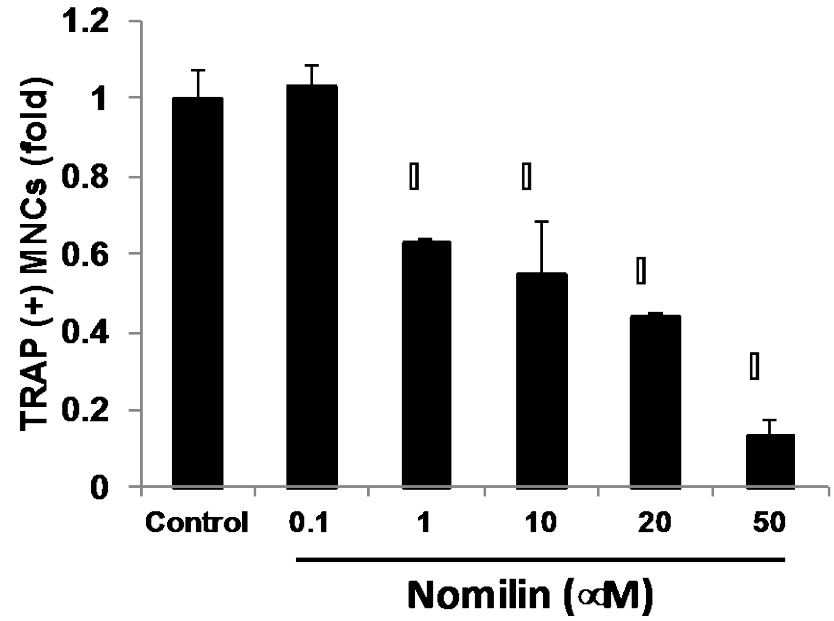
Fig. 3. Effect of nomilin on osteoclastic bone resorption and mRNA expression levels of *NFATc1* and *TRAP* in BMMs as analyzed by RT-PCR. BMMs were treated with various concentrations of nomilin (0.1, 1 or 10 μM) or DMSO vehicle control, with culture in α-MEM containing M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 14 days. (A) Photograph of pit formation areas on dentine slices. Scale bars represent 20 μm. (B) Pit formation areas on a dentine slice were analyzed qualitatively using Image J software. Cells were treated with various concentrations of nomilin (1 or 10 μM) or DMSO vehicle control, with culture in α-MEM containing M-CSF (20 ng/ml) and RANKL (20 ng/ml) for 6 h. RT-PCR analysis of the expression levels of *NFATc1* (C) and *TRAP* (D) mRNA are shown. Results are expressed as relative values to *β-actin*. Data are presented as means ± SD (n = 3), $*p < 0.05$.

Fig. 4. Effect of nomilin on MAPK pathways. RAW 264.7 cells were incubated with or without 10 μM nomilin for 1h and then stimulated with or without RANKL (20 ng/ml) for the indicated time. The cell lysates were analyzed by Western blotting. The data are representative of 3 independent experiments.

Figure 1


A
nomilin
(1-(acetyloxy)-1,2-dihydroobacunoic acid e-lactone)


B
Control
0.1 ∝M
1 ∝M
10 ∝M
20 ∝M
50 ∝M


C
TRAP (+) MNCs (fold)
1.2
1
0.8
0.6
0.4
0.2
0
Control
0.1
1
10
20
50
Nomilin (∝M)

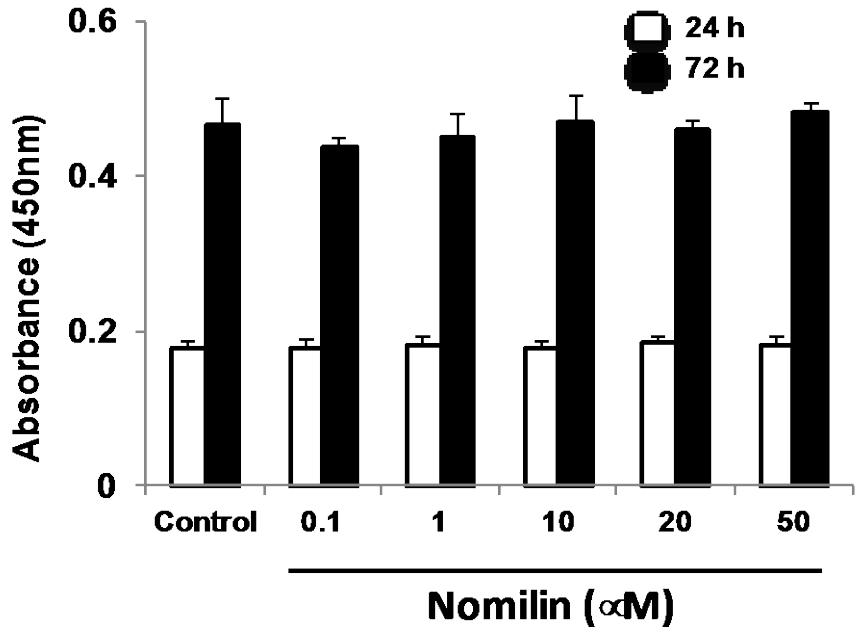
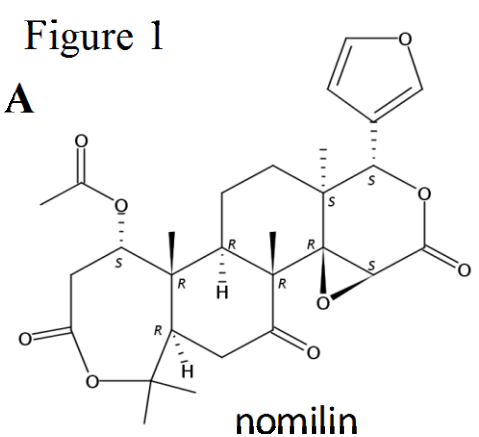
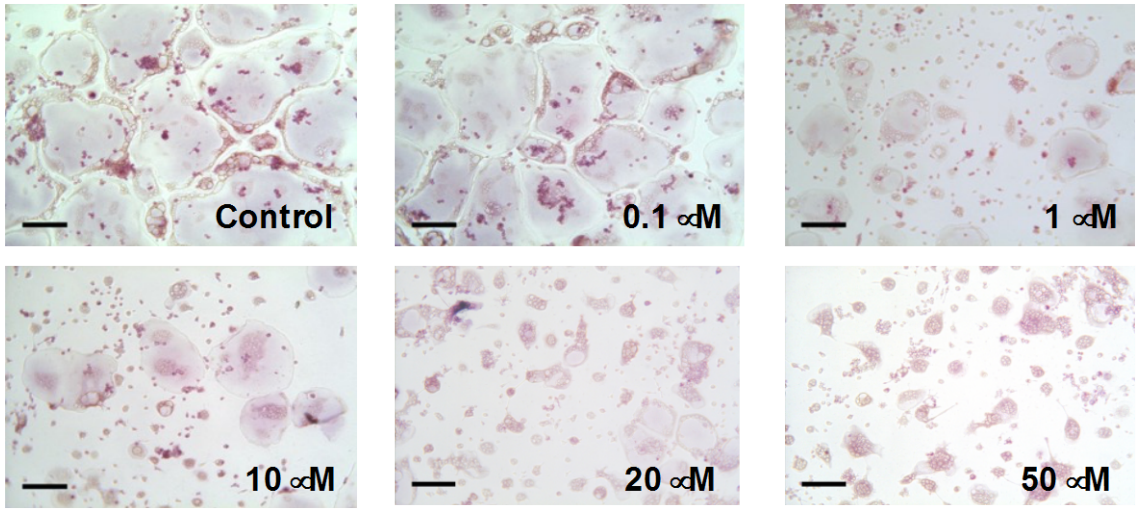
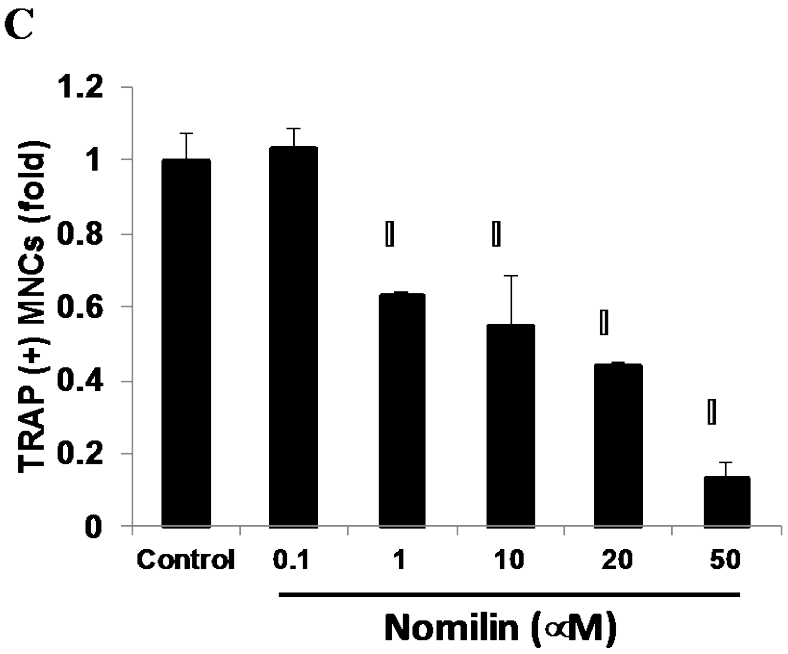

D
Absorbance (450nm)
0.6
0.4
0.2
0
24 h
72 h
Control
0.1
1
10
20
50
Nomilin (∝M)

Figure 1


A
nomilin
(1-(acetyloxy)-1,2-dihydroobacunoic acid e-lactone)


B
Control
0.1 ∝M
1 ∝M
10 ∝M
20 ∝M
50 ∝M


C
TRAP (+) MNCs (fold)
1.2
1
0.8
0.6
0.4
0.2
0
Control
0.1
1
10
20
50
Nomilin (∝M)

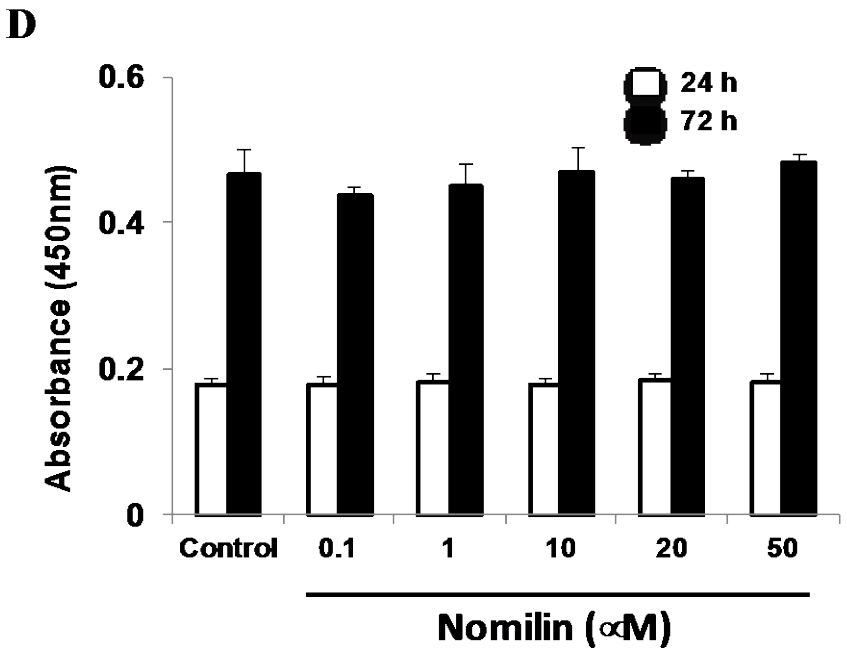

D
Absorbance (450nm)
0.6
0.4
0.2
0
24 h
72 h
Control
0.1
1
10
20
50
Nomilin (∝M)

Figure 2

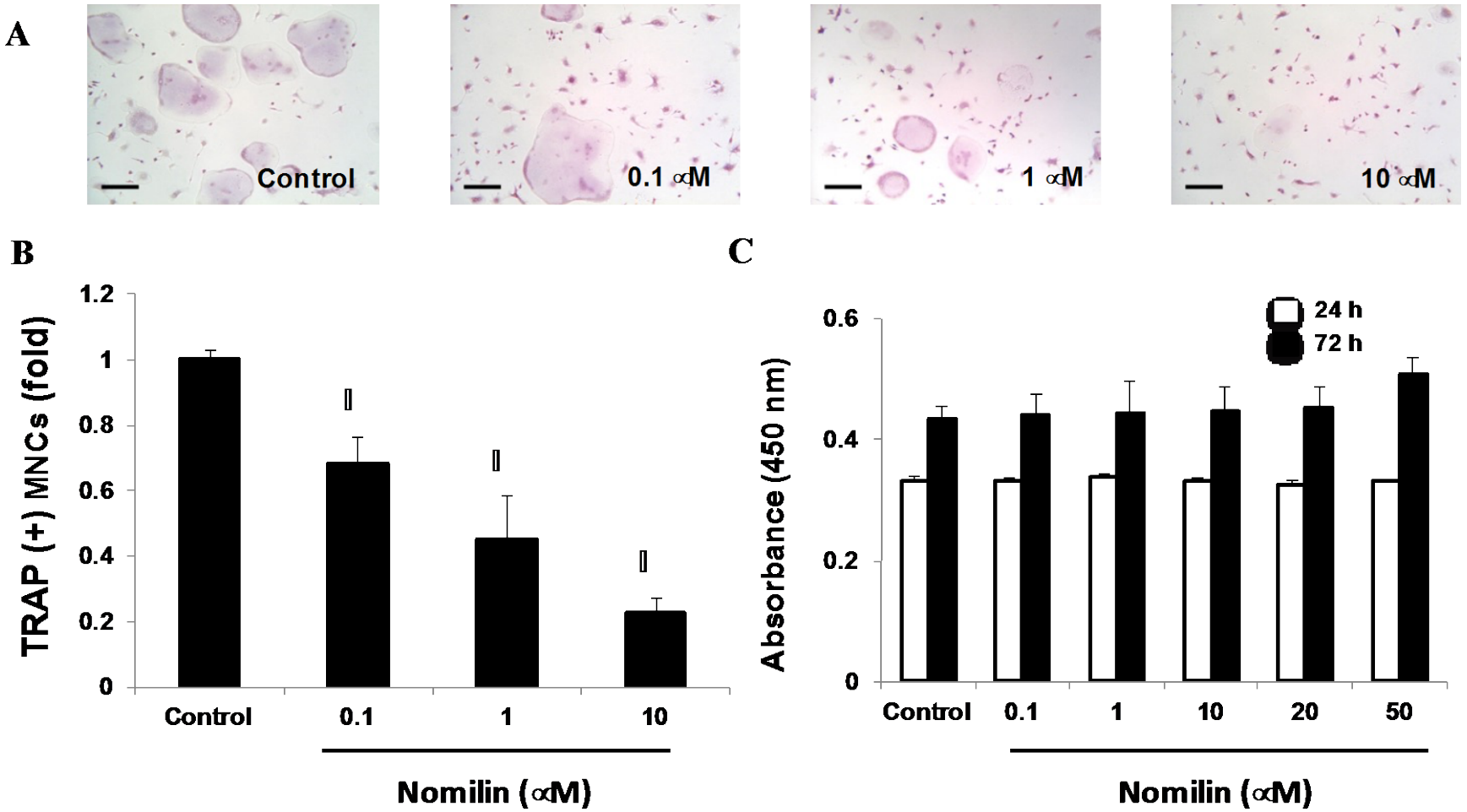

A
Control
0.1 ∝M
1 ∝M
10 ∝M
B
TRAP (+) MNCs (fold)
1.2
1
0.8
0.6
0.4
0.2
0
Control
0.1
1
10
Nomilin (∝M)
C
Absorbance (450 nm)
0.6
0.4
0.2
0
24 h
72 h
Control
0.1
1
10
20
50
Nomilin (∝M)

Figure 3

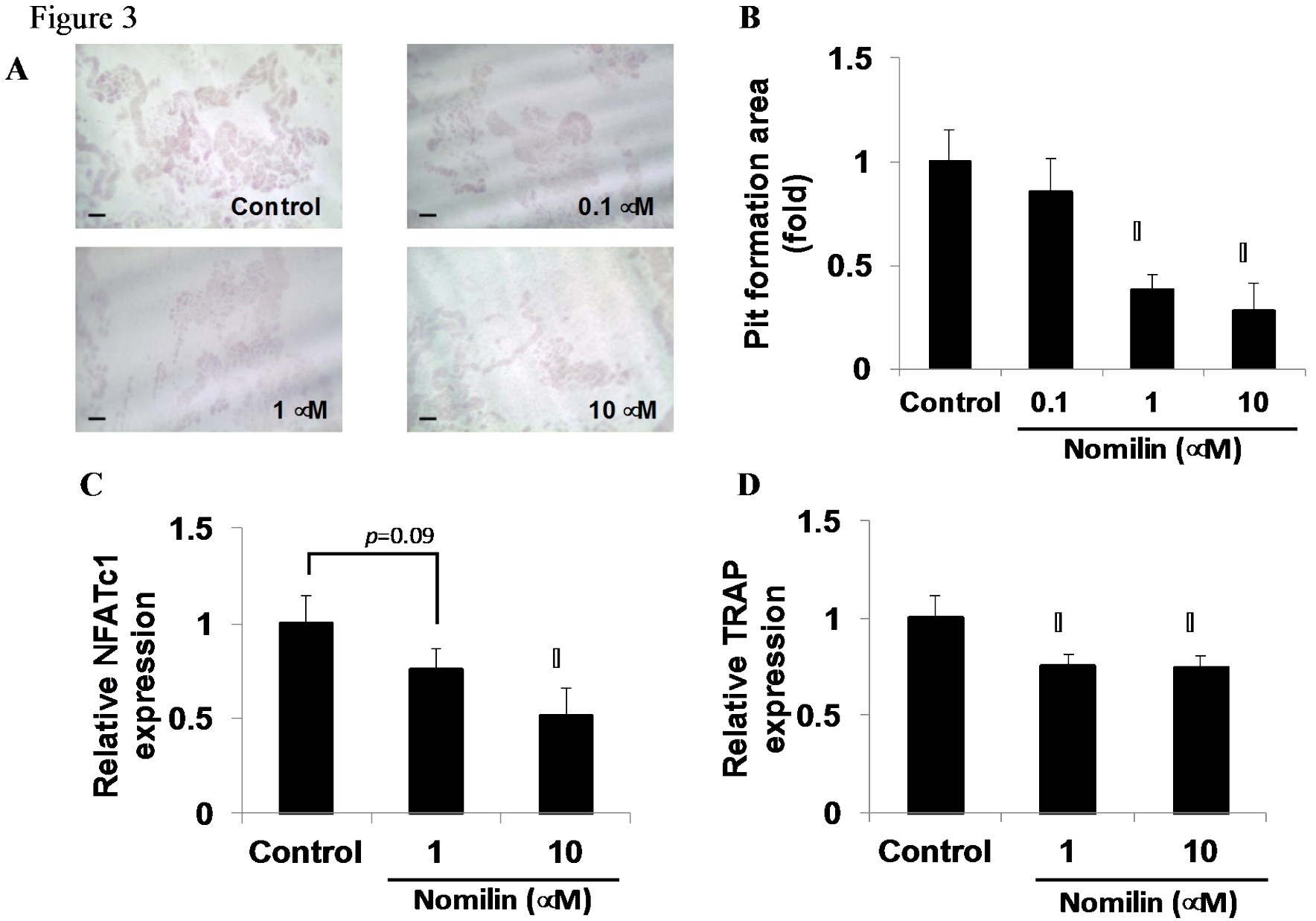
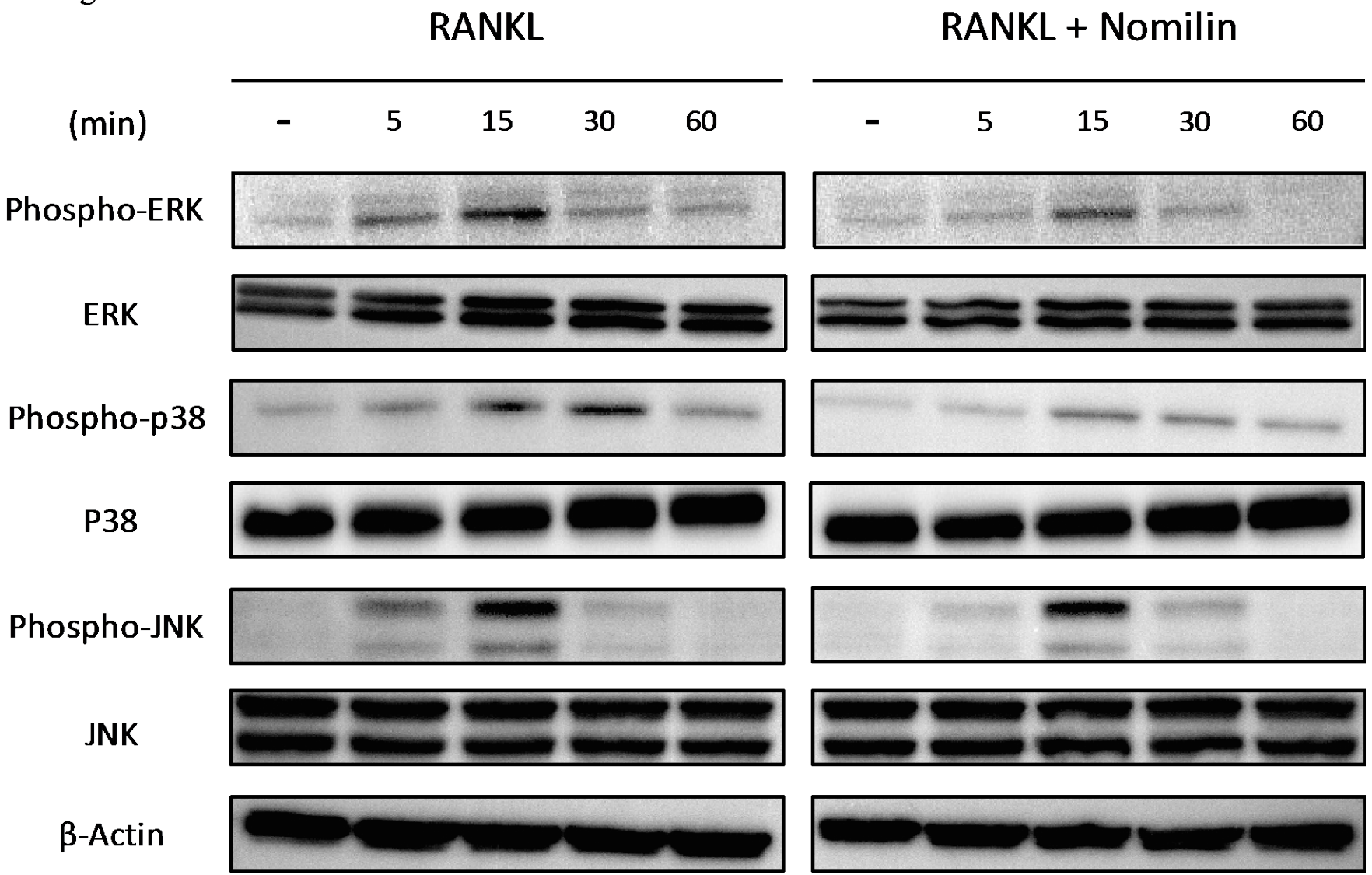

A
Control
0.1 ∝M
1 ∝M
10 ∝M
B
Pit formation area (fold)
1.5
1
0.5
0
Control
0.1
1
10
Nomilin (∝M)
C
Relative NFATc1 expression
p=0.09
1.5
1
0.5
0
Control
1
10
Nomilin (∝M)
D
Relative TRAP expression
1.5
1
0.5
0
Control
1
10
Nomilin (∝M)

Figure 4


RANKL
RANKL + Nomilin
(min)
-
5
15
30
60
-
5
15
30
60
Phospho-ERK
ERK
Phospho-p38
P38
Phospho-JNK
JNK
β-Actin