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Tetrandrine Prevents Bone Loss in Sciatic-Neurectomized Mice and Inhibits Receptor Activator of Nuclear Factor κB Ligand-Induced Osteoclast Differentiation

Tatsuo Takahashi,[a] Yusuke Tonami,[a] Mami Tachibana,[a] Masaaki Nomura,[b] Tsutomu Shimada,[c] Masaki Aburada,[c] and Shinjiro Kobayashi*,[a]

[a] *Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Hokuriku University;* [b] *Educational Center of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Hokuriku University; Kanazawa 920–1181, Japan: and* [c] *Research Institute of Pharmaceutical Sciences, Musashino University; Tokyo 202–8585, Japan.*
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One of the mediators of osteoclast differentiation is receptor activator of nuclear factor κB ligand (RANKL), which is produced by osteoblasts. Binding of RANKL to its receptor, RANK, activates several signaling pathways, including those involving mitogen-activated protein kinases (MAPKs), nuclear factor κB (NF-κB), nuclear factor of activated T cells c1 (NFATc1) and Ca^{2+}-calcineurin. In the present study, we found that tetrandrine, a bisbenzylisoquinoline alkaloid extracted from the root of *Stephania tetrandra* S. Moore, significantly ameliorated the decrease of bone mass in sciatic-neurectomized osteoporosis model mice. It appears that tetrandrine acts directly on osteoclast precursors, since tetrandrine inhibited osteoclast differentiation not only in mouse bone marrow cells, but also in monocultures of murine macrophage RAW 264.7 cells without osteoblasts. Tetrandrine suppressed RANKL-induced amplification of NFATc1, a master regulator of osteoclast differentiation. However, it did not affect other signaling molecules such as MAPKs and NF-κB. These results suggest that tetrandrine is a candidate for the treatment of bone-destructive diseases, or at least a suitable lead compound for further development.

Key words tetrandrine; osteoporosis; osteoclast; receptor activator of nuclear factor κB ligand; nuclear factor of activated T cell c1

Bone remodeling is a physiological process that involves resorption of bone by osteoclasts and synthesis of bone matrix by osteoblasts.[1] A relative increase of bone resorption over bone formation can result in osteoporosis, one of the most prevalent diseases in the aged population.[2,3] Osteoclasts are known to be formed by the fusion of hematopoietic cells of the monocyte-macrophage lineage during the early stage of the differentiation process.[4] Terminal differentiation in this lineage is characterized by acquisition of mature phenotypic markers, such as expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, matrix metalloproteinase 9 (MMP9), and cathepsin K, as well as morphological conversion into large multinucleated cells and formation of resorption lacunae on bone.[5–7]

The essential signaling molecules for osteoclast differentiation include receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), and both of them are produced by osteoblasts.[8] RANKL induces the signaling essential for precursor cells to differentiate into osteoclasts,[9] whereas M-CSF provides the survival signal for these cells.[10] RANKL binding to RANK, a receptor of RANKL that is expressed in osteoclast precursors, mediates the biological effects of RANKL and leads to the recruitment of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which in turn results in the activation of the downstream signaling pathway, including nuclear factor κB (NF-κB), c-Jun N-terminal protein kinase (JNK), p38, extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase/AKT.[11–14] RANKL activation of JNK phosphorylates the transcription factor c-Jun,[15] which forms activator protein-1 (AP-1) complex with c-Fos, an essential transcription factor for osteoclast differentiation.[16] Genetic disruption experiments have revealed that mice lacking both the p50 and p52 NF-κB subunits develop osteopetrosis owing to the arrested generation of osteoclasts.[17,18] Thus, NF-κB genes play an indispensable role in regulation of the differentiation and function of osteoclasts. It has been shown that NF-κB functions upstream of c-Fos expression during RANKL-induced osteoclastogenesis. RANKL-induced c-Fos expression is abolished in NF-κB p50/p52 double-knockout osteoclast precursors, and RANKL can induce osteoclast formation from NF-κB p50/p52 double-knockout osteoclast precursors when c-Fos is overexpressed.[19] Nuclear factor of activated T cells c1 (NFATc1), which also plays an important role in osteoclastogenesis, is upregulated by RANKL in osteoclast precursors through mechanisms that are dependent on NF-κB and c-Fos.[8,19,20] Furthermore, overexpression of NFATc1 in osteoclast precursors causes efficient induction of osteoclast formation even without RANKL stimulation.[20] These discoveries imply that NFATc1 may be a master transcription factor for osteoclast differentiation.

As bone and the immune system are closely intermingled, all factors that regulate immune cells should be investigated for their effect on bone. For this reason, treatment strategies for bone disease focus on the suppression of bone destruction and inflammation-associated bone loss. Bone-resorbing osteoclasts are important effector cells in diseases involving inflammation-induced bone loss, such as rheumatoid arthritis or periodontitis.[21,22] Inflammatory cytokines and prostaglandins upregulate RANKL in osteoblasts, synovial fibroblasts and activated T cells.[23,24] RANK-RANKL signaling was shown to be essential for osteoclast differentiation in inflammatory bone destruction.[25] In addition, many cytokines affected by inflammation, including the proinflammatory cytokines

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: s-kobayashi@hokuriku-u.ac.jp
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TNF-α and interleukin-1, may contribute to osteoclast differentiation and activation.[26)]

Tetrandrine, a bisbenzylisoquinoline alkaloid extracted from the root of *Stephania tetrandra* S. MOORE, has been widely used in China to treat patients with autoimmune disorders, inflammatory pulmonary diseases and cardiovascular diseases.[27–31)] It possesses a remarkable pharmacological profile, including immunosuppressive, anti-inflammatory and anti-tumor activities.[32–35)] In resting cells, an inhibitory protein, inhibitor of NF-κBα (IκBα), masks the nuclear localization signal of NF-κB, which is consequently retained in the cytosol. Stimulation results in the phosphorylation of IκBα by IκBα kinases (IKKs), resulting in ubiquitination and subsequent degradation of IκBα by proteosome.[36,37)] Recent studies have demonstrated that tetrandrine can inhibit NF-κB activation by suppression of IκBα degradation, and consequently it inhibits the production of proinflammatory cytokines.[34,38–41)] Therefore, we examined the anti-osteoporotic and anti-osteoclastogenic effects of tetrandrine. In the present study, we demonstrate for the first time that tetrandrine significantly ameliorates the decrease of bone mass in sciatic-neurectomized mice by inhibiting osteoclast differentiation. Mechanistic studies in murine macrophage RAW 264.7 cells indicated that tetrandrine had no effect on NF-κB activation, but suppressed RANKL-induced amplification of NFATc1.

MATERIALS AND METHODS

Mice and Reagents Seven- and 12-week-old male ddY mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed in accordance with the guidelines of the Committee on Animal Experiments in Hokuriku University.

The chemical structure of tetrandrine is shown in Fig. 1. Mouse monocyte/macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manasas, VA, U.S.A.). Alpha modification of Eagle's medium (α-MEM) was purchased from MP Biomedicals (Solon, OH, U.S.A.) and Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) and Fura Red/AM were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Penicillin/streptomycin was purchased from Lonza (Walkersville, MD, U.S.A.). Tetrandrine, glutamine, Naphthol AS-MX phosphate, Fast blue RR salt, Fast red violet LB salt and Pluronic F-127 were from Sigma (St. Louis, MO, U.S.A.). Ascorbic acid, β-glycerophosphate, dexamethasone, 1α,25-dihydroxyvitamin D_3, paraformaldehyde and soluble RANKL (sRANKL) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Primary antibodies against phospho-IκBα (Ser32), IκBα, phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK1/2/3 (Thr183/Tyr185), JNK1/2/3, phospho-p38 (Thr180/Tyr182), p38, phospho-c-Jun (Ser73) and c-Jun were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-c-Fos and anti-NFATc1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was obtained from Abcam (Cambridge, U.K.).

***In Vivo* Experiments Using Sciatic-Neurectomized Mice** Twelve-week-old mice were divided into four sciatic-neurectomized groups and one sham-operated group, each consisting of 6 mice. The mice were housed in a room maintained at

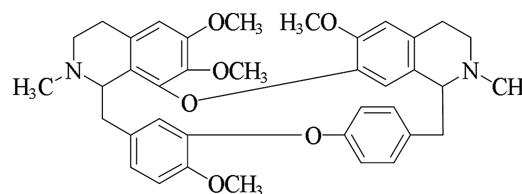

Fig. 1. Chemical Structure of Tetrandrine

24.5±0.5°C on a 12:12-h light–dark cycle and allowed free access to food and water. For the sciatic neurectomy procedure, the dorsal skin of the left thigh was cut and the posterior muscles were divided to expose the sciatic nerve. Chronic denervation was produced by excision of a 5 mm length of the sciatic nerve.[42)] Sciatic-neurectomized mice were injected intraperitoneally with tetrandrine (0.6, 2.0 or 6.0 mg/kg body weight) or phosphate-buffered saline (PBS; control) at 4 weeks after neurectomy. Tetrandrine or PBS was injected every other day for 4 weeks. All mice were sacrificed at 8 weeks post-neurectomy. The left femurs were removed and fixed in 70% ethanol for evaluation of the bone mineral density, bone volume (trabecular and cortical bone volume), trabecular volume, cortical volume, and cortical thickness. The femurs (between proximal and distal epiphysis) were scanned at 0.5 mm-intervals using an experimental X-ray CT instrument (LaTheta, Hitachi Aloka Medical, Tokyo, Japan). The trabecular bone density (Tb.V/TV; amount of trabecular bone volume per unit of tissue volume) was measured to asseses the trabecular bone microstructure of the femurs. Serum samples in all mice were collected before sacrifice, and alkaline phosphatase (ALP) and TRAP isoform 5b (TRAP5b) activities were determined (ALP and TRAP5b serve as markers of osteoblast activity and osteoclast activity, respectively). A 50 μL of volume of serum sample was combined with 250 μL of 10 mM *p*-nitrophenyl phosphate as a substrate in 1 M diethanolamine, pH 9.8, containing 1 mM magnesium chloride and 0.02 mM zinc chloride, and incubated at 37°C. The time-dependent increase in absorbance at 450 nm (reflecting *p*-nitrophenolate production) was measured on a plate spectrophotometer (Sunrise, TECAN, Austria). One unit of ALP activity was defined as the quantity of enzyme that catalyzed the hydrolysis of 1 μmol substrate in 1 min. Serum TRAP5b activity was measured using a mouse TRAP assay kit (Immunodiagnostic Systems, Boldon, U.K.) according to the manufacturer's instructions.

Mouse Bone Marrow Cell Culture Femurs and tibiae were excised from 7- to 9-week-old mice, and the connective and soft tissues were aseptically removed. The ends of the bones were cut off, and the bone marrow was flushed out into a 50 mL tube with α-MEM containing 10% heat-inactivated FBS and 1% penicillin/streptomycin (50 U/mL of penicillin and 50 μg/mL of streptomycin), using a 1 mL syringe. After centrifugation of the cell suspension at 1000 rpm for 5 min, the supernatant was removed, and the cells were resuspended in the medium to make 7×10^6 cells/mL. A half milliliter of the suspension was seeded into a 24-well plate. At 24 h after the seeding, the medium was changed to fresh medium supplemented with 0.2 mM ascorbic acid, 2.5 mM β-glycerophosphate and 10 nM dexamethasone for osteoblast differentiation, or supplemented with 10 nM 1α,25-dihydroxyvitamin D_3 for osteoclast differentiation. The cells were further cultured in the presence or absence of tetrandrine for 5 d; the medium was

replaced with fresh medium after 3 d. The cells were fixed with 4% paraformaldehyde in phosphate-buffered solution (pH 7.4) and stained for ALP or TRAP as described below.

Mouse Monocyte/Macrophage Cell Culture RAW 264.7 cells can differentiate into osteoclasts after RANKL treatment *in vitro*.[43] For osteoclastic differentiation, RAW 264.7 cells were suspended in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin and 2 mM glutamine, and then seeded at 2×10^3 cells/well in 96-well plate. At 24 h after the seeding, the medium was changed to fresh medium supplemented with 100 ng/mL of sRANKL. The cells were further cultured in the presence or absence of tetrandrine for 5 d; the medium was replaced with fresh medium after 3 d. The cells were fixed with 4% paraformaldehyde in phosphate-buffered solution (pH 7.4) and stained for TRAP as described below.

ALP Staining After cell culture, the fixed cells were incubated in 50 mM $MgCl_2$ and 0.1 M Tris–HCl (pH 7.4) for 30 min. Cells were then stained for ALP with a mixture of 0.1 mg/mL Naphthol AS-MX phosphate and 0.6 mg/mL Fast blue RR salt in 0.2 M Tris–HCl (pH 8.5). Cells were washed with distilled water and ALP-positive cell nodules were quantified using NIH Image software.

TRAP Staining After cell culture, the fixed cells were incubated in a mixture of ethanol and acetone (1 : 1, v/v) for 1 min. Cells were then stained for TRAP with a mixture of 0.1 mg/mL Naphthol AS-MX phosphate and 0.6 mg/mL Fast red violet LB salt in 50 mM sodium tartrate/0.1 M sodium acetate buffer (pH 5.0). Cells were washed with distilled water and TRAP-positive multinucleated cells (three or more nuclei) were counted under a microscope (Olympus, Tokyo, Japan).

Western Blot Analysis Cells were washed 3 times with ice-cold PBS and lysed in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 μg/mL aprotinin, 5 μg/mL leupeptin, 1 μg/mL microcystin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration of the cell lysate was determined by using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). An equal amount of protein (6 μg/lane) was resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was probed with the indicated primary antibodies. Blots were finally developed with horseradish peroxidase-conjugated secondary antibodies and visualized with ECL Plus kit (GE Healthcare, Buckinghamshire, U.K.).

Statistical Analysis Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer *post hoc* test. Differences were considered to be significant at $p<0.05$.

RESULTS

Preventive Effect of Tetrandrine against Bone Loss in Sciatic-Neurectomized Mice In the present animal model of hind limb immobilization by sciatic neurectomy, an imbalance between bone formation and resorption leads to bone loss with morphometric changes.[44] We first evaluated the effect of tetrandrine on bone loss of femurs in sciatic-neurectomized mice. At 8 weeks after neurectomy, the neurectomized mice displayed profound decreases in bone mineral density and bone volume compared with the sham-operated group (Figs. 2A–C). The decrease in bone volume was accompanied with reductions in both trabecular volume and cortical volume, though cortical thickness was not altered (Figs. 2D, E, G). The bone loss observed in the neurectomized mice was significantly ameliorated by tetrandrine. In particular, tetrandrine showed a remarkable protective effect in terms of the trabecular bone volume. The decrease of Tb.V/TV in neurectomized mice was significantly reversed by tetrandrine at 0.6 and 2.0 mg/kg (Fig. 2F). Serum TRAP5b activity increased in the neurectomized mice, and tetrandrine was significantly ameliorated the increased serum TRAP5b activity. In contrast, serum ALP activity was lower in the neurectomized mice than the sham-operated mice, and tetrandrine had no effect on the lowered ALP activity (Fig. 3). It was suggested that tetrandrine ameliorated the loss of bone volume in the neurectomized mice possibly by inhibiting osteoclastic bone resorption, but not enhancing osteoblastic bone formation.

Inhibitory Effect of Tetrandrine on Osteoclast Differentiation in Bone Marrow Cell Culture To investigate the mechanisms through which tetrandrine reduced bone loss, the effects of tetrandrine on osteoblast and osteoclast differentiation were examined in bone marrow cell culture. In the absence of tetrandrine, bone marrow cells differentiated into TRAP-positive multinucleated osteoclasts, but tetrandrine inhibited the differentiation into osteoclasts in a concentration-dependent manner (Figs. 4A, C). In contrast, tetrandrine had no apparent effect on osteoblast differentiation, at least up to 1 μM (Figs. 4A, B).

Inhibitory Effect of Tetrandrine on RANKL-Induced Osteoclast Differentiation It is possible that tetrandrine affects RANKL-induced signaling in osteoclast precursor cells, since osteoblasts support osteoclastogenesis from osteoclast precursor cells by expressing RANKL.[45] Therefore, we next examined the effects of tetrandrine on RANKL-induced osteoclast differentiation from osteoclast precursor RAW 264.7 cells. In the presence of sRANKL (100 ng/mL), RAW 264.7 cells differentiated into TRAP-positive multinucleated osteoclasts. Tetrandrine dose-dependently inhibited the sRANKL-induced differentiation of both RAW 264.7 cells and bone marrow cells into osteoclasts when present in the medium throughout the entire culture period (Fig. 5A). To explore the inhibitory effect of tetrandrine on osteoclast differentiation in more detail, we added tetrandrine at different time periods to sRANKL-treated RAW 264.7 cells in culture (Fig. 5B). Tetrandrine exposure during days 0–3 after sRANKL treatment, followed by wash-out and further culture in tetrandrine-free medium, resulted in inhibition of osteoclast differentiation. However, the inhibitory effect was decreased when tetrandrine was added at days 3–5. These findings suggest that tetrandrine inhibits early RANKL-induced cellular events. To exclude the possibility that the inhibition was due to cytotoxicity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out. Tetrandrine was confirmed to show little cytotoxicity to RAW 264.7 cells at the concentration at which it effectively inhibited osteoclast differentiation (Fig. 5C).

Effects of Tetrandrine on Phosphorylation of MAPKs, Degradation of IκB and c-Fos Expression in RANKL-Stimulated RAW 264.7 Cells A key signaling event induced by the binding of RANKL to RANK is the activation of MAPKs

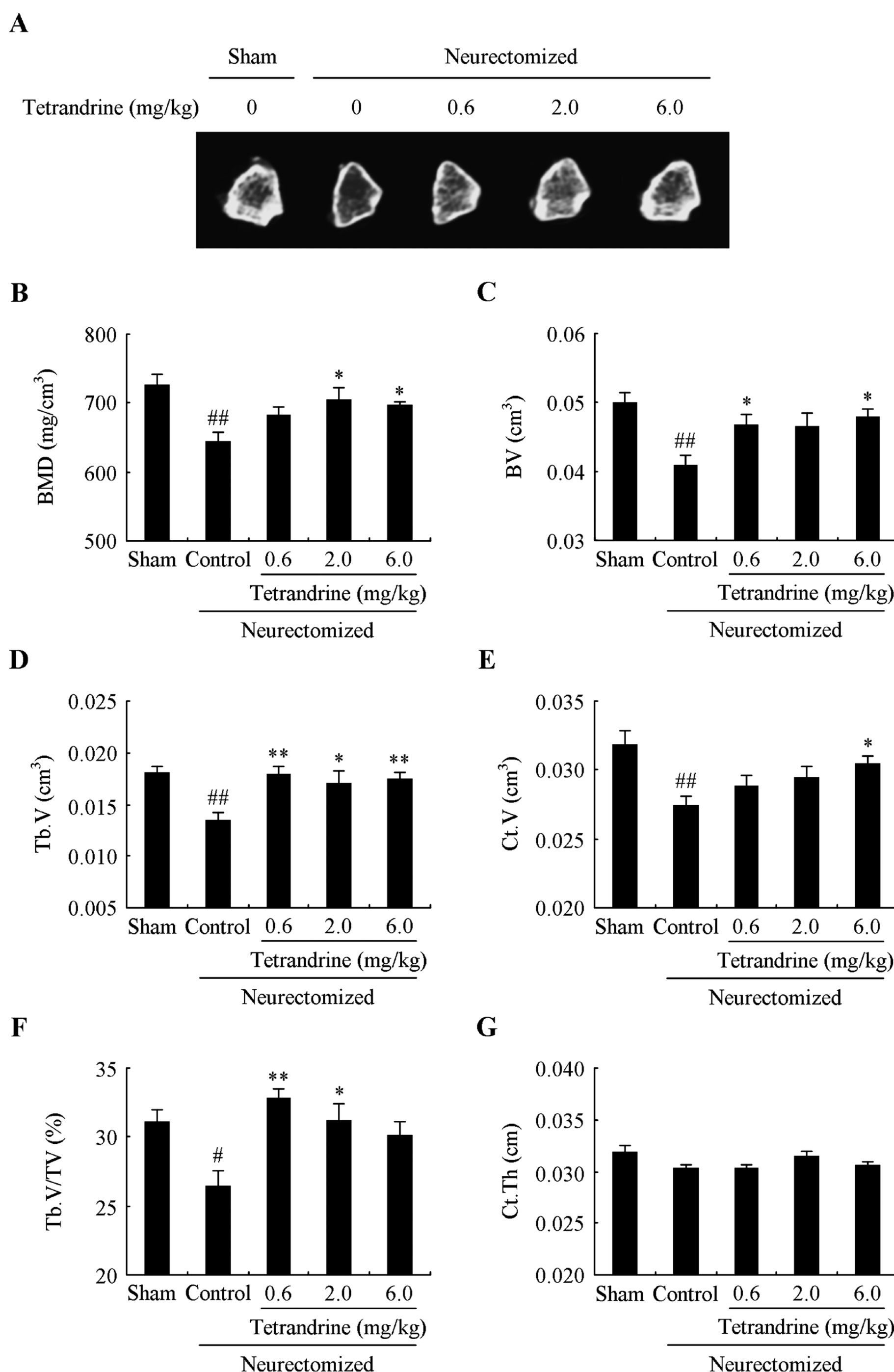

Fig. 2. Tetrandrine Ameliorated Bone Loss in Sciatic-Neurectomized Mice

Femurs of sciatic-neurectomized mice injected with PBS or tetrandrine (0.6, 2.0 or 6.0 mg/kg) and sham-operated mice were subjected to bone morphometric examination, including transverse section at 2 mm from the distal end (A), bone mineral density (BMD, B), bone volume (BV, C), trabecular volume (Tb.V, D), cortical volume (Ct.V, E), trabecular bone density (Tb.V/TV, F) and cortical thickness (Ct.Th, G). The results are expressed as means±S.E. of 6 mice. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *versus* sham-operated mice; $^{*}p<0.05$, $^{**}p<0.01$ *versus* PBS-injected control mice.

and NF-κB signaling. To elucidate the inhibitory mechanism and to identify the signaling pathway influenced by tetrandrine, RAW 264.7 cells were treated with sRANKL in the presence or absence of tetrandrine. The phosphorylation of three families of MAPKs, ERK, JNK and p38, as well as c-Jun, reached a maximal level within 15 min and then returned to the basal level in response to sRANKL. Tetrandrine did not affect the phosphorylation of MAPKs or c-Jun (Fig. 6A).

Activation of NF-κB involves initial phosphorylation and subsequent degradation of the inhibitory subunit IκB in response to an extracellular signal. The freed NF-κB is then translocated to the nucleus to bind target gene promoters. To examine whether NF-κB could be a target of tetrandrine, the protein level and phosphorylation of IκB were determined by Western blot analysis. The phosphorylation and the subsequent degradation of IκB were found to be activated in RAW 264.7 cells within 30 min after sRANKL treatment, and tetrandrine

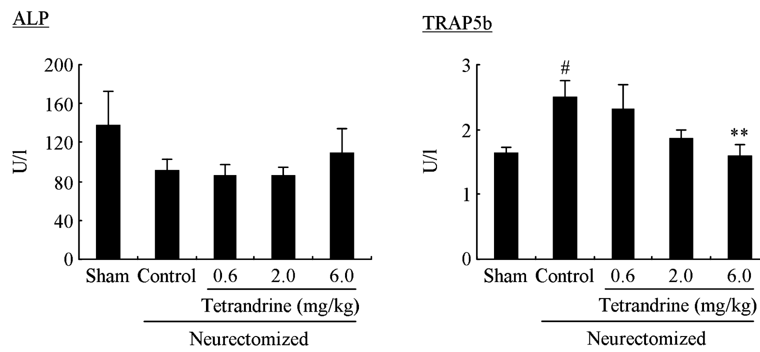

Fig. 3. Tetrandrine Attenuated Serum TRAP5b Activity Increased in Sciatic-Neurectomized Mice

Serum activities of ALP and TRAP5b of sciatic-neurectomized mice injected with PBS or tetrandrine (0.6, 2.0 or 6.0 mg/kg) and sham-operated mice were measured. The results are expressed as means±S.E. of 6 mice. $^{\#}p<0.05$ *versus* sham-operated mice; $^{**}p<0.01$ *versus* PBS-injected control mice.

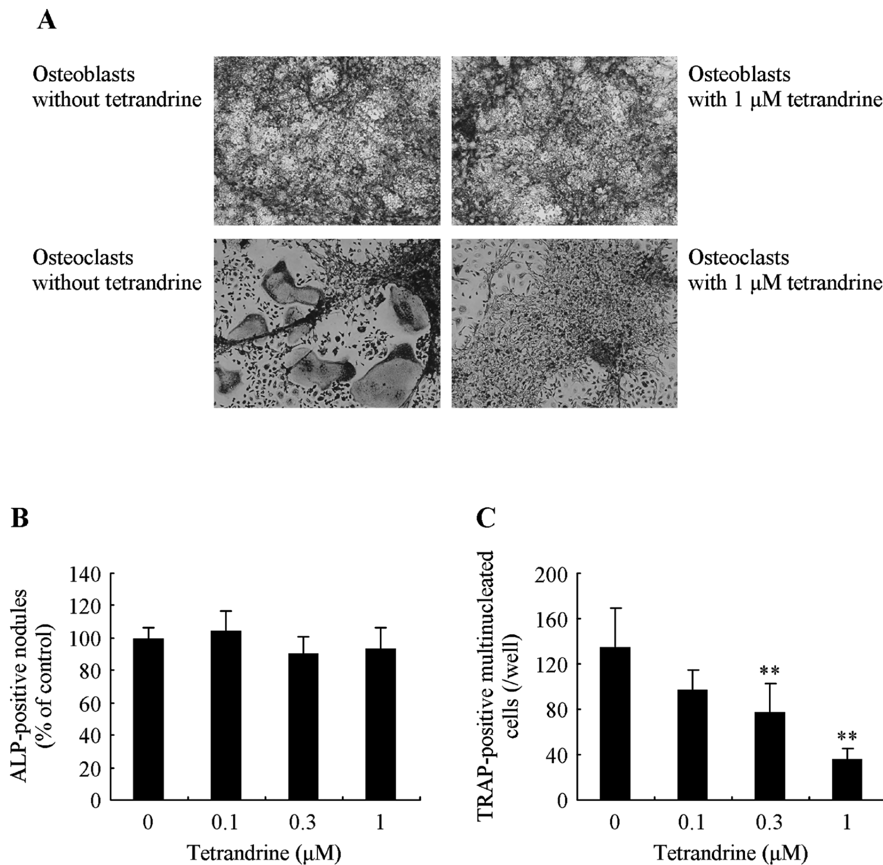

Fig. 4. Tetrandrine Inhibited Osteoclast Differentiation in Bone Marrow Cell Culture

Bone marrow cells were cultured with the indicated concentration of tetrandrine in the presence of 0.2 mM ascorbic acid, 2.5 mM β-glycerophosphate and 10 nM dexamethasone for osteoblast differentiation, or in the presence of 10 nM 1α,25-dihydroxyvitamin D_3 for osteoclast differentiation. After 5 d, cells were stained for ALP or TRAP, which are markers of osteoblasts and osteoclasts, respectively (A). ALP-positive cell nodules were quantified, and TRAP-positive multinucleated cells were counted (B). The results are expressed as means±S.D. of 6 cultures. $^{**}p<0.01$ *versus* control culture.

did not alter the RANKL-induced IκB phosphorylation or degradation (Fig. 6A). c-Fos is thought to be downstream from NF-κB and plays an important role in osteoclast differentiation by forming AP-1 complexes with c-Jun. We confirmed that sRANKL increased the protein level of c-Fos at 6 h in RAW 264.7 cells, and found that tetrandrine did not affect the expression of c-Fos (Fig. 6B).

Effect of Tetrandrine on Expression of NFATc1 in RANKL-Stimulated RAW 264.7 Cells Importantly, RANKL specifically and strongly induces NFATc1, a master regulator of osteoclast differentiation, and this induction is dependent on NF-κB and c-Fos, followed by auto-amplification of NFATc1. We next investigated whether tetrandrine reduced the expression of NFATc1 in RAW 264.7 cells. Protein expression of NFATc1 increased from 24 to 72 h in sRANKL-treated cells, but was not detected in the untreated control (Fig. 7A). Tetrandrine had little effect on the initial induction of NFATc1 by sRANKL. However, the subsequent increase of NFATc1, which is attributed to auto-amplification of NFATc1, was reduced by one-half in tetrandrine-treated cells (Fig. 7B). These

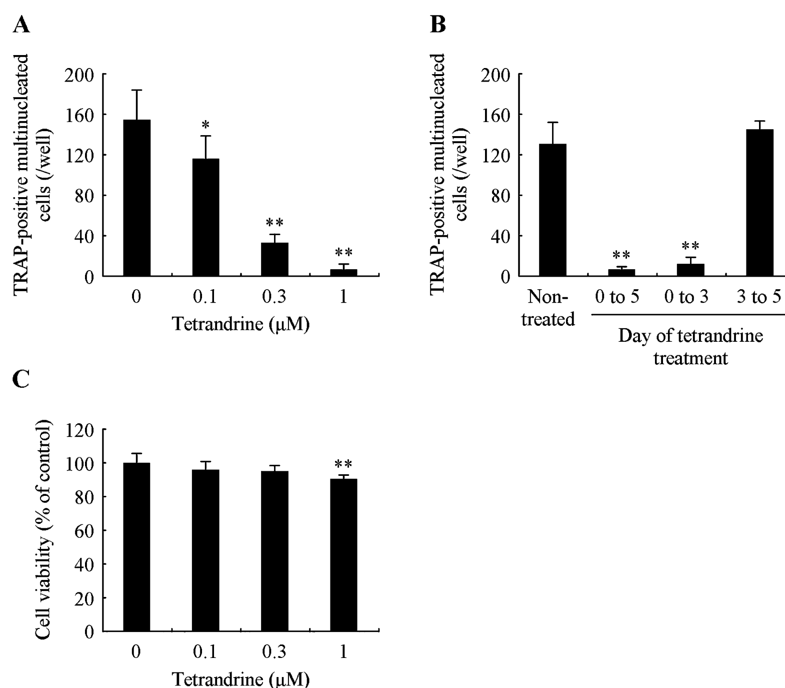

Fig. 5. Tetrandrine Inhibited Early Cellular Events Required for Osteoclast Differentiation in RAW 264.7 Cell Culture

RAW 264.7 cells were cultured with the indicated concentration of tetrandrine in the presence of 100 ng/mL sRANKL. After 5 d, TRAP-positive multinucleated cells were counted as osteoclasts (A). Tetrandrine (1 μM) was added to sRANKL-treated RAW 264.7 cell culture for the indicated time periods during the 5-d culture period (B). RAW 264.7 cells were cultured in the presence of the indicated concentration of tetrandrine, and treated with 250 μg/mL MTT for 1 h. The precipitated dye was solubilized in dimethylsulfoxide, and the absorbance at 570 nm was measured. The results are expressed as means±S.D. of 6 cultures. * $p<0.05$, ** $p<0.01$ *versus* control culture.

results suggest that the inhibitory effect of tetrandrine on osteoclast differentiation might be due to inhibition of NFATc1 auto-amplification, but not inhibition of initial induction of NFATc1.

DISCUSSION

Tetrandrine was discovered as a potent blocker of the reduction of bone volume in sciatic-neurectomized mice during a natural products screening program. It was found to inhibit osteoclast differentiation, while not affecting osteoblast differentiation or cell viability. Furthermore, it was a potent inhibitor when added at the early period of culture, but not when added later, suggesting that it acts on early events in osteoclast differentiation. Tetrandrine did not affect early RANKL-induced signaling pathways, including the phosphorylation of MAPKs and activation of NF-κB caused by RANKL-RANK interaction on osteoclast precursors. Consistently with this, c-Fos upregulation attributed to NF-κB activation was not also affected by tetrandrine.

Osteoclast differentiation and functions are reported to be mediated by RANKL-induced NF-κB activation.[46] RANKL stimulation triggers the formation of the RANK-TRAF6 complex, which leads to IKK activation and consequent degradation of IκBα. NFATc1 is one of the key target genes of NF-κB in the early phase of osteoclast differentiation.[8,47] NF-κB activates the NFATc1 promoter cooperatively with NFATc2, a member of the NFAT family of transcription factors, within minutes of RANKL stimulation.[48] As our results indicated that NF-κB is not a molecular target of tetrandrine, because IκBα degradation, c-Fos up-regulation and initial induction of NFATc1 were not inhibited by tetrandrine, we next focused on NFATc1, which directly regulates a number of osteoclast-specific genes, such as TRAP, calcitonin receptor, cathepsin K and β3 integrin.

Robust expression of NFATc1, attributed to auto-amplification, is important for osteoclast differentiation.[49,50] Our results indicated that, although tetrandrine had little effect on the initial induction of NFATc1 by sRANKL, it did inhibit the subsequent auto-amplification of NFATc1. All NFAT family members, including NFATc1, are mainly regulated by the serine/threonine phosphatase calcineurin, which is activated by intracellular Ca^{2+}. Dephosphorylation of the serine residues in NFATs by calcineurin leads to exposure of their nuclear-localization signal and translocation into the nucleus. In osteoclasts, NFATc1 undergoes efficient nuclear translocation in response to RANKL stimulation, suggesting that the Ca^{2+}-calcineurin signal is activated.[8] After RANKL stimulation, phospholipase Cγ (PLCγ) is activated and produces inositol-1,4,5-trisphosphate (IP_3), which evokes Ca^{2+} release from endoplasmic reticulum (ER) possibly through IP_3 receptor (IP_3R) 2 and IP_3R3, followed by long-lasting Ca^{2+} oscillation.[51] In addition, several studies have indicated that extracellular Ca^{2+} influx appeared to be necessary RANKL-induced osteoclast differentiation.[52,53] It is noteworthy that in most cell types, activation of receptors coupled to PLC by high concentrations of agonists triggers Ca^{2+} release from the ER followed by Ca^{2+} influx through store-operated Ca^{2+} (SOC) entry.[54,55] Lower and more physiological concentrations of receptor agonists induce repetitive Ca^{2+} oscillations, similar to those seen in RANKL-mediated osteoclast differentiation.[56,57] SOC entry is necessary to refill the store in order to maintain Ca^{2+} oscillations, which run down in the absence of SOC entry.[58] Indeed, a SOC channel blocker, Gd^{3+}, abolished RANKL-induced

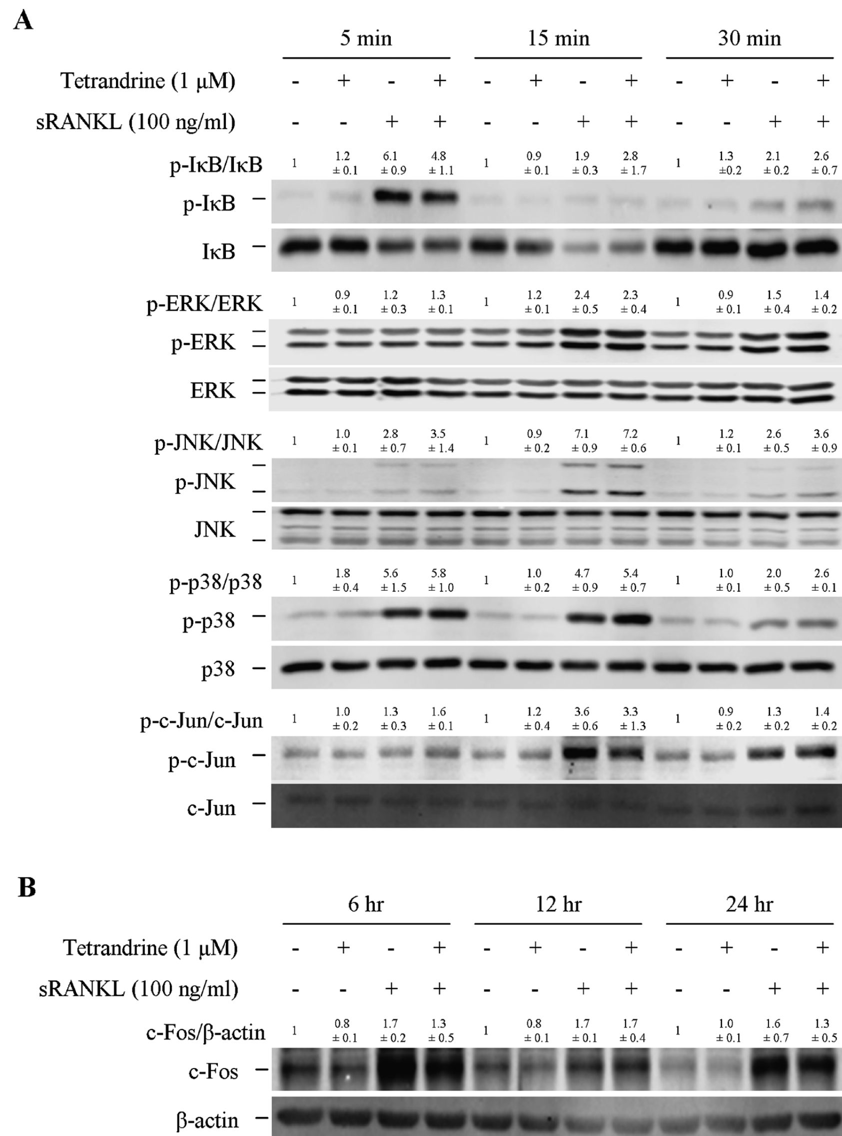

Fig. 6. Tetrandrine Had No Effect on RANKL-Induced Phosphorylation of IκB, MAPKs and c-Jun, or on the Expression of c-Fos

RAW 264.7 cells were pretreated with 1 μM tetrandrine for 1 h, and stimulated with 100 ng/mL sRANKL for 5 to 30 min to determine the phosphorylation of IκB, MAPKs and c-Jun (A), or for 6 to 24 h to determine the c-Fos expression (B). Cell extracts were analyzed by Western blotting using antibodies specifically directed against the phosphorylated and unphosphorylated forms, and against c-Fos protein. The intensities of protein bands were analyzed and normalized to unphosphorylated proteins or β-actin. The results are expressed as means±S.D. of 3 cultures.

Ca^{2+} oscillation.[53] Recent studies have focused on transient receptor potential (TRP) channels as candidates for the channels underlying Ca^{2+} influx in RANKL-induced Ca^{2+} oscillation. The vanilloid TRP4 (TRPV4) channel-deficient mice ($TRPV4^{-/-}$) displayed an increase in bone mass, which was attributed to impaired resorption activity of osteoclasts. The mRNA levels of NFATc1 were attenuated in cultured osteoclasts derived from $TRPV4^{-/-}$ mice, while osteoblast phenotypes were not affected, suggesting TRPV4 solely contributes to the differentiation and function of osteoclasts.[52] On the other hand, voltage-gated Ca^{2+} channels (VOCC) is characterized in plasma membrane and involved in transporting extracellular Ca^{2+} in osteoclasts as well as SOC channels and TRP channels. Augmented Ca^{2+} influx through VOCC resulted from locally increased extracellular Ca^{2+} during bone resorption induces osteoclast retraction and even apoptosis.[59,60] Although tetrandrine is known to be a blocker of VOCC, the blockade of VOCC is not though to contribute to the inhibitory effect on osteoclast differentiation.[61] Moreover, tetrandrine did not inhibit the serine/threonine phosphatase activity of calcineurin (data not shown). Taken together, these findings indicate that Ca^{2+}-calcineurin signaling may not be the molecular target of tetrandrine in osteoclasts. Interestingly, *in vivo* administration of FK506, a calcineurin inhibitor, results in a reduction in bone mass due to severe impairment of bone formation in mice, suggesting that calcineurin is also important for osteoblast function; this implies that inhibition of the Ca^{2+}-calcineurin signal would not be suitable therapeutic target for osteoporosis.[62,63]

It has been shown that NFATc1 forms an osteoclast-specific transcriptional complex containing AP-1, PU.1, MITF and CREB for the effective induction of osteoclast-specific genes and auto-amplification of NFATc1.[8,49,64] In osteoblasts, on the other hand, NFATc1 forms a complex with osterix, a zinc-finger transcription factor, and cooperatively regulates bone formation through induction of type I collagen.[65] These findings

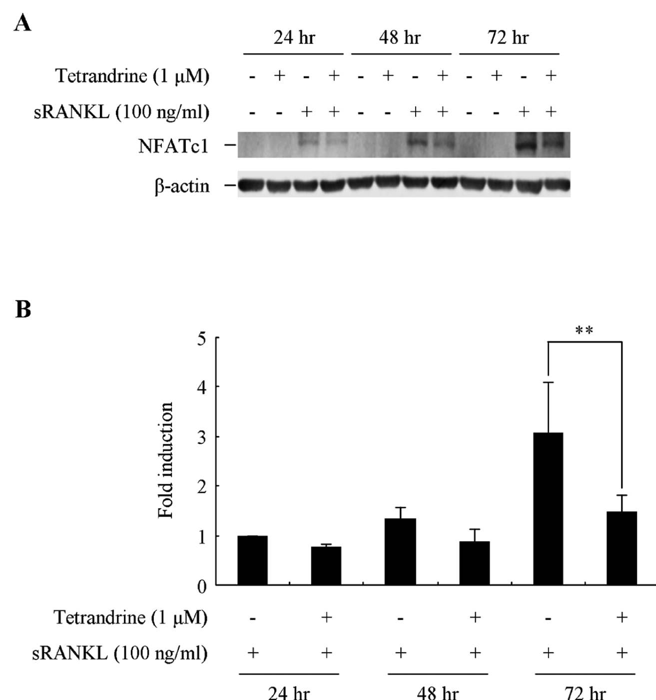

Fig. 7. Tetrandrine Inhibited RANKL-Induced NFATc1 Expression

RAW 264.7 cells were pretreated with 1 μM tetrandrine for 1 h, and stimulated with 100 ng/mL sRANKL for the indicated time periods. Cell extracts were analyzed by Western blotting using antibody directed against NFATc1 protein. Equal amounts of protein were loaded in all lanes as indicated by the level of β-actin (A). Band intensities were determined by densitometry and normalized with β-actin. The graph shows the fold induction of NFATc1 (B). The results are expressed as means±S.D. of 3 cultures. ** $p<0.01$ *versus* RANKL-treated cells.

raise the possibility that tetrandrine may act on the expression and/or function of transcription factors which form complexes with NFATc1 in osteoclasts. Further work will be required to clarify this possibility.

The identification of the RANK-RANKL pathway opened up the possibility of developing novel agents that would reduce osteoclastic bone resorption by inhibiting RANKL. Denosumab is a fully human monoclonal antibody that targets RANKL and prevents RANKL from binding to its receptor, thereby inhibiting the development, activation, and survival of osteoclasts.[66] Since tetrandrine inhibits RANKL-induced osteoclast differentiation, tetrandrine appears to achieve a similar goal as denosumab. Several clinical investigations showed that denosumab administration to the patients with osteoporosis resulted in a rapid increase of bone mineral density and a sustained decrease in bone turnover, characterized by the reductions in biomarkers of both bone resorption and bone formation.[66,67] Although tetrandrine inhibited osteoclast differentiation and decreased a bone resorption marker, TRAP 5b, in sciatic-neurectomized mice as well as denosumab, no evidence that tetrandrine attenuated osteoblast differentiation was observed *in vitro* and *in vivo*. This finding suggests tetrandrine may be worthy of an alternative to RANKL antibody therapy.

In the present study, we have demonstrated, for the first time, that tetrandrine has a preventive effect against bone loss in an animal model of osteoporosis, sciatic-neurectomized mice. We have also shown that tetrandrine inhibits RANKL-induced osteoclast differentiation, at least in part, by suppressing auto-amplification of NFATc1. Thus, tetrandrine is a candidate drug for treatment of diseases involving abnormal bone lysis, such as osteoporosis and rheumatoid arthritis, or at least a potential lead compound for drug development. Further studies, including elucidation of its direct molecular targets, will be needed to understand the effects of tetrandrine.

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REFERENCES

1) Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell*, **2**, 389–406 (2002).
2) Sambrook P, Cooper C. Osteoporosis. *Lancet*, **367**, 2010–2018 (2006).
3) Zaidi M. Skeletal remodeling in health and disease. *Nat. Med.*, **13**, 791–801 (2007).
4) Mohamed SG, Sugiyama E, Shinoda K, Taki H, Hounoki H, Abdel-Aziz HO, Maruyama M, Kobayashi M, Ogawa H, Miyahara T. Interleukin-10 inhibits RANKL-mediated expression of NFATc1 in part *via* suppression of c-Fos and c-Jun in RAW264.7 cells and mouse bone marrow cells. *Bone*, **41**, 592–602 (2007).
5) Reddy SV, Hundley JE, Windle JJ, Alcantara O, Linn R, Leach RJ, Boldt DH, Roodman GD. Characterization of the mouse tartrate-resistant acid phosphatase (TRAP) gene promoter. *J. Bone Miner. Res.*, **10**, 601–606 (1995).
6) Anusaksathien O, Laplace C, Li X, Ren Y, Peng L, Goldring SR, Galson DL. Tissue-specific and ubiquitous promoters direct the expression of alternatively spliced transcripts from the calcitonin receptor gene. *J. Biol. Chem.*, **276**, 22663–22674 (2001).
7) Motyckova G, Weilbaecher KN, Horstmann M, Rieman DJ, Fisher DZ, Fisher DE. Linking osteopetrosis and pycnodysostosis: regulation of cathepsin K expression by the microphthalmia transcription factor family. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 5798–5803 (2001).
8) Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell*, **3**, 889–901 (2002).
9) Theill LE, Boyle WJ, Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu. Rev. Immunol.*, **20**, 795–823 (2002).
10) Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*, **345**, 442–444 (1990).
11) Lee SE, Woo KM, Kim SY, Kim HM, Kwack K, Lee ZH, Kim HH. The phosphatidylinositol 3-kinase, p38, and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation. *Bone*, **30**, 71–77 (2002).
12) Darnay BG, Haridas V, Ni J, Moore PA, Aggarwal BB.

Characterization of the intracellular domain of receptor activator of NF-kappaB (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF-kappaB and c-Jun N-terminal kinase. *J. Biol. Chem.*, **273**, 20551–20555 (1998).

13) Darnay BG, Ni J, Moore PA, Aggarwal BB. Activation of NF-κB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-κB-inducing kinase. Identification of a novel TRAF6 interaction motif. *J. Biol. Chem.*, **274**, 7724–7731 (1999).
14) Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik P, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 1566–1571 (2000).
15) Kobayashi N, Kadono Y, Naito A, Matsumoto K, Yamamoto T, Tanaka S, Inoue J. Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *EMBO J.*, **20**, 1271–1280 (2001).
16) Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, Wagner EF. c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science*, **266**, 443–448 (1994).
17) Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, Leonardi A, Tran T, Boyce BF, Siebenlist U. Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev.*, **11**, 3482–3496 (1997).
18) Iotsova V, Caamaño J, Loy J, Yang Y, Lewin A, Bravo R. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat. Med.*, **3**, 1285–1289 (1997).
19) Yamashita T, Yao Z, Li F, Zhang Q, Badell IR, Schwarz EM, Takeshita S, Wagner EF, Noda M, Matsuo K, Xing L, Boyce BF. NF-kappaB p50 and p52 regulate receptor activator of NF-kappaB ligand (RANKL) and tumor necrosis factor-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. *J. Biol. Chem.*, **282**, 18245–18253 (2007).
20) Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ, Bachler MA, Amano H, Aburatani H, Ishikawa H, Wagner EF. Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. *J. Biol. Chem.*, **279**, 26475–26480 (2004).
21) Jimi E, Aoki K, Saito H, D'Acquisto F, May MJ, Nakamura I, Sudo T, Kojima T, Okamoto F, Fukushima H, Okabe K, Ohya K, Ghosh S. Selective inhibition of NF-κB blocks osteoclastogenesis and prevents inflammatory bone destruction *in vivo*. *Nat. Med.*, **10**, 617–624 (2004).
22) Mino T, Sugiyama E, Taki H, Kuroda A, Yamashita N, Maruyama M, Kobayashi M. Interleukin-1alpha and tumor necrosis factor alpha synergistically stimulate prostaglandin E2-dependent production of interleukin-11 in rheumatoid synovial fibroblasts. *Arthritis Rheum.*, **41**, 2004–2013 (1998).
23) Coon D, Gulati A, Cowan C, He J. The role of cyclooxygenase-2 (COX-2) in inflammatory bone resorption. *J. Endod.*, **33**, 432–436 (2007).
24) Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT, Martin TJ, Suda T. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.*, **103**, 1345–1352 (1999).
25) Anandarajah AP, Schwarz EM. Anti-RANKL therapy for inflammatory bone disorders: Mechanisms and potential clinical applications. *J. Cell. Biochem.*, **97**, 226–232 (2006).
26) Han KY, Yang D, Chang EJ, Lee Y, Huang H, Sung SH, Lee ZH, Kim YC, Kim HH. Inhibition of osteoclast differentiation and bone resorption by sauchinone. *Biochem. Pharmacol.*, **74**, 911–923 (2007).
27) Li D, Lu H, Li X, Quan Q, Li X, Lu W. Calcium channel blockers in cirrhotic patients with portal hypertension. *Chin. Med. J.* (Engl.), **108**, 803–808 (1995).
28) Kobayashi S, Inaba K, Kimura I, Kimura M. Inhibitory effects of tetrandrine on angiogenesis in adjuvant-induced chronic inflammation and tube formation of vascular endothelial cells. *Biol. Pharm. Bull.*, **21**, 346–349 (1998).
29) Kwan CY, Achike FI. Tetrandrine and related bis-benzylisoquinoline alkaloids from medicinal herbs: cardiovascular effects and mechanisms of action. *Acta Pharmacol. Sin.*, **23**, 1057–1068 (2002).
30) Lai JH. Immunomodulatory effects and mechanisms of plant alkaloid tetrandrine in autoimmune diseases. *Acta Pharmacol. Sin.*, **23**, 1093–1101 (2002).
31) Xie QM, Tang HF, Chen JQ, Bian RL. Pharmacological actions of tetrandrine in inflammatory pulmonary diseases. *Acta Pharmacol. Sin.*, **23**, 1107–1113 (2002).
32) Shen YC, Chou CJ, Chiou WF, Chen CF. Anti-inflammatory effects of the partially purified extract of radix *Stephaniae tetrandrae*: comparative studies of its active principles tetrandrine and fangchinoline on human polymorphonuclear leukocyte functions. *Mol. Pharmacol.*, **60**, 1083–1090 (2001).
33) Chen YJ. Potential role of tetrandrine in cancer therapy. *Acta Pharmacol. Sin.*, **23**, 1102–1106 (2002).
34) Ho LJ, Juan TY, Chao P, Wu WL, Chang DM, Chang SY, Lai JH. Plant alkaloid tetrandrine downregulates IκBα kinases-IκBα-NF-κB signaling pathway in human peripheral blood T cell. *Br. J. Pharmacol.*, **143**, 919–927 (2004).
35) Wu JM, Chen Y, Chen JC, Lin TY, Tseng SH. Tetrandrine induces apoptosis and growth suppression of colon cancer cells in mice. *Cancer Lett.*, **287**, 187–195 (2010).
36) Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science*, **278**, 860–866 (1997).
37) Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell*, **91**, 243–252 (1997).
38) Zhang H, Li YY, Wu XZ. Effect of Tetrandrine on LPS-induced NF-kappaB activation in isolated pancreatic acinar cells of rat. *World J. Gastroenterol.*, **12**, 4232–4236 (2006).
39) Wu SJ, Ng LT. Tetrandrine inhibits proinflammatory cytokines, iNOS and COX-2 expression in human monocytic cells. *Biol. Pharm. Bull.*, **30**, 59–62 (2007).
40) Lin ST, Wang Y, Xue Y, Feng DC, Xu Y, Xu LY. Tetrandrine suppresses LPS-induced astrocyte activation *via* modulating IKKs-IkappaBalpha-NF-kappaB signaling pathway. *Mol. Cell. Biochem.*, **315**, 41–49 (2008).
41) Kikuchi M, Kamimura S, Nomura M, Takahashi T, Hagino N, Kobayashi S. Inhibitory actions of tetrandrine on tumor necrosis factor α-induced NF-κB activation in neovascularization of cultured choroidal explants. *Chinese Medicine*, **1**, 75–83 (2010).
42) Musacchia XJ, Steffen JM, Fell RD. Disuse atrophy of skeletal muscle: animal models. *Exerc. Sport Sci. Rev.*, **16**, 61–87 (1988).
43) Bharti AC, Takada Y, Shishodia S, Aggarwal BB. Evidence that receptor activator of nuclear factor (NF)-kappaB ligand can suppress cell proliferation and induce apoptosis through activation of a NF-kappaB-independent and TRAF6-dependent mechanism. *J. Biol. Chem.*, **279**, 6065–6076 (2004).
44) Sakai A, Sakata T, Ikeda S, Uchida S, Okazaki R, Norimura T, Hori M, Nakamura T. Intermittent administration of human parathyroid Hormone(1–34) prevents immobilization-related bone loss by regulating bone marrow capacity for bone cells in ddY mice. *J. Bone Miner. Res.*, **14**, 1691–1699 (1999).
45) Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat. Rev. Immunol.*, **7**,

292–304 (2007).
46) Soysa NS, Alles N. NF-kappaB functions in osteoclasts. *Biochem. Biophys. Res. Commun.*, **378**, 1–5 (2009).
47) Takatsuna H, Asagiri M, Kubota T, Oka K, Osada T, Sugiyama C, Saito H, Aoki K, Ohya K, Takayanagi H, Umezawa K. Inhibition of RANKL-induced osteoclastogenesis by (−)-DHMEQ, a novel NF-kappaB inhibitor, through downregulation of NFATc1. *J. Bone Miner. Res.*, **20**, 653–662 (2005).
48) Asagiri M, Sato K, Usami T, Ochi S, Nishina H, Yoshida H, Morita I, Wagner EF, Mak TW, Serfling E, Takayanagi H. Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. *J. Exp. Med.*, **202**, 1261–1269 (2005).
49) Kim Y, Sato K, Asagiri M, Morita I, Soma K, Takayanagi H. Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. *J. Biol. Chem.*, **280**, 32905–32913 (2005).
50) Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, McHugh KP. NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. *Gene*, **372**, 92–102 (2006).
51) Kuroda Y, Hisatsune C, Nakamura T, Matsuo K, Mikoshiba K. Osteoblasts induce Ca^{2+} oscillation-independent NFATc1 activation during osteoclastogenesis. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 8643–8648 (2008).
52) Masuyama R, Vriens J, Voets T, Karashima Y, Owsianik G, Vennekens R, Lieben L, Torrekens S, Moermans K, Vanden Bosch A, Bouillon R, Nilius B, Carmeliet G. TRPV4-mediated calcium influx regulates terminal differentiation of osteoclasts. *Cell Metab.*, **8**, 257–265 (2008).
53) Kim MS, Yang YM, Son A, Tian YS, Lee SI, Kang SW, Muallem S, Shin DM. RANKL-mediated reactive oxygen species pathway that induces long lasting Ca^{2+} oscillations essential for osteoclastogenesis. *J. Biol. Chem.*, **285**, 6913–6921 (2010).
54) Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.*, **4**, 517–529 (2003).
55) Parekh AB, Putney JW Jr. Store-operated calcium channels. *Physiol. Rev.*, **85**, 757–810 (2005).
56) Berridge MJ, Galione A. Cytosolic calcium oscillators. *FASEB J.*, **2**, 3074–3082 (1988).
57) Thomas AP, Bird GS, Hajnóczky G, Robb-Gaspers LD, Putney JW Jr. Spatial and temporal aspects of cellular calcium signaling. *FASEB J.*, **10**, 1505–1517 (1996).
58) Bird GS, Putney JW Jr. Capacitative calcium entry supports calcium oscillations in human embryonic kidney cells. *J. Physiol.*, **562**, 697–706 (2005).
59) Miyauchi A, Hruska KA, Greenfield EM, Duncan R, Alvarez J, Barattolo R, Colucci S, Zambonin-Zallone A, Teitelbaum SL, Teti A. Osteoclast cytosolic calcium, regulated by voltage-gated calcium channels and extracellular calcium, controls podosome assembly and bone resorption. *J. Cell Biol.*, **111**, 2543–2552 (1990).
60) Ritchie CK, Maercklein PB, Fitzpatrick LA. Direct effect of calcium channel antagonists on osteoclast function: alterations in bone resorption and intracellular calcium concentrations. *Endocrinology*, **135**, 996–1003 (1994).
61) King VF, Garcia ML, Himmel D, Reuben JP, Lam YK, Pan JX, Han GQ, Kaczorowski GJ. Interaction of tetrandrine with slowly inactivating calcium channels. Characterization of calcium channel modulation by an alkaloid of Chinese medicinal herb origin. *J. Biol. Chem.*, **263**, 2238–2244 (1988).
62) Stern PH. The calcineurin-NFAT pathway and bone: intriguing new findings. *Mol. Interv.*, **6**, 193–196 (2006).
63) Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, Crabtree GR. Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev. Cell*, **10**, 771–782 (2006).
64) Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S, Hisatake K, Nogi Y. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. *J. Biol. Chem.*, **279**, 45969–45979 (2004).
65) Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K, Takayanagi H. NFAT and Osterix cooperatively regulate bone formation. *Nat. Med.*, **11**, 880–885 (2005).
66) McClung MR, Lewiecki EM, Cohen SB, Bolognese MA, Woodson GC, Moffett AH, Peacock M, Miller PD, Lederman SN, Chesnut CH, Lain D, Kivitz AJ, Holloway DL, Zhang C, Peterson MC, Bekker PJ; AMG 162 Bone Loss Study Group. Denosumab in postmenopausal women with low bone mineral density. *N. Engl. J. Med.*, **354**, 821–831 (2006).
67) Kendler DL, Roux C, Benhamou CL, Brown JP, Lillestol M, Siddhanti S, Man HS, San Martin J, Bone HG. Effects of denosumab on bone mineral density and bone turnover in postmenopausal women transitioning from alendronate therapy. *J. Bone Miner. Res.*, **25**, 72–81 (2010).