**Modulating calcium-mediated NFATc1 and MAPK deactivation
underlies the inhibitory effects of kavain on osteoclastogenesis and
bone resorption**

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Running title: Kavain inhibits RANKL-induced osteoclastogenesis

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Abstract:

Osteoclasts are responsible for bone resorption during the process of bone rebuilding. Increased osteoclast numbers and bone resorption activity are the main factors contributing to bone loss related diseases such as osteoporosis. Therefore, modulating the formation and function of osteoclasts is critical for the effective treatment of osteolysis and osteoporosis. We decided to assess the potency of natural compounds in regulating osteoclast formation and function. Kavain is the active ingredient extracted from the root of the kava plant, which possesses known anti-inflammatory properties. However, the effects of kavain on osteoclastogenesis and bone resorption remain unclear. In this study, we found that kavain inhibits receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclast differentiation and fusion using tartrate-resistant acid phosphatase staining and immunofluorescence. Furthermore, kavain inhibited resorption pit formation by osteoclasts. Using reverse transcription-polymerase chain reaction and western blot analysis, we found that kavain downregulated the expression of osteoclast marker genes, such as nuclear factor of activated T-cells, cytoplasmic 1 (*Nfatc1*), v-ATPase d2, cathepsin K (*Ctsk*) and *acp5*. Additionally, kavain repressed RANKL-induced calcium oscillations, NFAT activation, and mitogen-activated protein kinase (MAPK) phosphorylation, while leaving NF- κ B unaffected. We found no effects of kavain on either osteoblast proliferation or differentiation. Collectively, our data indicate that kavain inhibits osteoclast formation and function by suppressing calcium-mediated NFATc1 activation and phosphorylation of MAPK. These data suggest a potential use for kavain as a candidate drug for the treatment of osteolytic diseases.

Keywords:

Kavain; Osteoclast; Bone resorption; MAPK; Calcium

Introduction

Bone remodeling collectively refers to the processes of bone resorption and bone formation [1]. The balance between these two processes maintains normal bone homeostasis [2]. Bone resorption outstripping bone synthesis is the pathological feature of osteolysis and osteoporosis [3,4]. Osteoclasts are the primary cells in the bone that serve the function of bone resorption. Therefore, exploring the regulatory mechanisms underlying osteoclast differentiation and function is of great significance for the treatment of osteoporosis [5,6]. Currently, several antiresorption drugs which inhibit the osteoclast-mediated bone resorption are widely used in the clinic, limitations to the treatment of osteoporosis still exist [7,8]. In this respect, further alternative treatments are still required.

Osteoclasts are giant, multinucleated cells that are derived from the monocyte/macrophage hematopoietic lineage [9,10]. Receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) play critical roles in mediating osteoclast differentiation and maturation [5]. M-CSF activates ERK1/2 and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling in early osteoclastogenesis [11]. RANKL activates its receptor RANK and triggers several signaling cascades including nuclear factor kappa-B (NF- κ B)-associated signaling cascades, mitogen-activated protein kinase (MAPK) pathway [12,13], calcium-signaling pathway. These pathways further co-regulate the expression of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) which can be auto-amplified and is one of key transcription factors involved in terminal osteoclast differentiation [14].

Natural compounds and their derivatives play indispensable roles in the development of new therapeutic treatments [15,16]. One such compound is derived from the root of the perennial shrub plant, *Piper methysticum* (known as kava). Kava root is already being utilized for the treatment of neurological disorders, such as anxiety and epilepsy [17]. The primary active ingredients of kava are liposoluble resins called kavalactones. One such kavalactone, called kavain, possesses anti-inflammatory and anti-oxidant effects. Kavain reduces lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF- α) secretion in mouse macrophages and human peripheral blood mononuclear cells [18,19]. Furthermore, kavain protects neural cells against amyloid beta peptide-induced neurotoxicity by inducing protective gene expression mediated by NRF2 activation [20]. However, the effects of kavain on bone homeostasis, specifically on the differentiation of osteoclasts and osteoblasts, have not been reported. In this study, we found that kavain inhibited RANKL-induced osteoclastogenesis by blocking the activation of both calcium-mediated NFAT and MAPK pathways. In addition, bone resorption by osteoclasts was also suppressed by kavain; however, it had no discernible effects on the differentiation and proliferation of osteoblasts. Together, our data demonstrates osteoclast-specific potency of the natural compound kavain, suggesting its potential therapeutic value in the treatment of osteolytic diseases.

Materials and methods

Materials

Alpha modified minimal essential medium (α -MEM) and fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Scoresby, Australia). Kavain was provided by Prof. Renxiang Tan at Nanjing University, and prepared at a stock concentration of 1 mM in phosphate buffered saline (PBS). Antibodies specific for Integrin- β 3, CTSK, NFATc1, I κ B- α , ERK, JNK, p38, phosphorylated (p) ERK, p-p38, p-JNK and β -actin were obtained from Santa Cruz Biotechnology (San Jose, CA). Antibodies to V-ATPase d2 were produced as previously described [21]. The MTS and luciferase assay system were obtained from Promega (Sydney, Australia). Recombinant macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Minneapolis, MN). Leucocyte acid phosphatase staining kits were obtained from Sigma-Aldrich (Sydney, Australia). Recombinant GST-rRANKL protein was expressed and purified as previously described [22].

Cell culture

RAW264.7 mouse macrophage cells and MC3T3-E1 preosteoblast cells were obtained from the American Type Culture Collection (Manassas, VA). RAW264.7 and MC3T3-E1 were cultured in α -MEM and DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin respectively. Bone marrow monocytes (BMMs) were obtained from 6-week-old C57BL/6J mice, which were euthanized according to procedures approved by the Animal Ethics Committee of the University of Western Australia (RA/3/100/1244). Long bones were dissected free of soft tissues and the bone marrow was flushed from the femur and tibia, and then cultured in complete medium in the presence of M-CSF (50 ng/mL).

106 *Osteoblast differentiation assay*

107 MC3T3-E1 cells were plated into 6-well culture plates at a density of 5×10^5 cells/well, and treated with
108 osteoblast differentiation medium. The medium was composed of dexamethasone (10 nmol/L), ascorbate (50
109 $\mu\text{g/ml}$) and β -glycerophosphate (5 mmol/L) replaced every 3 d.

110

111 *Osteoclastogenesis assay*

112 Drug concentration screening assays were conducted using BMMs isolated as described above to evaluate
113 RANKL-induced osteoclastogenesis. BMMs were plated into 96-well culture plates at a density of 6×10^3
114 cells/well, and treated with complete medium containing M-CSF and GST-rRANKL (50ng/mL) in the presence
115 of varying concentration of kavain. The cell culture medium was changed every 2 days. After 5 days, cells were
116 fixed with 4% paraformaldehyde for 10 min, washed three times with PBS, and then stained for tartrate resistant
117 acid phosphatase (TRAcP) enzymatic activity using the leucocyte acid phosphatase staining kit, following the
118 manufacturer's procedures. TRAcP-positive multinucleated cells (>3 nuclei) were counted as osteoclasts.

119

120 *Cytotoxicity assays*

121 BMMs and MC3T3-E1 were seeded into a 96-well plate at 6×10^3 cells/well and left overnight to adhere. The
122 following day, the cells were incubated with varying concentrations of kavain. After a further 48 hours MTS
123 solution (20 $\mu\text{L/well}$) was added and incubated with cells for 2 h. The absorbance at 490 nm was read with a
124 microplate reader (Multiscan Spectrum, ThermoLabsystem, Chantilly, VA).

125

126 *Immunofluorescent staining*

BMMs were seeded at a density of 6×10^3 cell/well in the presence of M-CSF (50 ng/mL) overnight. Cells were stimulated with M-CSF and GST-rRANKL (50 ng/mL) until mature osteoclasts formed. The osteoclasts were then treated with different concentrations of kavain for 48 h before being fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 PBS, and then blocked with 3% BSA in PBS. Next, cells were incubated with Rhodamine-conjugated phalloidin for 45 minutes in the dark to stain F-actin. Cells were then washed with PBS, nuclei were counterstained with DAPI, and mounted for confocal microscopy.

Hydroxyapatite resorption assay

To measure osteoclast activity, BMMs were cultured onto 6-well collagen-coated plates (1×10^5 cells per well) (BD Biocoat, ThermoFisher, Scoresby, Australia) and stimulated with 50 ng/mL GST-rRANKL and M-CSF until mature osteoclasts were generated [23]. Cells were gently detached from the plate using cell dissociation solution (Sigma-Aldrich, Sydney, Australia) and equal numbers of mature osteoclasts were seeded into individual wells in hydroxyapatite coated 96-well plates (Corning Osteoassay, Corning, NY). Mature osteoclasts were incubated in medium containing GST-rRANKL and M-CSF with or without kavain at the indicated concentrations. After 48 h, half of the wells were histochemically stained for TRAcP activity as above to assess the number of multinucleated cells per well. The remaining wells were bleached for 10 min to remove the cells and allow measurement of the resorbed areas. Resorbed areas were photographed under standard light microscopy and ImageJ software (NIH, Bethesda, MD) was used to quantify the percentage area of hydroxyapatite surface resorbed by the osteoclasts.

Luciferase reporter assays

To investigate NF- κ B and NFATc1 transcriptional activation, RAW264.7 cells were stably transfected with either an NF- κ B responsive luciferase reporter construct or an NFATc1 responsive luciferase reporter construct [24-26]. Transfected cells were cultured in 48-well plates at a density of 1.5×10^5 cells/well and pretreated with various concentrations of kavain for 1 h. Following pre-treatment cells were subsequently stimulated with GST-rRANKL (50 ng/mL) for 6 h (NF- κ B luciferase report gene assay) or 24 h (NFAT luciferase reporter gene assay) and luciferase activity was detected using the luciferase reporter assay system according to the manufacturer's protocol (Promega, Sydney, Australia).

Measurement of intracellular calcium oscillation

Calcium oscillations were investigated using the calcium binding dye Fluo4-AM according to the manufacturer's method (Molecular probes, Thermo Fisher Scientific, Scoresby, Australia). Briefly, BMMs were seeded into a 48-well plate at a concentration of 1×10^4 cells/well. The following day, the medium was replaced with complete medium containing 15 μ M kavain supplemented with GST-rRANKL and M-CSF for 24 h. Cells were then washed in Assay buffer (HANKS balanced salt solution supplemented with 1mM probenecid and 1% FBS) and then incubated with 4 mM Fluo4 staining solution for 45 min at 37°C. Cells were washed once in Assay buffer and incubated at room temperature for 20 min followed by a further two washes in Assay buffer and visualization of fluorescence using an inverted fluorescent microscope (Nikon, Tokyo, Japan) at an excitation wavelength of 488 nm. Images were captured every 2 sec for 2 min and calcium flux analyzed using Nikon Basic Research Software.

Alkaline phosphatase (ALP) staining assay

The MC3T3-E1 cells were seeded into 48-well plates at a density of 2×10^4 cells/well and were treated with kavain for 7 days with a complete medium change every 3 days. After that, the cells were fixed with 4% paraformaldehyde for 20 minutes and stained with 100 μ L BCIP (Sigma-Aldrich, Sydney, Australia) for 30 minutes at 37 °C in the dark. The staining solution was then discarded, and the cells were washed with phosphate-buffered saline (PBS) and photographed using a digital camera as previously described.

Quantitative RT-PCR analysis

Total RNA was isolated from cells using Trizol reagent according to the manufacturer's protocol (ThermoFisher Scientific, Scoresby Australia). The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with 1 μ g of RNA template and oligo-dT primers. Polymerase chain reaction amplification of specific sequences was performed using the following cycle: 94°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 60°C for 40 sec, and 72°C for 40 sec, and a final extension step of 5 min at 72°C. The detailed information of specific primers is shown in **Table 1**. The relative mRNA level was calculated by normalization to *Hmbs* and *Hprt*.

Western blotting

BMMs were cultured in complete medium with M-CSF in 6-well plates and stimulated with 50 ng/mL GST-rRANKL for the stated times. Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer, and proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Silverwater, Australia). The membranes were blocked in 5% skim milk for 1 h, and then probed with various specific primary antibodies with gentle shaking overnight at 4°C. Membranes

190 were washed and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies.

191 Antibody reactivity was then detected with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia

192 Biotech, Piscataway, NJ), visualized on an Image-quant LAS 4000 (GE Healthcare, Silverwater, Australia).

193
194 *Statistics*

195 All data are representative of at least three experiments of similar results performed in triplicate unless otherwise

196 indicated. Data are expressed as mean $\pm$ SD. One-way ANOVA followed by Student-Newman-Keuls post hoc

197 tests was used to determine the significance of differences between results, with $p < 0.05$ being regarded as

198 significant.

Results

Kavain inhibits RANKL-induced osteoclast formation and fusion

To determine the effect of kavain on osteoclastogenesis, BMMs were cultured in complete medium with varying concentrations of kavain in the presence of 50 ng/mL M-CSF and 100 ng/mL RANKL for 5 days. The results of tartrate-resistant acid phosphatase (TRAP) staining showed that the addition of 15 μ M kavain reduced the number of mature osteoclasts (Figure 1A and B). Kavain-induced cell toxicity was assessed using the MTS assay. No significant changes in cell viability were observed, even in the presence of 25 μ M kavain (Figure 1C). We also performed immunofluorescent staining to assess the effect of kavain on osteoclast fusion. The results demonstrated that 15 μ M kavain reduced the average number of nuclei per osteoclast, suggesting an impairment in osteoclast fusion (Figure 1D and E).

Kavain attenuates osteoclastic hydroxyapatite resorption

To test the effect of kavain on osteoclast function, BMMs were first plated in a six-well plate in the presence of 50 ng/mL M-CSF and 100 ng/mL RANKL. After differentiation (at day 4), equal numbers of osteoclasts were plated on a hydroxyapatite substrate for an osteoresorption assay and treated with different concentrations of kavain. Compared to the control group, the pit area was much less pronounced in the groups treated with kavain (Figure 2A and C); while osteoclast numbers were comparable across groups (Figure 2B).

Kavain represses the expression of osteoclast marker genes and proteins

Next, we measured the expression of osteoclast-specific genes, *Nfatc1*, TRAcP (acid phosphatase 5; *Acp5*), cathepsin K (*Ctsk*), and *Atp6v0d2*, in BMMs treated with kavain following induction with 100 ng/ml RANKL

for 5 days. As expected, RT-PCR results indicated that RANKL stimulated the expression of these osteoclast-specific genes. However, 15 μ M kavain decreased RANKL-induced expression of these genes (Figure 3A–D). In addition, western blot analysis was performed to determine whether kavain regulated the expression of NFATc1 and associated downstream proteins. Consistent with the results above, kavain treatment downregulated NFATc1 protein expression on days 3 and 5 compared with the RANKL-treated PBS control groups (Figure 3E). Furthermore, the expression of bone NFATc1-associated, resorption-related downstream proteins, including V-ATPase-d2, CTSK, and integrin β 3, was also repressed in groups treated with 15 μ M kavain on days 3 and 5 compared to the RANKL-treated PBS control groups (Figure 3E).

Kavain affects activation of NFATc1 by modulating intracellular calcium levels

To directly evaluate the effects of kavain on NFATc1 activity, we treated RAW264.7 cells stably transfected with an Nfatc1-luciferase reporter construct with RANKL in the presence of varying concentrations of kavain. The results revealed that 15 μ M kavain significantly decreased Nfatc1-luciferase activity (Figure 4A). To further investigate the mechanism by which kavain regulates NFATc1 activation, we studied its effects on calcium signaling pathways. BMMs were stimulated with GST-rRANKL (100 ng/mL) for 24 h, and then calcium flux was assessed using the Fluo4 calcium indicator. As expected, the intensity of calcium oscillations in the RANKL-treated groups was significantly increased compared with the control groups (Figure 4B and C). Treatment with kavain significantly decreased the intensity of RANKL-mediated calcium oscillations (Figure 4D and E).

Kavain inhibits specific MAPK signaling pathway

We next examined its effects on MAPK activation, which is one of the key RANKL-mediated signaling pathways for osteoclast differentiation and activation. Western blot analysis showed that kavain decreased significantly the phosphorylation of MAPK downstream targets P38 and JNK1/2, but did not affect the phosphorylation of ERK1/2 (Figure 5).

Kavain has little effect on RANKL-induced activation of NF- κ B

We also measured the effects of kavain on RANKL-induced NF- κ B activity. Using luciferase reporter assays, we found that NF- κ B luciferase intensity was increased upon RANKL activation. However, no obvious changes in NF- κ B luciferase intensity were observed following kavain treatment compared to groups treated with RANKL alone (Figure S1A). Furthermore, the results of western blot analysis indicated that kavain did not affect the degradation of I κ B- α induced by RANKL (Figure S1B). Therefore, these data suggest that kavain had no significant influence on NF- κ B signaling during osteoclastogenesis.

Kavain does not affect osteoblast proliferation and differentiation

Next, we wanted to test the effects of kavain on osteoblast differentiation and proliferation. Before exploring the roles of kavain in osteoblast differentiation, the toxicity of kavain was evaluated by the MTS assay. MC3T3-E1 osteoblasts were plated in a 96-well plate with different concentration of kavain for 24 and 48 h. The results showed that kavain did not affect osteoblast proliferation, even at high concentrations (20 μ M) of kavain (Figure S2A and S2B). Next, we performed alkaline phosphatase (ALP) staining to directly assess the effect of kavain on osteoblast differentiation. ALP staining showed no differences between control groups and groups treated with kavain (Figure S2C). The results of RT-PCR showed that the expression levels of osteoblast marker genes

263 such as *Alp*, runt-related transcription factor 2 (*Runx2*), and collagen type I alpha 1 (*Col1a1*) in groups treated
264 with kavain were not changed relative to control untreated groups (FigureS2D–F). In summary, these results
265 demonstrate that osteoblast proliferation and differentiation were unaffected by kavain.
266

Discussion

Osteoclasts can resorb bone and cartilage, thereby regulating bone remodeling and mineral homeostasis. However, in some bone-loss diseases, such as osteoporosis, rheumatoid arthritis, or periodontitis, a significant increase in either osteoclastogenesis or osteoclast function is found [27-29]. Thus, reducing osteoclast viability is a classic antiresorptive therapeutic strategy for patients suffering from these osteolytic diseases. Kavain is an active ingredient extracted from the root of the kava plant. Kavain and its derivatives are currently used as relaxants, sedatives, and anxiolytics [30]. Here, we demonstrated for the first time that kavain inhibits osteoclastogenesis and bone resorption, potentially through the suppression calcium - mediated NFATc1 and MAPK signaling pathways. Our results are consistent with recent finding with new mechanistic insights that kavain displayed promise in the treatment of bone loss and inflammation in an experimental model of periodontitis [31].

It is well established that M-CSF and RANKL are the major cytokines that can initiate osteoclast differentiation and stimulate their bone resorptive function [32]. NFATc1, the master transcription factor promoting osteoclast differentiation, is induced following the activation of the RANKL/RANK axis. Expression of NFATc1 is dependent on TNF receptor-associated factor 6 (TRAF6) and kinds of pathways such as NF- κ B, MAPK and calcium-signaling pathways [33]. In turn, NFATc1 regulates the expression of target genes that enable osteoclast attachment to the bone and mediate their bone resorptive functions [34-36]. In a previous study, kavain and other derivative molecules were suggested to modulate LPS-induced signaling pathways, such as NF- κ B, activator protein 1 (AP-1), and ERK [19,37]. However, the precise mechanism by which kavain regulates osteoclast activity is unclear. Interestingly, we showed that kavain can suppress the expression of an

Nfatc1-luciferase fusion gene and inhibit downstream effectors ACP5 (also known as TRAcP), CTSK, and integrin β 3 based on RNA and protein analyses. These findings suggest one mechanism by which kavain can inhibit osteoclastogenesis and bone resorption. However, we saw no changes in the activation of the NF- κ B pathway upon the addition of kavain. The difference of the effect of kavain on NF- κ B pathway might be due to different cell types and culture systems used.

Calcium signaling, another critical pathway regulating osteoclastogenesis, can be activated by RANKL/RANK and phospholipase C gamma, which facilitate the activation of NFATc1. Particularly, NFATc1 can be auto-amplified during active calcium signaling [38,39]. In our study, kavain inhibits intracellular calcium oscillations. Therefore, the inhibition calcium signaling pathway resulting in deactivation of NFATc1 could contribute to the observed decrease in osteoclast formation and activity.

MAPK pathways act downstream of RANKL signaling. MAPK activity in turn activates JNK, P38, ERK via major specific Mitogen-activated protein kinase kinase (MAP2Ks) responsible for MAPK phosphorylation. For example, MEK1 and MEK2 are responsible for ERKs; the MKK3 and MKK6 activate the P38; MKK4 and MKK7 are in charge of JNKs though MKK4 also takes part in P38 phosphorylation. Activation of the MAPK pathway and downstream components promotes osteoclastogenesis [2]. In our study, we provide evidence that kavain reduces the phosphorylation of JNK and P38 but ERK. In contrast, kavain was previously reported to inhibit TNF- α expression via the dephosphorylation of ERK2 and myeloid differentiation in mouse bone marrow macrophages. TNF- α can, in turn, promote osteoclastogenesis by activating RANKL signaling in osteoclast precursors [40]. We speculate that in the same cell line under different stimulation conditions, the

309 downstream pathways involved may act divergently. Apart from this, kavain may exert different influence in
310 the specific MAP2Ks. The mechanism underlying these differences warrants further investigation.
311
312 Mesenchymal stem cell-derived osteoblasts also contribute to bone remodeling. Osteoblasts can express the key
313 osteoclast differentiation factor RANKL and counterbalance its downstream effects by producing
314 osteoprotegerin [41]. Bone loss can also be caused by an imbalance between osteoblast and osteoclast numbers
315 or impaired communication between these two cell populations. Our results reveal that kavain has no effect on
316 the differentiation and function of osteoblasts *in vitro*, indicative of its cell type specific effect on osteoclasts.
317 In summary, these results suggest that kavain inhibits osteoclast formation and function by modulating
318 intracellular calcium- mediated NFATc1 and MAPK deactivation, while not affecting osteoblasts. While future
319 studies need to confirm the comparable activity of kavain against osteoclasts *in vivo*, our data suggest a potential
320 role for this naturally derived compound in the treatment of osteolytic diseases.
321

322 **Competing Interests**

323 The authors declare no conflict of interest.

324

325 **Acknowledgments**

326 It is supported in part by Guangxi Scientific Research and Technology Development Plan Project

327 (GKG13349003, 1598013-15), Western Australia Medical & Health Research Infrastructure Fund, Arthritis

328 Australia foundation, The University of Western Australia (UWA) Research Collaboration Awards, and the

329 Australian Health and Medical Research Council (NHMRC, No 1107828, 1027932).

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Figure legends

Figure 1. Kavain inhibits RANKL-induced osteoclast formation and fusion. **(A)** Representative images of BMMs stained for TRAcP treated with kavain at the different dosages indicated. Scale bar represents 200 μ m. **(B)** Quantification of osteoclast numbers (TRAcP positive, $\geq$ three nuclei). **(C)** Survival of BMMs in the presence of kavain as assessed by MTS cell viability assay. **(D)** Representative confocal images of osteoclasts stained for F-actin and nuclei. Scale bar represents 200 μ m. **(E)** Average nuclei number per osteoclast in each field counted. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($P < 0.05$) or ** ($P < 0.01$).

Figure 2. Kavain attenuates osteoclastic hydroxyapatite resorption. **(A)** Representative images of osteoassay surface after removal of osteoclasts (middle and lower panels) with corresponding TRAP-stained osteoclasts (upper panels). Scale bar represents 200 μ m. **(B)** Quantification of osteoclasts in each well. **(C)** Quantification of bone resorption pit area per well. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as ** ($P < 0.01$) or *** ($P < 0.001$).

Figure 3. Kavain represses the expression of osteoclast marker genes and proteins. BMMs were treated with M-CSF (50 ng/mL) and GST-rRANKL (100 ng/mL) in the presence or absence of the indicated concentrations of kavain. **(A-D)** Relative mRNA expression levels of *Nfatc1*, *Atp6v0d2*, *Ctsk*, and *Acp5* during osteoclastogenesis. Gene expression was normalized to the housekeeping gene *Hmbs*. **(E)** Representative western blot images of NFATc1, Integrin β 3, CTSK, V-ATPase-d2 and ACTB protein levels in lysates from BMMs. The ratio of above proteins relative to ACTB was determined. The data in the figures represent the

mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

Figure 4. Kavain affects activation of NFATc1 and intracellular calcium levels. **(A)** Luciferase activity in RANKL-stimulated RAW 264.7 cells transfected with an Nfatc1-luciferase construct. Transfected cells were pretreated with indicated concentrations of kavain and subsequently stimulated with GST-rRANKL (100 ng/mL) for 24 h. **(B)** Representative calcium oscillations within three cells treated with M-CSF only (negative control). **(C)** Representative calcium oscillations within three cells treated with M-CSF and RANKL (positive control). **(D)** Representative calcium oscillations within three cells treated with M-CSF, RANKL, and kavain. **(E)** Average change in intensity of calcium oscillations per cell. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as *** ($P < 0.001$).

Figure 5. Kavain inhibits specific MAPK signaling pathway. Representative western blot images of p-P38, P38, p-JNK1/2, and JNK p-ERK1/2, and ERK from BMMs induced with RANKL (100 ng/mL) and M-CSF (50 ng/mL) in the absence or presence of kavain. The ratio of phosphorylated proteins relative to unphosphorylated proteins was determined.

Figure 6. Schematic diagram of the inhibitory effect of kavain on osteoclast differentiation. Kavain suppresses NFATc1 expression and phosphorylation of P38 and JNK, while leaving ERK and NF- κ B unaffected. Besides, kavain attenuates NFATc1 activation via modulating intracellular calcium oscillations, hence inhibits osteoclastogenesis.

Table 1: Primer sequences for qPCR

Genes	Forward (5'-3')	Reverse (5'-3')	Tm (°C)
<i>Hmbs</i>	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG	60
<i>Hprt</i>	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	61
<i>Runx2</i>	AGAGTCAGATTACAGATCCCAGG	TGGCTCTTCTTACTGAGAGAGG	60
<i>Alp</i>	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT	61
<i>Colla1</i>	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG	62
<i>Nfatc1</i>	GACCCGGAGTTCGACTTCG	TGACACTAGGGGACACATAACTG	60
<i>Atp6v0d2</i>	CTGGTTCGAGGATGCAAAGC	GTTGCCATAGTCCGTGGTCTG	61
<i>Ctsk</i>	GGGAGAAAAACCTGAAGC	ATTCTGGGGACTCAGAGC	61
<i>Acp5</i>	TGTGGCCATCTTTATGCT	GTCATTTCTTTGGGGCTT	61

Hmbs, hydroxymethylbilane synthase; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Runx2*, runt related transcription factor 2; *Alp*, alkaline phosphatase, liver/bone/kidney; *Colla1*, collagen, type I, alpha 1; *Nfatc1*, nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1; *Atp6v0d2*, ATPase, H⁺ transporting, lysosomal V0 subunit D2; *Ctsk*, cathepsin K; *Acp5*, acid phosphatase 5.

Figure 1

Figure 2

Figure S1. Kavain has little effect on RANKL-induced activation of NF- κ B and phosphorylation of ERK. **(A)** Luciferase activity in RANKL stimulated RAW264.7 cells transfected with an NF- κ B luciferase construct. Transfected cells were pre-treated with indicated concentrations of kavain, and subsequently stimulated with GST-rRANKL (100 ng/mL) for 6 h. **(B)** Representative western blot images of I κ B- α and ACTB from BMMs which were induced with RANKL (100 ng/mL) and M-CSF (50 ng/mL) in the presence of kavain.

Figure S2. Kavain does not affect osteoblast proliferation and differentiation. MTS analysis of cell viability of MC3T3-E1 cells treated with different concentrations of kavain for **(A)** 24 h and **(B)** 48 h. **(C)** Representative ALP staining images of MC3T3-E1 cells in kavain-treated and -untreated groups (day 7). **(D)** Relative mRNA expression levels of *Alp*, **(E)** *Runx2*, and **(F)** *Col1a1* from MC3T3-E1 cells in different groups (days 0 and 7). The data in the figures represent the mean $\pm$ SD.

Figure 3

Figure 4

Figure 5

A **B**

Figure S1

Figure S2

Schematic diagram of the inhibitory effect of kavain on osteoclast differentiation. Kavain suppresses NFATc1 expression by inhibiting phosphorylation of P38 and JNK, while leaving ERK and NF-κB unaffected. Besides, kavain attenuates NFATc1 activation via modulating intracellular calcium oscillations, hence inhibits osteoclastogenesis.