**Lumichrome inhibits osteoclastogenesis and bone resorption through
suppressing RANKL-induced NFAT activation and calcium signaling**

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Running title: Lumichrome blocks RANKL-induced osteoclastogenesis

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Abstract:

The dynamic balance between bone resorption and bone formation is crucial to maintain bone mass. Osteoclasts are key cells that perform bone resorption while osteoblasts and osteocytes function in bone formation. Osteoporosis, a bone metabolism disease characterized by bone loss and degradation of bone microstructure, occurs when osteoclastic bone resorption outstrips osteoblastic bone synthesis. The interaction between receptor activator of nuclear factor κ B ligand (RANKL) and RANK on the surface of bone marrow macrophages promotes osteoclast differentiation and activation. In this study, we found that lumichrome, a photodegradation product of riboflavin, inhibits RANKL-induced osteoclastogenesis and bone resorption as determined by tartrate resistant acid phosphatase staining, immunofluorescence, RT-PCR, and western blot. Our results showed that lumichrome represses the expression of osteoclast marker genes, including cathepsin K (*Ctsk*) and *Nfatc1*. In addition, lumichrome suppressed RANKL-induced calcium oscillations, NFATc1, NF- κ B and MAPK signaling activation. Moreover, lumichrome promoted osteoblast differentiation at an early stage, as demonstrated by upregulated expression of osteoblast marker genes *Alp*, *Runx2*, and *Col1a1*. We also found that lumichrome reduces bone loss in ovariectomized (OVX) mice by inhibiting osteoclastogenesis. In summary, our data suggest the potential of lumichrome as a therapeutic drug for osteolytic diseases.

Keywords:

Lumichrome; Osteoclast; Osteoporosis; RANKL; Calcium signaling

45 **Introduction**

46 Bone is a hard connective tissue that possesses important functions, such as protection of various organs,
47 storage of minerals, and harboring of bone marrow (Florencio-Silva et al., 2015; Kobayashi and Kronenberg,
48 2014). Continuously remodeling makes bone a highly dynamic organ. The balance of bone remodeling is
49 orchestrated by bone resorption performed by osteoclasts and bone formation performed by osteoblasts
50 (Raggatt and Partridge, 2010; Teitelbaum, 2000). Derived from the monocyte/macrophage hematopoietic
51 lineage, osteoclasts are specialized cells that are rich in mitochondria and lysosomes (Boyle et al., 2003).
52 Receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony stimulating factor
53 (M-CSF) are the critical cytokines for osteoclast differentiation and activation (Teitelbaum and Ross, 2003).
54 Binding of RANKL to RANK on the osteoclast cell surface induces intracellular signaling pathways such as
55 NF- κ B, MAPK and calcium oscillation, thereby upregulating the expression of NFATc1, an essential
56 transcription factor involved in osteoclastogenesis (Ishida et al., 2002). The osteoblast is derived from
57 mesenchymal stem cells and is the main cell involved in bone formation (Khan and Hardingham, 2012). After
58 proliferation, maturation, and mineralization, osteoblasts differentiate into osteocytes to regulate bone growth
59 and maintain bone mass (Nakahama, 2010).

60

61 Osteoporosis is a common metabolic bone disease manifested as bone loss and microstructure damage, which
62 results in bone pain and increased fracture risk (McCormick, 2007). The development of osteoporosis is
63 associated with low estrogen levels, high parathyroid hormone levels, and many other factors (Dalsky, 1990;
64 Tobias et al., 1990). Excessive bone resorption mediated by osteoclasts is the crucial mechanism leading to
65 osteoporosis (Manolagas, 1998). Therefore, bone resorption inhibitors are commonly utilized as

anti-osteoporosis drugs. Since natural compounds are abundant and have many biological functions such as anti-oxidation and anti-inflammation, the identification of natural inhibitors of osteoclast formation and bone resorption to treat osteoporosis has become a priority (An et al., 2016).

Lumichrome is one of the photodegradation products of riboflavin which is important in cellular oxidation and energy metabolism (Bro-Rasmussen, 1958). In addition, riboflavin has been shown to have a positive effect on bone formation (Yazdanpanah et al., 2008; Yazdanpanah et al., 2007) and upregulation of osteoblast-related gene expression *in vitro* (Chaves Neto et al., 2010). It has been reported that lumichrome and riboflavin protected rat heart from ischemic reperfusion damage (Kotegawa et al., 1994). However, the role of lumichrome in the skeletal system, especially in osteoporosis has not been reported. Therefore, we evaluated the effects of lumichrome on osteoclastogenesis, osteoclastic bone resorption, and osteoblast differentiation. The results revealed that lumichrome represses RANKL-induced osteoclast formation and bone resorption via suppressing RANKL-induced NFAT activation and calcium signaling. Interestingly, osteoblast differentiation was also promoted by lumichrome. Besides, lumichrome administration prevented bone loss in ovariectomized (OVX) mice by decreasing the osteoclast number *in vivo*. The results of this study suggest that lumichrome may serve as a potential drug candidate to treat osteoporosis.

Materials and methods

Materials

Alpha modified minimal essential medium (α -MEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Scoresby, Australia). Lumichrome was provided by Professor Renxiang Tan from Nanjing University, and was prepared at a stock concentration of 1 mM in phosphate buffered saline (PBS). Antibodies specific for Integrin- β 3, c-Fos, CTSK, NFATc1, STAT3, I κ B- α , ERK, JNK, p38, phosphorylated (p) ERK, p-p38, p-JNK p-STAT3 and β -actin were obtained from Santa Cruz Biotechnology (San Jose, CA). The MTS and luciferase assay systems 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 (Xu et al., 2000).

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).

104 *Mice*

105 The 12-week-old female **C57BL/6J** mice were provided by the animal center of Third Military Medical
106 University. **The animal protocol in this study was approved by the Laboratory Animal Welfare and Ethics**
107 **Committee of the Third Military Medical University. All the animal procedures in the current study were**
108 **strictly performed according to the approved guidelines.** All efforts were made to reduce the number of
109 animals tested and their suffering. Mice were divided into three groups: sham operated mice (n=4),
110 ovariectomized mice (n=4), and lumichrome treated ovariectomized mice (n=4). PBS or lumichrome
111 was injected intraperitoneally three times a week for 6 weeks. All treated mice were sacrificed by
112 cervical dislocation one day after last administration.

113

114 *Osteoblast differentiation assay*

115 MC3T3-E1 cells were plated into 6-well culture plates at a density of 5×10^5 cells/well, and treated with
116 osteoblast differentiation medium. The medium, composed of dexamethasone (10 nmol/L), ascorbate (50
117 $\mu\text{g/ml}$) and β -glycerophosphate (5 mmol/L), was replaced every 3 days as previously described.

118

119 *Osteoclastogenesis assay*

120 Drug screening assays were conducted using BMMs isolated as described above to evaluate RANKL-induced
121 osteoclastogenesis. BMMs were plated into 96-well culture plates at a density of 6×10^3 cells/well. The cells
122 were treated with complete medium containing M-CSF (10 ng/mL) and GST-rRANKL (100 ng/mL) in the
123 presence or absence of varying concentrations of lumichrome. The cell culture medium was changed every 2
124 days. After 5 days, cells were fixed with 4% paraformaldehyde for 10 min, washed three times with PBS. Then

the cells were stained for tartrate resistant acid phosphatase (TRAcP) enzymatic activity using the leucocyte acid phosphatase staining kit, following the manufacturer's procedures. TRAcP-positive multinucleated cells (> 3 nuclei) were counted as osteoclasts.

Cytotoxicity assays

BMMs and MC3T3-E1 were seeded into a 96-well plate at 6×10^3 cells/well and left overnight to adhere. The following day the cells were incubated with varying concentrations of lumichrome. After a further 48 hours and 120 hours MTS solution (20 μ L/well) was added and incubated with cells for 2 hours. The absorbance at 490 nm was read with a microplate reader (Multiscan Spectrum, Thermo Labsystem, Chantilly, VA).

Immunofluorescent staining

BMMs were seeded at a density of 6×10^3 cells/well in the presence of M-CSF (10 ng/mL) overnight. Cells were stimulated with M-CSF and GST-rRANKL (100 ng/mL) until mature osteoclasts formed. The osteoclasts were treated with different concentrations of lumichrome for 48 hours. Then the cells were 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, osteoclasts were first generated from BMMs (1×10^5 cells/well) cultured in 6-well collagen coated plates (BD Biocoat, Thermo Fisher, Scoresby, Australia). And the cells were stimulated

with 10 ng/mL M-CSF and 100 ng/mL GST-rRANKL until mature osteoclasts were generated (Zhou et al., 2016). Cells were gently detached from the plate using cell dissociation solution (Sigma-Aldrich, Sydney, Australia). 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 lumichrome at the indicated concentrations. After 48 hours, 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. 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, RAW 264.7 cells were stably transfected with either an NF- κ B responsive luciferase reporter construct or an NFATc1 responsive luciferase reporter construct (Cheng et al., 2017; van der Kraan et al., 2013; Wang et al., 2003). Transfected cells were cultured in 48-well plates at a density of 1.5×10^5 cells/well and pretreated with various concentrations of lumichrome for 1 hour. Following pre-treatment cells were subsequently stimulated with GST-rRANKL (100 ng/mL) for 6 hours (NF- κ B luciferase report gene assay) or 24 hours (NFAT luciferase reporter gene assay). 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) (Song et al., 2018). 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 10 μ M lumichrome supplemented with GST-rRANKL and M-CSF for 24 hours. 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. Then the fluorescent of cells was detected 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. The cells were treated with lumichrome for 7 days with a complete medium change every 3 days. After that, the cells were fixed with 4% paraformaldehyde for 20 minutes. Then the cells were stained with 100 μ L BCIP (Sigma-Aldrich, Sydney, Australia) for 30 minutes at 37 °C in the dark. The staining solution was discarded, and the cells were washed with PBS and photographed using a digital camera.

Alizarin red staining assay

The MC3T3-E1 cells were seeded at a density of 2×10^4 cells/well into 48-well plates. After treatment for 28 days with lumichrome, the cells were gently washed twice with PBS, then fixed with 4% paraformaldehyde for

20 minutes. Next the cells were post-fixed with 70% ethanol and stained with 1% Alizarin red solution for 15 minutes at room temperature. Then the cells were washed with 50% ethanol. The images of extracellular matrix mineralisation were captured using a digital camera.

Quantitative RT-PCR analysis

Total RNA was isolated from cells using Trizol reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Scoresby Australia). The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with 1 µg 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* or *Hprt*.

Western blotting

BMMs were cultured in complete medium with M-CSF in 6-well plates and stimulated with 100 ng/mL GST-rRANKL for the stated times. Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. 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 hour, and then probed with various specific primary antibodies with gentle shaking overnight at 4°C. Membranes were washed and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Antibody reactivity was then detected with enhanced chemiluminescence (ECL) reagent

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

MicroCT analysis and histological analysis

The Bruker MicroCT Skyscan 1272 system (Kontich, Belgium) with an isotropic voxel size of 10.0 μm was used to image the whole femur fixed in 4 % paraformaldehyde. The scanning images were obtained using an X-ray tube potential of 60 kV, an X-ray intensity of 166 μA , and an exposure time of 1700 ms. The threshold for the trabecular bones was set at 86–255 (8-bit gray scale bitmap). MicroCT scans of whole bodies of mice (except the skulls) were obtained using an isotropic voxel size of 148 μm . Reconstruction was accomplished using NRecon (Ver. 1.6.10). 3D images were obtained from contoured 2D images using methods based on the distance transformation of the original gray scale images (CTvox, Ver. 3.0.0). 3D and 2D analyses were performed using CT Analyzer software (Ver. 1.15.4.0). All images presented are representative of their respective groups.

For the histological analysis of the bones, the femurs were dissected and fixed in 4 % paraformaldehyde-PBS for 48 hours. The femurs were then decalcified by daily changes of a 15 % tetrasodium EDTA soaking solution for 2 weeks. The decalcified femurs were dehydrated by passing them through a series of increasing concentrations of ethanol, cleared in xylene twice, embedded in paraffin. Then the femurs were sectioned into 8- μm -thick sections along the coronal plate from anterior to posterior. The decalcified femoral sections were stained with TRAcP.

230 *Statistics*

231 All data are representative of at least three experiments performed in triplicate unless otherwise indicated. Data
232 are expressed as mean $\pm$ SD. One-way ANOVA followed by Student-Newman-Keuls post hoc tests was used to
233 determine the significance of differences between results, with $p < 0.05$ being regarded as significant.
234

Results

Lumichrome inhibits RANKL-induced osteoclastogenesis and osteoclast fusion

To assess the effects of lumichrome on osteoclast formation, BMMs were cultured with RANKL and M-CSF for five days together with different concentrations of lumichrome. We found that lumichrome has an inhibitory effect on the formation of TRAcP positive cells when the concentration of lumichrome reached to 7.5 μ M (Figure 1A, 1B). To determine if the effects of lumichrome were due to cytotoxicity, we performed an MTS assay. The results showed lumichrome has no cytotoxicity at the concentration of 10 μ M and lower (Figure 1C, S1A). In addition, to evaluate the effects of lumichrome on osteoclast fusion, we stained osteoclasts formed in the presence or absence of lumichrome with rhodamine-phalloidin to visualize F-actin, and DAPI to identify nuclei (Figure 2A). The results showed that lumichrome at the concentration of 10 μ M significantly suppresses both osteoclast formation and the average number of nuclei per osteoclast (Figure 2B, 2C). Taken together, these data reveal that lumichrome has an inhibitory effect on RANKL-induced osteoclast formation and osteoclast fusion but has no cytotoxic effects in BMMs.

Lumichrome inhibits osteoclastic hydroxyapatite resorption

To investigate the effect of lumichrome on osteoclastic bone resorption, we seeded equal numbers of mature osteoclasts onto hydroxyapatite plates. The mature osteoclasts were then exposed to 7.5 μ M or 10 μ M lumichrome for 24 hours. TRAcP staining indicated that the number of mature osteoclasts was not affected by treatment with lumichrome (Figure 3A, 3B). However, resorption area of groups treated with lumichrome was significantly reduced in a dose-dependent manner when compared to untreated groups (Figure 3A, 3C).

Therefore, these data show that lumichrome suppresses osteoclastic hydroxyapatite resorption, but has no cytotoxic effect in mature osteoclasts.

Lumichrome attenuates RANKL-induced gene expression

We next assessed the effects of lumichrome on RANKL-induced gene expression during osteoclastogenesis. Gene expression levels in BMMs treated with RANKL and M-CSF for 5 days with different dosages of lumichrome were analyzed by RT-PCR. The mRNA expression levels of osteoclast related genes *Nfatc1*, *Fos*, *Ctsk* and *Acp5* were significantly down-regulated following lumichrome treatment (Figure 4). These results were consistent with the inhibitory effects of lumichrome on osteoclastogenesis and hydroxyapatite resorption.

Lumichrome represses NFATc1 activation and associated downstream protein expression

To detect the effect of lumichrome on RANKL-induced NFATc1 activity, RAW264.7 cells that had been transfected with an NFATc1 luciferase reporter construct were stimulated with RANKL in combination with different concentrations of lumichrome. The results showed that the NFATc1 luciferase activity was decreased by treatment with lumichrome at the concentration of 7.5 and 10 μ M (Figure 5A). Western blot analysis of protein levels was used to confirm the results of the luciferase reporter assay. Consistent with the inhibition of NFATc1 activation, the expression of NFATc1 protein levels was notably blocked by lumichrome treatment on days 3 and 5 (Figure 5B). Additionally, NFATc1 associated downstream protein expression, including c-Fos, matrix metalloproteinase 9 (MMP9), cathepsin K (CTSK) and Integrin- β 3 were also down-regulated in the presence of lumichrome at the concentration of 10 μ M (Figure 5B).

276

277 *Lumichrome suppresses RANKL-induced activation of NF- κ B and MAPK pathways*

278 It is known that RANKL-induced NF- κ B and MAPK signaling pathways play vital roles in osteoclastogenesis.

279 Thus we employed luciferase reporter assays to detect the effect of lumichrome on RANKL-induced NF- κ B

280 activity. NF- κ B luciferase activation was reduced following treatment with lumichrome at the concentration of

281 7.5 and 10 μ M (Figure 6A). Western blotting revealed that RANKL induced I κ B- α degradation was inhibited in

282 the presence of lumichrome (Figure 6B). Interestingly, lumichrome also repressed the phosphorylation of

283 ERK1/2 and p38 (Figure 6B) and had little effect on STAT3 and JNK1/2 phosphorylation (Figure S2). Taken

284 together, these data indicate that lumichrome blocks RANKL-induced activation of NF- κ B and MAPK

285 signaling pathways.

286

287 *Lumichrome inhibits RANKL-induced calcium oscillations*

288 To explain the inhibitory effects of lumichrome on NFAT activation, we further investigated the ability of

289 lumichrome to affect RANKL-induced calcium signaling. As expected, calcium oscillations were activated by

290 RANKL compared with control groups (Figure 7A, 7B). Noticeably, lumichrome significantly attenuated the

291 amplitude of calcium oscillations induced by RANKL (Figure 7C, 7D). These results indicate that

292 lumichrome abrogates RANKL-induced calcium signaling pathway, consistent with its inhibitory effect on

293 NFAT activation and RANKL-induced osteoclastogenesis.

294

295 *Lumichrome promotes ALP expression in osteoblasts.*

To further test the effect of lumichrome on osteogenic differentiation and proliferation, we performed MTS assay to evaluate the toxicity of lumichrome on osteoblastic cells. The osteoblast cell line MC3T3-E1 was treated with different dosages of lumichrome for 24, 48, and 120 hours. The results revealed that lumichrome had no cytotoxicity at the concentration of 12 μ M and lower (Figure 8A, 8B, S1B). To assess the impact of lumichrome on osteoblast differentiation, alkaline phosphatase (ALP) activity and gene expression were assessed on day 7. We found that the activity and gene expression of ALP in groups treated with lumichrome was increased compared with control groups (Figure 8C-D). Osteoblastic mineralisation was assessed on day 28 in cultures by alizarin red staining. The results revealed that mineralization, as assessed by calcium deposition, was not changed following treatment with lumichrome (Figure 8E-F). In addition, the expression of osteoblast specific genes *Runx2* and *Col1a1* was promoted by lumichrome treatment during the early stages of osteoblast differentiation (day 7) but had normalized by day 21 (Figure 8G, 8H). Collectively, lumichrome had positive effects on osteoblast differentiation at early stages

Lumichrome prevents ovariectomy (OVX) -induced bone loss.

Next, we investigated the effect of lumichrome on OVX-induced bone loss in mice. After sham operated or ovariectomized, the mice were then treated with lumichrome (7.5 mg/kg) or PBS by intraperitoneal injection every 2 days for 6 weeks. The femurs were then collected for microCT analysis. The reconstructed region was contoured as showed in the dashed red box. The results revealed that lumichrome treatment inhibited bone loss in OVX mice (Figure. 9A). Quantitative analysis showed that the bone mineral density (BMD) and trabecular bone volume fraction (BV/TV) were significantly increased in the lumichrome treated OVX mice compared with the control group (Figure 9C). To further confirm the inhibitory effect of lumichrome on

317 osteoclastogenesis *in vivo*, we also performed histological TRAcP staining. The results showed that osteoclast
318 surface/bone surface (Oc.S/BS) was increased in OVX mice compared to the control group and lumichrome
319 considerably reduced the ratio of Oc.S/BS (Figure 9B, 9D). Therefore, these data indicate that lumichrome
320 inhibits OVX-induced bone loss by suppressing osteoclastogenesis.
321

Discussion

Lumichrome is a photodegradation product of riboflavin. Lumichrome and riboflavin have previously been shown to have protective effects against ischemic reperfusion damage of rat heart (Kotegawa et al., 1994). However, there has been very few studies investigating the role of lumichrome in bone biology. Understanding the mechanism and effects of lumichrome on osteoclastogenesis and osteogenesis might provide a new perspective for the treatment of bone disease (Deng et al., 2017; Wang et al., 2017). Our study shows that lumichrome could prevent ovariectomy-induced bone loss through suppressing osteoclastogenesis. Besides, lumichrome represses RANKL-induced osteoclast formation and bone resorption by suppressing NFAT activation and calcium signaling. We also found that lumichrome could promote osteoblast differentiation at an early stage.

At the initial stage of osteoclastogenesis, RANKL interacts with RANK. Tumor necrosis factor receptor-associated factor (TRAF) is then recruited to bind to the intracellular domain of RANK to induce the activation of NFATc1 (Kobayashi et al., 2001). As an important transcription factor during osteoclastogenesis, NFATc1 regulates the expression of osteoclast specific genes, such as MMP9, CTSK, Calcitonin receptor (CTR) and Integrin β 3 (Balkan et al., 2009; Crotti et al., 2008; Shen et al., 2007). It has been reported that NFATc1 deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL stimulation (Takayanagi et al., 2002). Our study demonstrated that lumichrome inhibits RANKL-induced NFATc1 activation and calcium signaling and then suppressed the expression of downstream genes such as MMP9 and CTSK (Kanzaki et al., 2016).

343 Along with inhibition of NFATc1 by lumichrome, we further examined its impact on RANKL signaling
344 including NF- κ B and MAPK. NF- κ B signaling plays an important role in regulating osteoclastogenesis
345 (Wong et al., 1998). RANKL activates NF- κ B-inducible kinase (NIK) to dissociate NF- κ B and I κ B- α . Then,
346 NF- κ B translocates rapidly into the nucleus to regulate osteoclast differentiation and maturation (Boyce et al.,
347 2015; Franzoso et al., 1997). Interestingly, lumichrome was found to reduce RANKL-induced NF- κ B activity
348 through inhibiting the degradation of I κ B- α , which is consistent with the inhibitory effects of lumichrome on
349 RANKL-induced osteoclast formation and bone resorption. MAPK signaling, including the ERK, JNK, and
350 p38 pathways, also regulates osteoclast differentiation by activating key transcription factors such as c-Fos
351 and NFATc1 (Huang et al., 2006; Park et al., 2017). Studies have found that p38 mutant mice developed
352 osteoporosis, which was associated with an increase in osteoclastogenesis and bone resorption (Cong et al.,
353 2017). It has been demonstrated that sophoridine attenuates ovariectomy-induced osteoporosis via inhibition
354 of ERK phosphorylation (Zhao et al., 2017). From our data, lumichrome suppresses RANKL-induced
355 activation of p38 and ERK and has no influence on JNK phosphorylation. These results suggest that
356 lumichrome inhibits NFATc1 expression by regulating p38 and ERK pathways.

357

358 In addition to these signaling pathways, RANKL also promotes calcium release and increases intracellular
359 calcium levels (Komarova et al., 2005). The activation of NFATc1 is dependent on the continuous low
360 concentration of calcium and the threshold of activation is induced by calcium oscillations (Hwang and
361 Putney, 2011; Takayanagi et al., 2002). A recent study confirmed that impaired calcium mobilization
362 aggravated posttraumatic bone loss in osteoporotic mice (Fischer et al., 2017). We found that the average

amplitude of calcium oscillations was significantly attenuated by lumichrome, consistent with its inhibitory effects on osteoclastogenesis and bone resorption.

In addition to osteoclasts, osteoblasts are important components of bone tissue and are the major functional cells in bone formation (Dirckx et al., 2013; Mackie et al., 1990). Osteoblasts play key roles in regulating bone mineralization and maintaining bone mass (Watrous and Andrews, 1989). Osteoblasts gradually express specific marker genes such as *Alp*, osteocalcin (*Ocn*), and *Colla1* during differentiation and maturation, which are regulated by a series of transcription factors (Karsenty, 1998; Komori, 2010). The osteoblast-specific transcription factor, Runx2, is indispensable for osteoblast differentiation and bone formation (Drissi et al., 2000; Komori, 2017). Calvarial osteoblasts derived from *Runx2* knockout mice presented with decreased bone nodule formation and bone marker gene expression (Tu et al., 2008). In our study, we found that lumichrome promotes ALP activity, and the expression of *Alp*, *Runx2*, and *Colla1* during osteoblast differentiation. However, bone mineralization was not affected by treatment with lumichrome.

In conclusion, our results show that lumichrome treatment prevents bone loss in OVX mice. Lumichrome significantly suppresses RANKL-induced osteoclastogenesis and bone resorption by inhibiting RANKL-induced activation of calcium signaling, NFATc1, NF- κ B, and MAPK while stimulating osteoblast differentiation (Figure 10). Our findings suggest the potential of lumichrome as a therapeutic agent for osteoporosis and other osteolytic bone diseases.

383 **Competing Interests**

384 The authors declare no conflict of interest.

385

386 **Acknowledgments**

387 This work was supported by the Natural Science Foundation of China (81572164), the National Key
388 Technology Research and Development Program of China (2017YFC1103300). It is also supported in part by
389 Guangxi Scientific Research and Technology Development Plan Project (GKG13349003, 1598013-15),
390 Western Australia Medical & Health Research Infrastructure Fund, Arthritis Australia foundation, The
391 University of Western Australia (UWA) Research Collaboration Awards, and the Australian Health and
392 Medical Research Council (NHMRC, No 1107828, 1027932).

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Figure legends

Figure 1. Lumichrome abrogates RANKL-induced osteoclastogenesis. **(A)** Representative images of BMMs stained for TRAcP treated with lumichrome at different dosages. Scale bar represents 200 μ m. **(B)** Quantification of osteoclast numbers (TRAcP positive, ≥ 3 nuclei). **(C)** Survival of BMMs in the presence of lumichrome as assessed by MTS cell viability assay. 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. lumichrome represses RANKL-induced osteoclast fusion. **(A)** Representative confocal images of osteoclasts stained for F-actin and nuclei. Scale bar represents 200 μ m. **(B)** Quantification of osteoclasts in each field. **(C)** Average nuclei number per osteoclast in each field. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$).

Figure 3. Lumichrome inhibits osteoclastic hydroxyapatite resorption. **(A)** Representative images of osteoassay surface after removal of osteoclasts (down) with corresponding TRAcP stained osteoclasts (up). Scale bar represents 200 μ m. **(B)** Quantification of osteoclasts in each well. **(C)** Quantification of hydroxyapatite resorption area in each well. 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 4. Lumichrome attenuates RANKL-induced gene expression. BMMs were treated with M-CSF (10 ng/mL) and GST-rRANKL (100 ng/mL) in the presence or absence of indicated concentrations of lumichrome. Gene expression was normalized to *Hmbs*. Relative mRNA expression levels of *Nfatc1* **(A)**, *Fos*

(B), *Ctsk* (C) and *Acp5* (TRAcP) (D) during osteoclastogenesis. 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 5. Lumichrome represses NFATc1 activation and associated downstream protein expression. (A) Luciferase activity in RANKL stimulated RAW 264.7 cells transfected with an NFATc1 luciferase construct. Transfected cells were pre-treated with indicated concentrations of lumichrome, and subsequently stimulated with GST-rRANKL (100 ng/mL) for 24 hours. (B) Representative western blot images of c-Fos, NFATc1, MMP9, CTSK, Integrin β 3 and ACTB from BMMs which were induced with and M-CSF (10 ng/mL) and RANKL (100 ng/mL) in the presence of lumichrome. 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 6. Lumichrome suppresses RANKL-induced activation of NF- κ B and MAPK pathways. (A) Luciferase activity in RANKL stimulated RAW 264.7 cells transfected with an NF- κ B luciferase construct. Transfected cells were pre-treated with indicated concentrations of lumichrome, and subsequently stimulated with GST-rRANKL (100 ng/mL) for 6 hours. (B) Representative western blot images of p-P38, P38, p-ERK1/2, ERK, I κ B- α and ACTB from BMMs which were induced with M-CSF (10 ng/mL) and RANKL (100 ng/mL) in the presence of lumichrome. 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 7. Lumichrome inhibits RANKL-induced calcium oscillations. BMMs were stimulated with GST-rRANKL (100 ng/mL) for 24 hours and then calcium flux was assessed using Fluo4 calcium indicator. (A) Representative calcium fluctuations within three cells treated with M-CSF only (negative control). (B) Representative calcium fluctuations within three cells treated with M-CSF and RANKL (positive control). (C) Representative calcium fluctuations within three cells treated with M-CSF, RANKL and lumichrome. (D) Average change in intensity per cell. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as ** ($p < 0.01$).

Figure 8. Lumichrome promotes ALP expression and has no effect on mineralization of osteoblasts. (A-B) MTS analysis of cell viability of MC3T3-E1 cells treated with different concentrations of lumichrome for 24 hours and 48 hours. (C) Representative ALP staining images of MC3T3-E1 cells in different groups (day 7). (D) Relative mRNA expression levels of *Alp* from MC3T3-E1 cells in different groups (day 0 and 7). (E) Representative alizarin red staining images of MC3T3-E1 cells in different groups (day 28). (F) Quantification of mean intensity of alizarin red staining. (G-H) Relative mRNA expression levels of *Runx2* and *Colla1* from MC3T3-E1 cells in different groups (day 0, 7, 14 and 21). 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 9. Lumichrome prevents bone loss in OVX mice. (A) Representative microCT images of the longitudinal section of the femurs, cross-sectional view of the distal femurs, and reconstructed trabecular structure of the ROI (red dashed box). (B) Representative images of TRAcP-stained histological slides

589 focusing on the metaphyseal region of the distal femur from mice of different groups. Scale bar represents 200
590 μm and 400 μm . (C) Quantitative microCT analysis of distal femoral volumetric bone mineral density (BMD)
591 and trabecular bone volume fraction (BV/TV) in each group. (D) Quantitative analysis of osteoclast
592 surface/bone surface ratio. The data in the figures represent the averages $\pm$ SD. Statistically significant
593 differences between the treatment and control groups are indicated as $** (p < 0.01)$ or $*** (p < 0.001)$.

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