

Piperine alleviates osteoclast formation through the p38/c-Fos/NFATc1 signaling axis

Vishwa Deepak¹, Marlena C. Kruger^{2, 3, 4}, Annie Joubert¹, Magdalena Coetzee^{1, 4*}

¹Department of Physiology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

²School of Food and Nutrition, Massey Institute of Food Science and Technology, Massey University,
Palmerston North, New Zealand

³Department of Human Nutrition, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

⁴Associate of the Institute for Food, Nutrition and Well-being, University of Pretoria, Pretoria, South Africa

*Author to whom correspondence should be addressed:

Dr. Magdalena Coetzee, Department of Physiology, University of Pretoria, Private Bag X323, Arcadia 0007,
Pretoria, South Africa

E-Mail: magdalena.coetzee@up.ac.za; Tel.: +27-12-319-2445; Fax: +27-12-321-1679

Abstract

Increased bone fracture is one of the health risk factors in patients with bone loss related disorders such as osteoporosis and breast cancer metastasis to bone. Over activity of osteoclasts leads to uncoupling of bone remodeling favoring bone loss over bone formation. Receptor activator of nuclear factor- κ ligand (RANKL) triggers the differentiation pathway leading to multinucleated osteoclast formation. Modulation of RANKL or its downstream signaling pathways involved in osteoclast formation is of significant interest in the development of anti-resorptive agents. In this study, we investigated the effects of piperine, an alkaloid present in *Piper nigrum L.* on osteoclast formation. Piperine inhibited tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclast formation in murine RAW264.7 macrophages and human CD14⁺ monocytes induced by RANKL and breast cancer cells. Piperine attenuated the p38-mitogen activated protein kinase (MAPK) pathway activation, while the extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) or NF- κ B pathways downstream of RANKL remained unaffected. Concomitantly, expression of c-Fos and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), the key transcription factors involved in osteoclastogenesis were remarkably inhibited by piperine. Furthermore, piperine disrupted the actin ring structure and bone resorption, a characteristic hallmark of osteoclasts. Collectively, these results suggest that piperine inhibits osteoclast differentiation by suppressing the p38/NFATc1/c-Fos signaling axis.

Keywords: RANKL, osteoclast, breast cancer, bone, RAW264.7, CD14⁺ monocytes

1. Introduction

Osteoclasts are specialized bone resorbing multinucleated cells derived from hematopoietic cells of the monocyte/macrophage lineage (1). The receptor activator of nuclear factor kappa- (RANK) and its ligand (RANKL) are key molecules involved in differentiation and activation of osteoclasts from precursor cells (2). On the other hand, osteoblasts arising from progenitor cells of the mesenchymal lineage are responsible for bone formation and mineral deposition (3). The interplay among osteoblasts and osteoclasts are coupled in a manner that the processes of bone formation and bone resorption termed bone remodeling does not supersede each other. Intriguingly, bone remodeling is often aggravated during certain diseases such as osteoporosis, cancer metastasis to bone, rheumatoid and osteo-arthritis (4, 5). Excessive bone loss as seen in aforementioned pathological conditions correlate either with elevated osteoclast formation or augmented osteoclast activity. In line with this, ageing also constitutes to the negative effects seen in bone-loss related diseases as stemness and differentiation potential of mesenchymal progenitors towards osteoblast lineage is afflicted (6, 7).

RANKL and its receptor RANK are key factors involved in the initiation of osteoclast formation (8). RANKL, a member of the tumor necrosis factor (TNF) family triggers the formation of osteoclasts and plays an important role in their survival (8). RANKL is produced in membrane-bound form by cells of osteoblast lineage and activated T-cells (9). Both, the soluble as well as membrane bound form of RANKL are produced by activated T-cells and certain type of cancer cells (prostate- and breast cancer), although production of soluble RANKL indicates pathological conditions (9-11).

In the bone microenvironment, physical interaction between osteoblasts and osteoclast precursors activates RANK resulting in initiation of the downstream signaling cascade involved in osteoclast formation. During rheumatoid arthritis, production of soluble RANKL by activated T-lymphocytes can also trigger osteoclastogenesis from osteoclast precursors causing joint destruction (12). Osteoblasts also produce and secrete a decoy receptor of RANK, osteoprotegerin (OPG) (2). The decoy receptor binds to all forms of RANKL and neutralizes its functions. The RANKL/RANK/OPG system is instrumental in bone remodeling.

Macrophage-colony stimulating factor (M-CSF) is a cytokine produced by various cells including osteoblasts and acts in a paracrine manner to maintain the survival and proliferation of osteoclast progenitors (13). Trimerization of RANK occurs when RANKL binds to the receptor leading a subsequent step that involves binding to an adaptor molecule, TNF receptor-associated factor 6 (TRAF6) (14). This activates a sequential chain of events comprising activation of I B / kinase (IKK), NF- , MAPKs, c-Fos and NFATc1 resulting in osteoclast formation.

Since, RANKL-induced signaling pathway is critical for osteoclast formation, attenuation of RANKL or effectors downstream of RANKL is a promising therapeutic option against osteoclastogenesis (15). As reported by Newman et al, approximately 48.6% of currently approved drugs by FDA are either natural products or are directly derived from them and thereby serve as an essential source of lead compounds (16). Piperine is one of the major constituents of black pepper, *Piper nigrum* Linn. and long pepper, *Piper longum* Linn. Piper species have been used as an oriental medicinal plant for curing a wide range of diseases such as diarrhoea, gastric ailments and cholera in Chinese-, Indian- and pacific islands traditional medicines (17). Piperine is a major bioactive compound reported to possess anti-cancer-, analgesic, anti-inflammatory- and anti-oxidant properties and is also used to increase bioavailability of drugs and nutrients (18-20). Oral supplementation of piperine has been reported to

suppress lung carcinogenesis in mice (21). Vijayakumar et al. observed that piperine can reduce high-fat diet induced oxidative stress (22). Piperine has been shown to increase plasma levels of orally supplemented coenzyme Q₁₀ (23). Moreover, piperine has also been demonstrated to inhibit cancer stem cell self-renewal properties (24). Park et al. have demonstrated antiadipogenic properties of piperine in 3T3-L1 cells *in vitro* (25). Although it is widely used and has an extensive range of pharmacological activities, little is known about the effects of piperine on osteoclastogenesis. Hence, we sought to analyze the potential effects of piperine on osteoclast formation. We provide first line of evidence that piperine has potential anti-osteoclastogenic effects by disruption of the p38/c-Fos/NFATc1 signaling axis and suppression of osteoclast function by disruption of cytoskeletal rearrangement.

2. Methods

2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM), α -MEM and heat-inactivated fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY). Antibiotic-antimycotic solution containing 100 U/ml, penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml fungizone was supplied by Highveld Biological (Johannesburg, South Africa). Piperine, phalloidin-Atto-488, TRAP kit and all other chemicals of research grade were obtained from Sigma-Aldrich Inc. (St Louis, MO). Human M-CSF was supplied by Insight Biotechnology (Middlesex, UK). Mouse- and human RANKL were acquired from R&D Systems (Minneapolis, MN) respectively. All components for the magnetic separation of CD14⁺ monocytes were supplied by Miltenyi Biotec (San Diego, CA). Alamar blue reagent, cell extraction buffer, NBT/BCIP Western Detection Chromogenic Kit were provided by Life Technologies (Carlsbad, CA). Osteoassay surface multiwell plates were acquired from Corning Inc. (New York, NY). The bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (Rockford, IL). Primary rabbit antibodies against phosphorylated forms of I κ B^{ser32/36}, p38^{thr180/tyr182}, JNK^{thr183/tyr185}, ERK^{thr202/tyr204} and total-c-Fos antibodies were obtained from Signalway Antibody LLC (College Park, MD) and IKK β ^{ser176/180} and total-NFATc1 antibodies were supplied by Cell Signaling Technology (Beverly, MA). Primary rabbit antibody against GAPDH was obtained from Abcam (Cambridge, MA) and goat anti-rabbit ALP-conjugated secondary antibody was procured from Life Technologies (Carlsbad, CA).

2.2 Stock Solution

A 200 mM stock solution of piperine was prepared in DMSO (vehicle) and frozen as aliquots in -80°C until further use. Stock solutions were freshly diluted to working concentrations in complete culture medium before experiments. The final DMSO concentration in the culture medium did not exceed 0.05% (v/v).

2.3 Cell culture

RAW264.7 murine macrophages (#TIB-71), MDA-MB-231 (#HTB-26), MCF-7 (#HTB-22) human breast cancer cell lines and MC3T3-E1 cells (#2593) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in DMEM with 10% FBS. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

2.4 Isolation of human CD14+ monocytes and cell culture

This article does not contain any studies with animals performed by any of the authors. All the procedures and experimental protocols were approved by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Protocol approval number: S154/2012) and in accordance with the 1964 Helsinki declaration and its later amendments. Eligible participants were asked to provide an additional written informed consent for enrolment. Peripheral blood (40–60 ml) was collected from healthy male donors (aged 18–35) into heparinized tubes. Human peripheral blood CD14+ monocytes were isolated as described elsewhere using CD14+ magnetic beads as per manufacturer's instructions (Miltenyi Biotec, San Diego, CA) (26). Cells were cultured in α -MEM supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere with 7% CO₂.

2.5 Alamar blue assay

Cells were seeded in 96-well plates and after 12h of attachment were exposed to increasing concentrations of piperine (20 μ M, 40 μ M, 80 μ M and 100 μ M). Alamar blue assay was conducted as per manufacturer's instructions (Life Technologies). Absorbance was measured at 570 nm with 600 nm as reference wavelength on a microplate reader (BioTek Instruments Inc., Winooski, VT).

2.6 Osteoclast differentiation and TRAP staining

RAW264.7 macrophages were differentiated into osteoclasts under the influence of RANKL for 5 days as described previously (27). Briefly, cells were suspended in DMEM containing 10% FBS and seeded into sterile 96-well culture plates at a density of 5×10^3 cells per well. Cells were stimulated with RANKL alone (15 ng/ml) or in combination with increasing concentrations of piperine (20 μ M, 40 μ M, 80 μ M and 100 μ M). Cell culture media and factors were replaced every third day and differentiation was terminated on the fifth day unless otherwise stated.

Breast cancer cell conditioned medium (CM) was prepared by culturing the cells in T-75 flasks until confluency followed by collection of supernatant and filter sterilization. Resultant CM was stored as aliquots at -80°C until further use. CM at 10% was added to the RAW264.7 culture medium and cells were differentiated for 7 days.

CD14+ monocytes were differentiated in the presence of M-CSF (25 ng/ml) and RANKL (30 ng/ml) for 14 days as described previously (26).

Osteoclast specific TRAP staining was performed using a leucocyte acid-phosphatase kit as per manufacturer's directions (Sigma Aldrich, St Louis). TRAP+ cells with 3 or more nuclei were scored as osteoclasts. Photomicrographs were taken with a Zeiss AxioCam MRc5 camera attached to a Zeiss Axiovert 40 CFL microscope (Carl Zeiss AG, Oberkochen, Germany).

2.7 Pit formation assay

The bone resorption function of RANKL-induced osteoclasts derived from RAW264.7 cells was analyzed using the osteoassay plates as per manufacturer's instructions (Corning Inc, New York). Resorption pits were observed under a light microscope and quantified by ImageJ software (28).

2.8 Actin ring formation assay

Actin rings of osteoclasts were detected by staining actin filaments with Atto-conjugated phalloidin as described elsewhere (29). Images were acquired using a fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

2.9 Western blot analysis

Cell lysates were prepared using cell extraction buffer (Life Technologies, Carlsbad), supplemented with protease and phosphatase inhibitors (Sigma Aldrich, St Louis) and resolved on 12% SDS-PAGE gels. Purified proteins were quantified using a BCA protein assay kit as per manufacturer's directions (Sigma Aldrich, St Louis). Proteins were electrotransferred to nitrocellulose membranes with Tris-glycine transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol (v/v)] probed with each antibody and detected by NBT/BCIP substrate. Digital images of the blots were acquired using a flatbed scanner (Ricoh Aficio, Johannesburg, South Africa). Band intensities were calculated using ImageJ software (28).

2.10 Statistical analysis

Data are representative of three independent experiments unless otherwise stated and are represented as mean \pm standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc multiple comparison test using Graph Pad Prism Software (GraphPad Software Inc., CA). $P < 0.05$ was regarded as statistically significant.

3. Results

This study was conducted to analyze the effects of piperine (Fig. 1A) on osteoclast formation induced by breast cancer cells and RANKL, as well as to investigate the effects of piperine on RANKL signaling involved in osteoclastogenesis. RAW264.7 murine macrophages and human CD14⁺ monocytes (henceforth referred as CD14⁺ monocytes) were used as osteoclast differentiation models for *in vitro* and *ex vivo* studies respectively.

3.1 Piperine does not affect the cell viability of RAW264.7 macrophages and CD14⁺ monocytes

RAW264.7 macrophages and CD14⁺ monocytes were treated with 20-100 μ M of piperine for 48h, 72h and 96h to analyze cell viability by alamar blue assay. At tested concentrations, piperine did not exert cytotoxic effects on the studied cell lines (Fig. 1B, C). Hence, these concentrations were chosen to study the effects of piperine in downstream experiments.

3.2 Piperine inhibits RANKL-induced osteoclastogenesis

RANKL is a major cytokine involved in the induction of osteoclastogenesis. Therefore, we investigated whether piperine can modulate osteoclast differentiation induced by RANKL. The osteoclast progenitor RAW264.7 macrophage cell line was treated with various concentrations (20-100 μ M) of piperine in the presence or absence of RANKL and allowed to differentiate into osteoclasts for 5 days. As shown in Figure 2A, RANKL-treated cells differentiated into TRAP⁺ giant multinucleated osteoclasts. However, co-presence of piperine significantly reduced the differentiation of cells into osteoclasts. Furthermore, piperine mediated inhibition of osteoclastogenesis occurred in a dose-dependent manner (Fig. 2B). As low as 80 μ M piperine had a significant effect on RANKL-induced osteoclastogenesis in RAW264.7 macrophages.

We next examined whether the anti-osteoclastogenic effects of piperine observed in murine macrophages *in vitro* could also be interpolated on human cells *ex vivo* in peripheral blood CD14⁺ monocytes. Results indicated that RANKL robustly induced osteoclast formation, whereas piperine inhibited this differentiation in a dose-dependent manner in CD14⁺ monocytes (Fig. 2C, D).

3.3 Piperine mediated inhibition of RANKL-induced osteoclastogenesis correlates with the onset of osteoclast formation

To determine the onset of RANKL-induced osteoclast formation and analyze piperine mediated inhibition of osteoclastogenesis we followed the differentiation from days 3, 4 and 5. Stimulation of RAW264.7 macrophages with RANKL induced their differentiation into TRAP⁺ osteoclasts as early as day 3 as evidenced through morphological observations (Fig. 3A). On the contrary, piperine exposure significantly attenuated RANKL-induced osteoclastogenesis. The extent of suppression was measured by counting the number of TRAP⁺ osteoclasts per well (Fig. 3B) after days 3, 4, and 5. RANKL-induced osteoclast formation occurred in a time-dependent manner with highest number of osteoclasts seen at day 5 (Fig. 3B). However, compared to cells treated with RANKL alone, piperine dose-dependently reduced the number of TRAP⁺ osteoclasts with maximum inhibition observed at 100 μ M at all days examined.

3.4 Piperine inhibits RANKL-induced osteoclastogenesis at an early stage of differentiation

As seen in earlier experiments, osteoclasts first began to appear from the third day of RANKL exposure in RAW264.7 macrophages and completely differentiated into osteoclasts by the fifth day. To assess whether piperine-mediated inhibition of RANKL-induced osteoclastogenesis is time-dependent, we either treated cells together with RANKL and piperine at day 0 or initially treated the cells with RANKL alone, and piperine was added after days 1, 2, 3, or 4 (Figure 4A, B). As seen in figure 4A, piperine suppressed osteoclast formation when added at day 0 or even after 1 or 2 days of RANKL addition. However, piperine did not affect osteoclast formation when supplemented after days 3 and 4 of RANKL stimulation (Fig.4), indicating inhibitory effects of piperine at early stages of osteoclast differentiation.

3.5 Piperine inhibits actin ring formation and bone resorption

Actin ring formation and bone resorption are characteristic hallmarks of osteoclast. Treatment with RANKL induced fusion of RAW264.7 macrophages generating large osteoclasts with actin rings clearly seen as green fluorescence in figure 5A. On the contrary and in line with the osteoclast formation results RAW264.7 macrophages co-treated with piperine (100 μ M) failed to fuse into osteoclasts and develop actin rings (Fig. 5A). Furthermore, owing to the attenuation of osteoclastogenesis and actin ring formation, RANKL and piperine (100 μ M) co-treated cells did not form resorption pits (Fig. 5B, C). However, RANKL treated cells clearly demonstrated bone resorptive activity (Fig. 5B, C).

3.6 Piperine inhibits RANKL-induced activation of p38 MAP kinase pathway

To delineate the molecular mechanisms involved in piperine mediated inhibition of osteoclastogenesis we analysed activation of MAPKs and NF- κ B pathways at various time-points after RANKL stimulation. Treatment of RAW264.7 macrophages with RANKL led to a steep activation of these pathways as seen by western blot

through phosphorylation-specific antibodies (Fig. 6). Piperine markedly inhibited p38 activation whereas other signaling pathways including JNK, ERK and NF- pathways remained unaffected (Fig. 6).

3.7 Piperine suppresses RANKL-induced c-Fos and NFATc1 expression

c-Fos and NFATc1 are crucial transcription factors induced at later stages of osteoclast signaling pathway. To analyse the effects of piperine on RANKL-mediated expression of these transcription factors western blotting was performed. As shown in figure 7A and B, RANKL potently induced the expression of c-Fos and NFATc1 as early as 12h and 24h respectively. However, co-exposure with piperine led to a drastic decrease in the expression levels of c-Fos and NFATc1 (Fig. 7A, B).

3.8 Piperine inhibits osteoclastogenesis induced by breast cancer cells

Breast cancer metastasis to bone is associated with osteoclastogenesis and bone loss. To assess whether piperine can modulate breast cancer-induced osteoclastogenesis, we exposed RAW264.7 macrophages to 10% CM derived from MDA-MB-231 and MCF-7 human breast cancer cells. As shown in Fig. 8A, B incubation of RAW264.7 macrophages with CM derived from the breast cancer cells led to formation of osteoclasts after 7 days, and piperine suppressed this differentiation.

3.9 Piperine did not affect the viability of MC3T3-E1 osteoblast-like cells

Bone remodeling is a closely coupled process involving bone formation and resorption by osteoblasts and osteoclasts respectively. An ideal anti-osteoclastogenic compound should not affect the viability of osteoblasts. To analyze the effects of piperine on the viability of murine MC3T3-E1 osteoblast like cells we further conducted an alamar blue assay. Treatment of MC3T3-E1 cells with piperine (20-100 μ M) did not affect the viability of these cells (Fig. 9).

4. Discussion

Osteoclasts play an important role in bone remodeling by resorbing the old and deteriorating bone. However, during certain conditions such as osteoporosis and cancer metastasis of bone, overactivity of osteoclasts results in uncoupling of the remodeling process leading to excessive osteoclast activity and increased risk of fracture (30). In this study we assessed the effects of piperine on RANKL and breast cancer induced osteoclastogenesis in murine macrophage RAW264.7 cells and human CD14⁺ monocytes. Our results indicate that piperine inhibits the RANKL and breast cancer-induced osteoclastogenesis by attenuating the RANKL-mediated p38 activation and suppressing the expression of c-Fos and NFATc1 transcription factors. Furthermore, we also show that piperine acts at an early stage of osteoclast differentiation and does not affect the viability of osteoblast-like cells.

Osteoclasts originate from the monocyte/macrophage cells of the hematopoietic lineage (13). Osteoclasts attach to the bone matrix and degrade the mineral and organic components of bone. Since, the last decade considerable information has been accumulated regarding signaling pathways and mechanisms involved in differentiation and activation of osteoclasts (31). RANKL is a major cytokine implicated in osteoclast formation and differentiation. Unregulated RANKL signaling has been reported during various conditions such as ageing, osteoporosis, arthritis and cancer-induced bone loss (30, 5). Natural products are a great source of efficacious and affordable lead

compounds for drug development (16). Piperine, an alkaloid found in *Piper nigrum* Linn. is such a compound with several bioactive properties (32).

Osteoclast formation is a multi-step process tightly regulated by the MAPKs (p38, JNK and ERK) and NF- pathways leading to the induction and expression of c-Fos and NFATc1 (33, 8). Especially, NFATc1 plays a key role at terminal stages of osteoclast differentiation (34). Abrogation of any of these pathways severely alleviates osteoclast formation and bone resorption (35-37). Our results demonstrate that piperine perturbed the RANKL-induced phosphorylation and activation of p38 MAPKs without affecting the activation of JNK, ERK and NF- pathways. Earlier published studies have shown that p38-mediated pathway plays an indispensable role in osteoclast formation and pharmacological inhibition of p38 activation severely blunts osteoclastogenesis (35, 38). Moreover, p38 has been reported to be crucial for the induction of c-Fos and NFATc1 during RANKL-induced osteoclast formation (39).

c-Fos is a critical transcription factor involved in osteoclastogenesis and induction of downstream genes related with osteoclast differentiation (40). c-Fos knockout mice develop severe osteopetrotic phenotype with complete deficiency of osteoclasts (41, 42). In the present study, we found that piperine drastically attenuated the RANKL-induced expression of c-Fos in osteoclast progenitors. It has been reported elsewhere that downregulation of c-Fos by 1, 25-dihydroxyvitamin D3 results in suppression of osteoclastogenesis (43). Thus c-Fos is a crucial target for inhibiting osteoclastogenesis. NFATc1 is one of the most downstream transcription factors involved in osteoclast formation (34). Expression of NFATc1 has been reported to occur downstream of c-Fos followed by auto-amplification of NFATc1 by its own activity (44, 34). NFATc1 knockout mice completely lack osteoclasts and are osteopetrotic (34). Our results in this study demonstrate that piperine negatively regulated the NFATc1 expression. Hence, the anti-osteoclastogenic effects of piperine could be due to its inhibition of p38 signaling pathway that consecutively led to the downregulated expression of c-Fos and NFATc1.

Breast cancer cells are reported to frequently migrate to bone and express proteins that aid in attachment to bone including chemokines (CXCR4) and increase osteolysis via osteoclasts (45). Osteoclastogenesis has also been reported to be triggered by pathways alternative to RANKL such TNF- induced osteoclastogenic pathway (46). Earlier lines of evidence suggest that breast cancer cell lines employed in this study (MDA-MB-231 and MCF-7) do not express RANKL (47). However, secretion of TNF- in the conditioned medium of these cell lines has been reported (48). Additionally, Kwak et al. and Zwerina et al., in separate studies reported that p38 signaling pathway plays a critical role downstream of TNF- in osteoclast differentiation and inflammatory bone loss (49, 50). Inhibition of p38 significantly reduced clinical signs of TNF- mediated arthritis (50). Our results from this study indicate that piperine inhibited osteoclastogenesis induced by the conditioned-medium derived from the breast cancer MDA-MB-231 and MCF-7 cells. Hence, it is possible that piperine suppressed osteoclast formation induced by breast cancer cells through inhibition of p38 activation.

Anti-resorptive and anabolic therapies are often prescribed in conjunction to patients for increasing bone mass and preventing bone loss. Our results indicate that piperine did not exert cytotoxic effects on osteoblast-like cells supporting its possible efficacy in combination with anabolic agents. Current therapeutic options available for bone loss treatments either involves usage of anti-osteoclastogenic therapies such as bisphosphonates, denosumab or anabolic therapies such as parathyroid hormone (PTH) (51). Long-term usage of bisphosphonates results in

toxic side effects such as renal impairment and osteonecrosis of the jaw which outweigh the positive effects (52, 53). Furthermore, denosumab a humanized monoclonal antibody to RANKL is used as a therapy for patients with post-menopausal osteoporosis and cancer induced bone loss. Usage of this therapy is often implicated with skin problems (eczema, infections), hypocalcemia and osteonecrosis of the jaw (54). Additionally, usage of antibody for therapeutic purposes is costly, unaffordable and often out of reach to many patients. Therefore, cheaper, affordable therapeutic options with minimal or no side effects are much warranted for treating bone loss.

Hence, our study demonstrates that the plant-derived small molecule piperine alleviates osteoclast formation induced by RANKL and breast cancer cells in RAW264.7 macrophages *in vitro* and by RANKL in human CD14⁺ monocytes *ex vivo*. Piperine could be a potential lead compound and a novel source for synthesizing analogues for anti-osteoclastogenic drug development.

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Figure Legends

Figure 1. Effect of piperine on the viability of RAW264.7 macrophages and CD14+ monocytes. **A)** Molecular structure of piperine. **B)** Cell viability of piperine-treated RAW264.7 macrophages (5×10^3 /well) and **C)** CD14+ monocytes (1×10^4 /well with 25 ng/ml M-CSF). Cells were treated with indicated concentrations of piperine for 48h, 72h, 96h and cell viability was measured using alamar blue assay. Data are expressed as mean \pm SD percent of control and are representative of three independent experiments performed in triplicate.

Figure 2. Piperine inhibits osteoclastogenesis in RAW264.7 macrophages and CD14+ monocytes. **A)** RAW 264.7 macrophages (5×10^3 /well) were differentiated with RANKL (15 ng/ml) alone or in combination with piperine at indicated concentrations for 5 days. TRAP+ cells containing three or more nuclei were counted as osteoclasts. Osteoclasts stain purple/pink in the presence of TRAP (scale bars: 50 μ m). **B)** The numbers of TRAP+ multinucleated osteoclasts/well from RAW264.7 macrophages were counted and represented in a graphical format. **C)** CD14+ monocytes (4×10^4 /well) were differentiated with RANKL (15 ng/ml) and M-CSF (25 ng/ml) or in combination with piperine at indicated concentrations for 14 days. **D)** The number of TRAP+ osteoclasts formed were counted and represented in a graphical format (scale bars: 50 μ m). The results are mean \pm SD and are representative of three independent experiments performed in triplicate. (* P <0.05, ** P <0.01, *** P <0.001 versus RANKL).

Figure 3. Piperine attenuates osteoclastogenesis from the onset of the differentiation. **A)** RAW 264.7 macrophages (5×10^3 /well) were differentiated with RANKL (15 ng/ml) alone or in combination with piperine (100 μ M) for 3, 4 or 5 days and stained for TRAP (scale bars: 50 μ m). **B)** Quantification of multinucleated osteoclasts (containing three or more nuclei) after treatment with RANKL alone or RANKL plus indicated concentrations of piperine for 3, 4, or 5 days. Values represent mean \pm SD and are representative of three independent experiments performed in triplicate. (* P <0.05, ** P <0.01, *** P <0.001 versus RANKL).

Figure 4. Piperine inhibits osteoclastogenesis at an early stage of differentiation. **A)** RAW 264.7 macrophages (5×10^3 /well) were differentiated with RANKL (15 ng/ml) alone (topmost panel) or in combination with piperine (100 μ M) as indicated and stained for TRAP on the fifth day of differentiation. **B)** Multinucleated osteoclasts containing three or more nuclei were counted (scale bars: 50 μ m). Values represent mean \pm SD and are representative of three independent experiments performed in triplicate. (* P <0.05, *** P <0.001 versus RANKL).

Figure 5. Piperine inhibits actin ring formation and bone resorption. RAW264.7 macrophages were differentiated into osteoclasts in the presence of RANKL alone or with piperine (100 μ M) and, **A)** were stained for actin ring formation with phalloidin (scale bars: 10 μ m) or **B)** were tested for resorption pit formation on osteoassay plates (scale bars: 50 μ m). **C)** Resorption pit formation was quantified with ImageJ software. Arrows represent resorbed area. Results are representative of two independent experiments.

Figure 6. Piperine suppresses the phosphorylation and activation of p38. RAW264.7 macrophages were pretreated with or without piperine (1000 μ M) for 4h prior to RANKL (35 ng/ml) stimulation at the indicated time

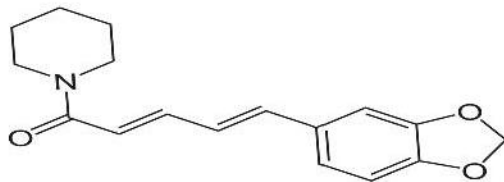
points. Cell lysates were analyzed by western blot analysis with indicated antibodies. Results are representative of three independent experiments. Band intensities were calculated using ImageJ software.

Figure 7. Piperine suppresses RANKL-induced c-Fos and NFATc1 expression. **A,B)** RAW264.7 macrophages were cultured in the presence of RANKL alone or with piperine (100 μ M) for the indicated time periods and whole cell lysates were subjected to western blot analysis with indicated antibodies. Results are representative of two independent experiments. Band intensities were calculated using ImageJ software.

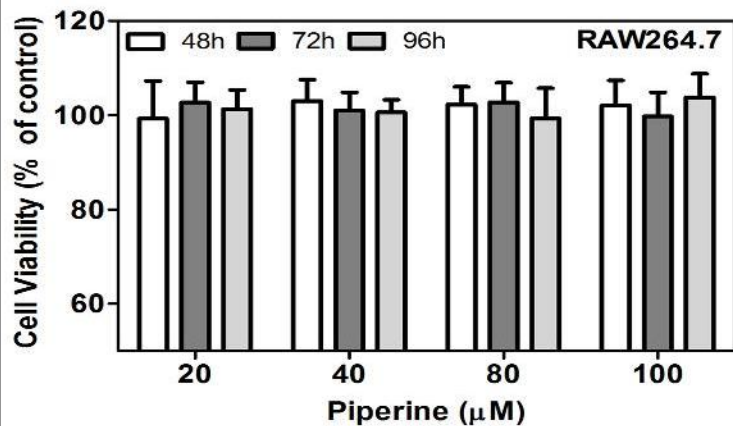
Figure 8. Piperine suppresses osteoclastogenesis induced by breast cancer cells. RAW 264.7 macrophages (5×10^3 /well) were differentiated for 7 days in the presence of 10% CM from **A)** MCF-7 or **B)** MDA-MB-231 breast cancer cells alone or in the co-presence of piperine (100 μ M). Resultant TRAP+ osteoclasts containing three or more nuclei were counted (scale bars: 50 μ m). Values represent mean \pm SD and are representative of three independent experiments performed in triplicate. (***) $P < 0.001$ versus CM).

Figure 9. Effect of piperine on the viability of MC3T3-E1 cells. Cells were treated with the indicated concentrations of piperine for 48 h, cell viability was measured using alamar blue assay. Data are expressed as mean \pm SD percent of control and are representative of three independent experiments performed in triplicate.

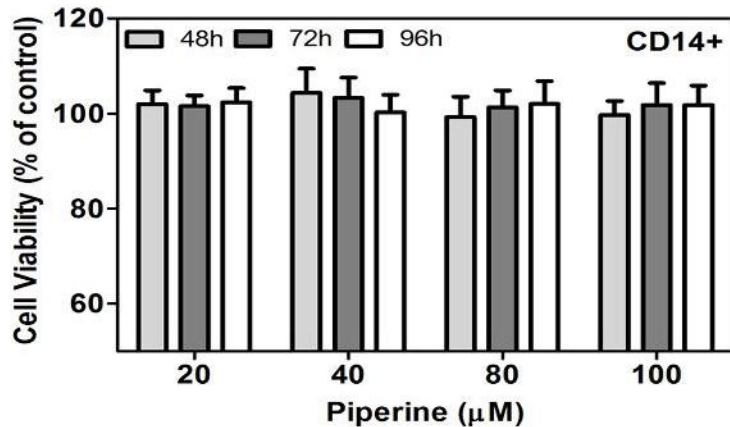
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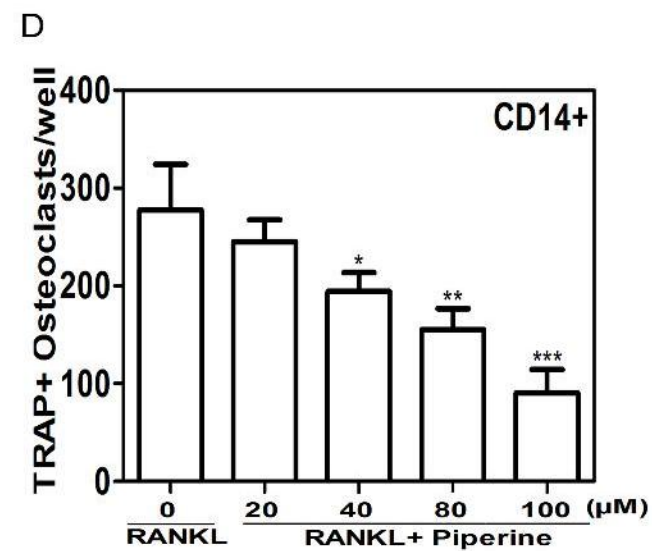
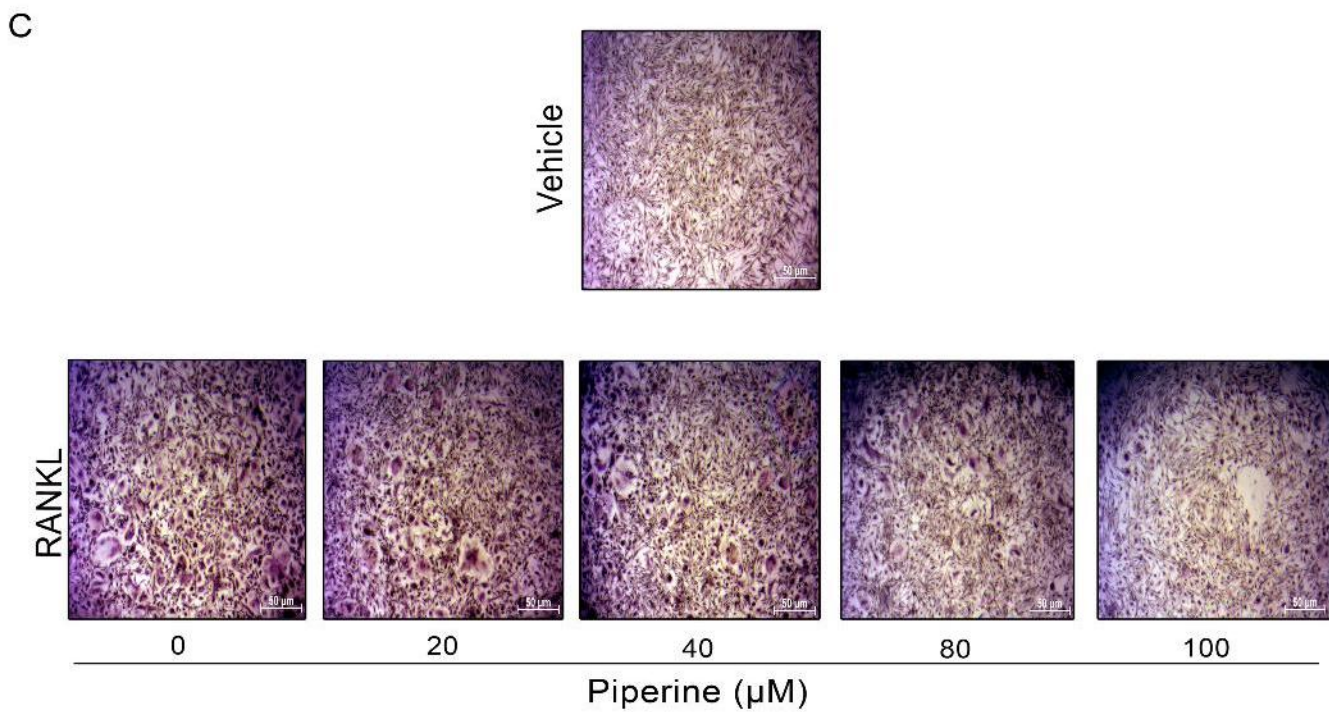
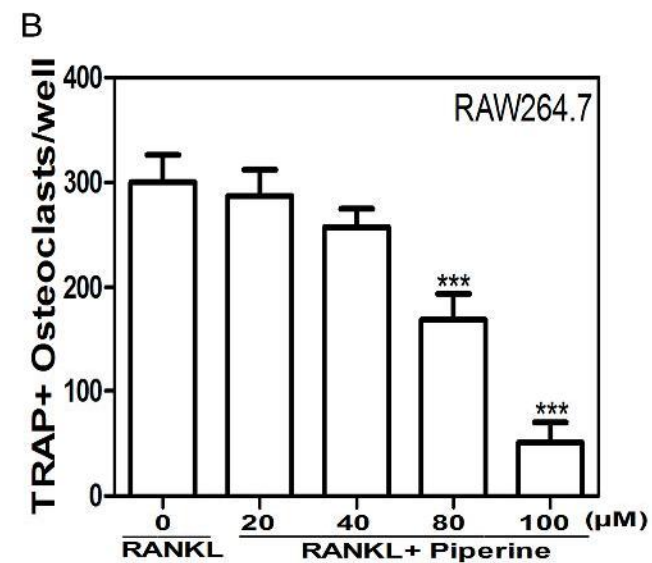
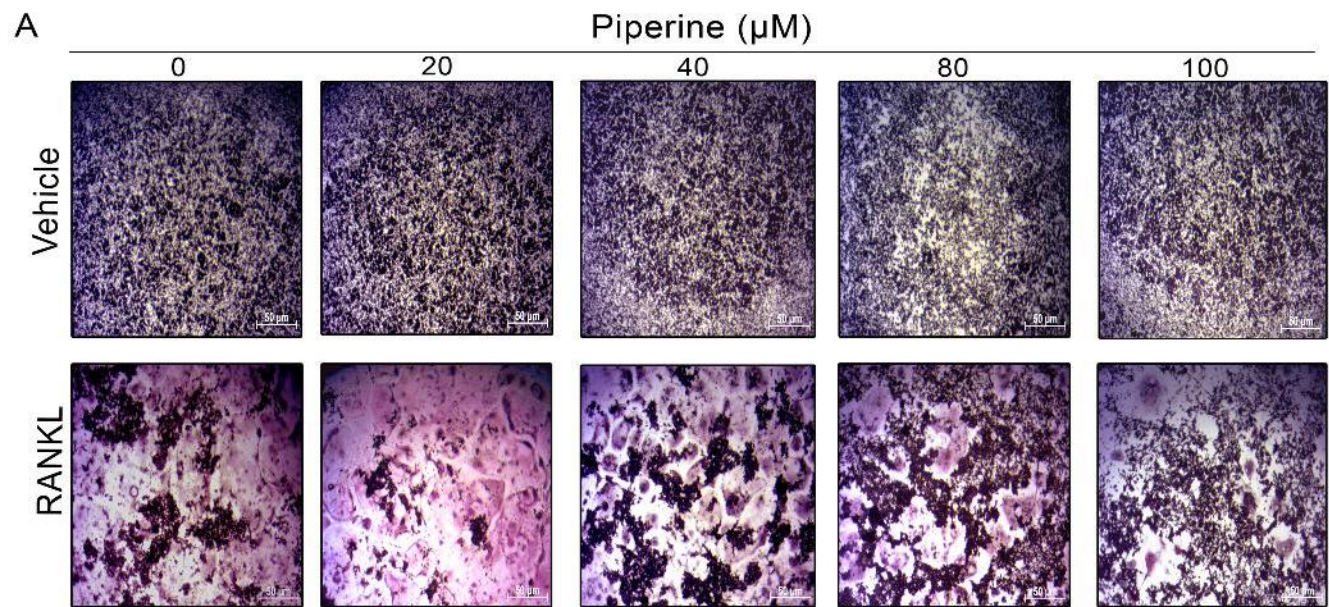


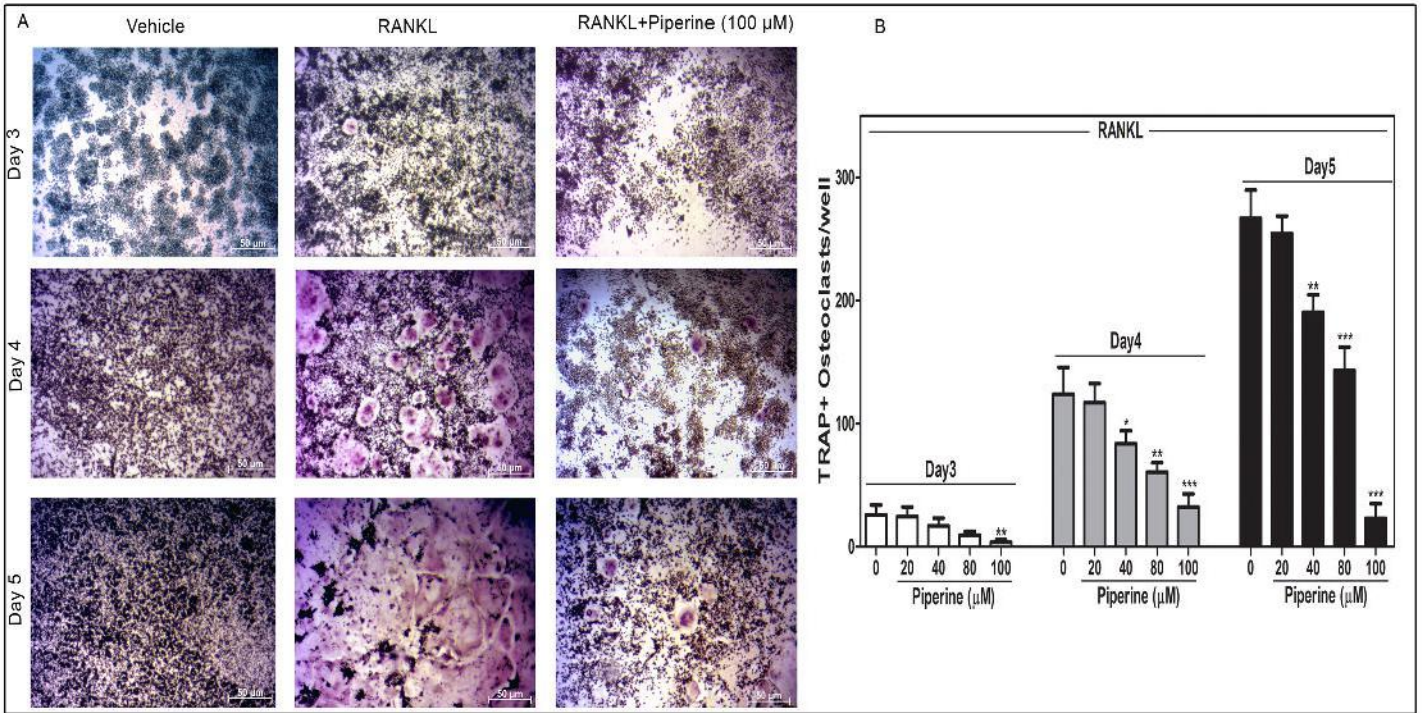
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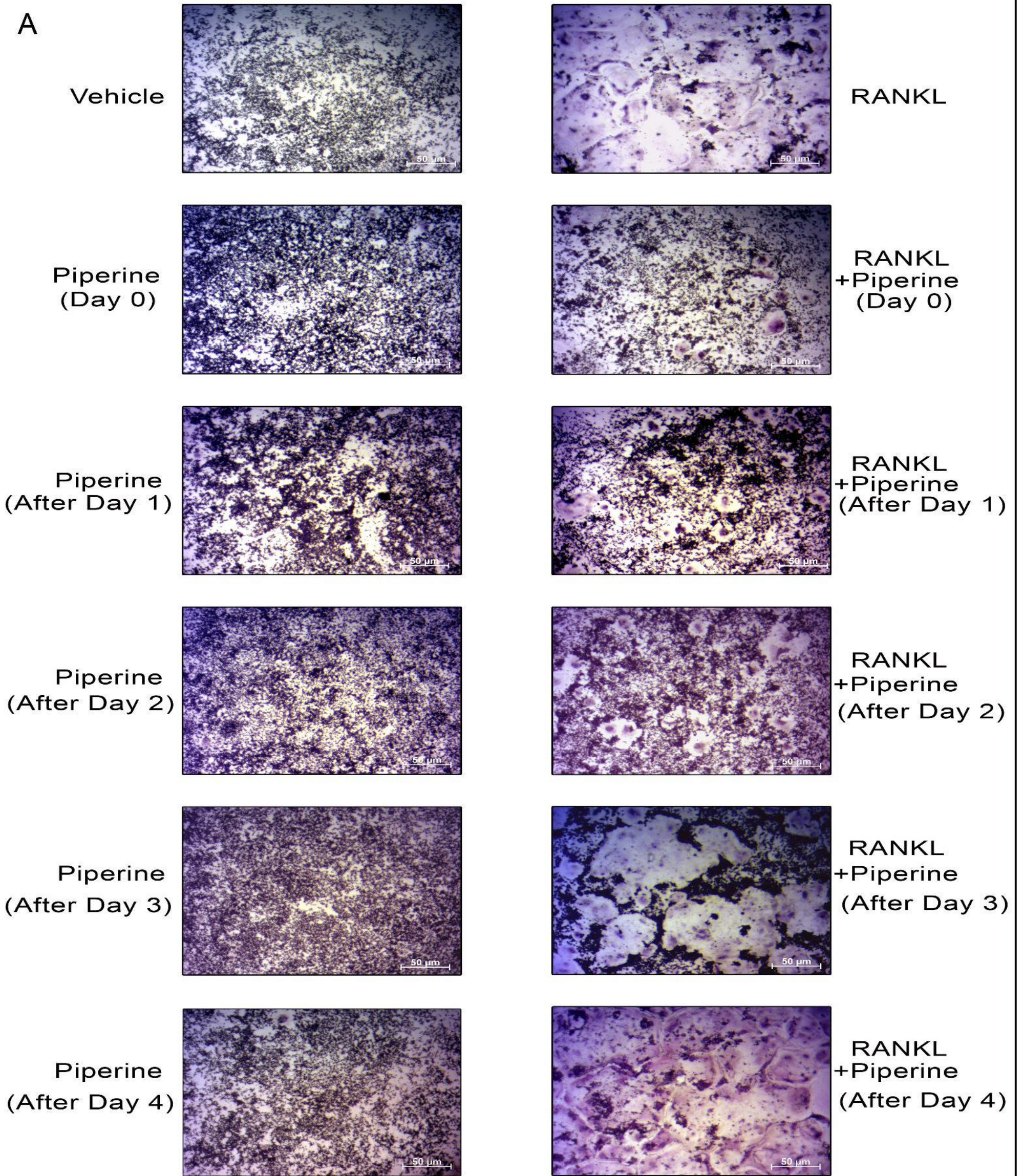
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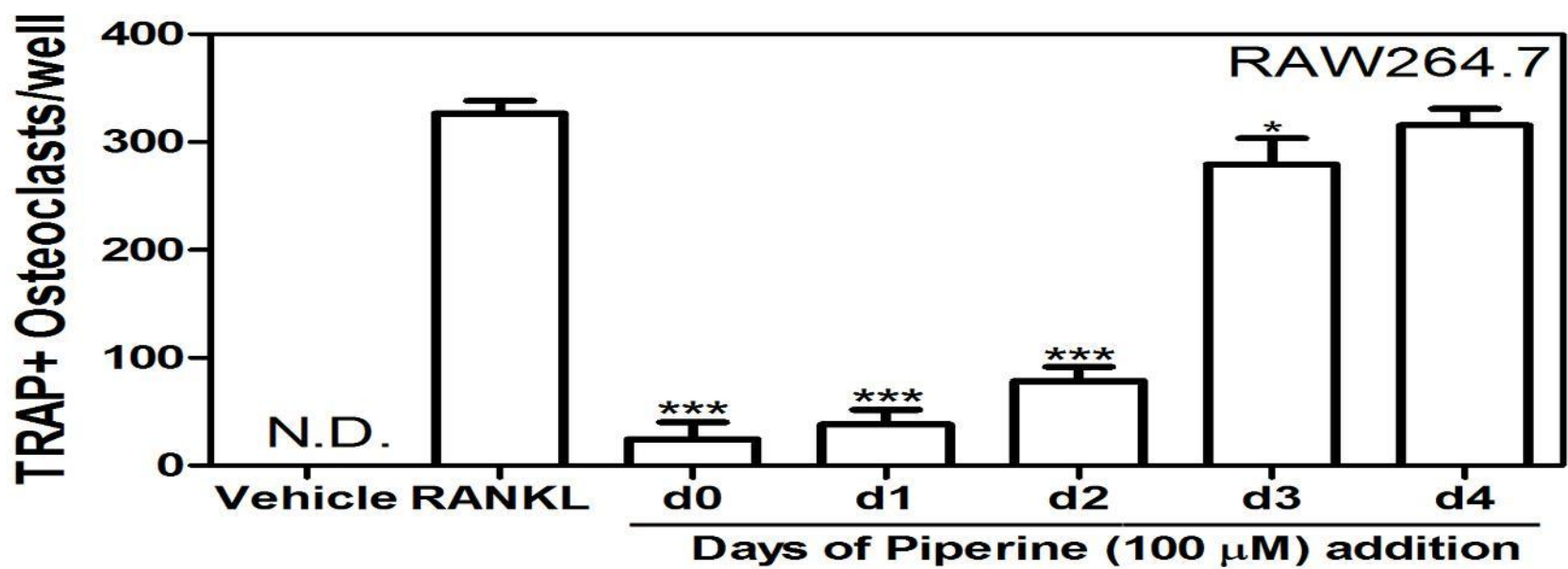


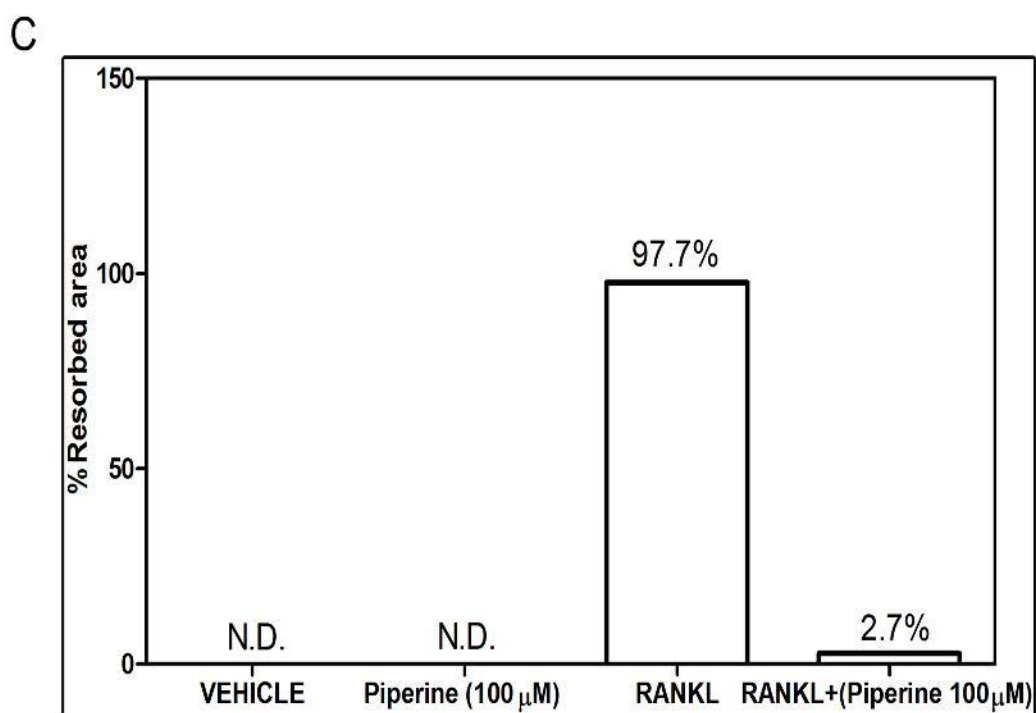
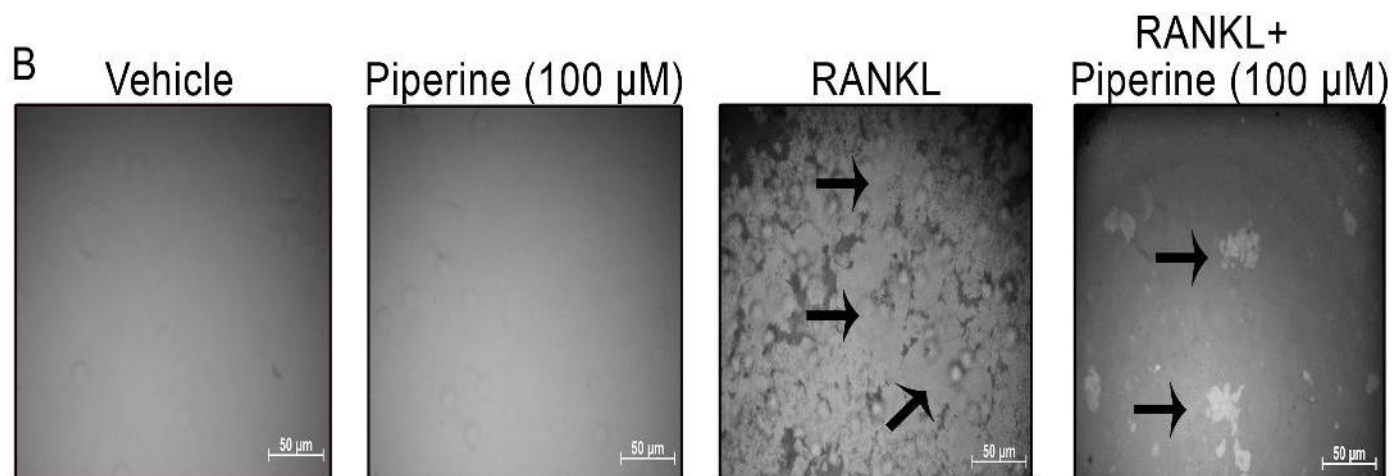
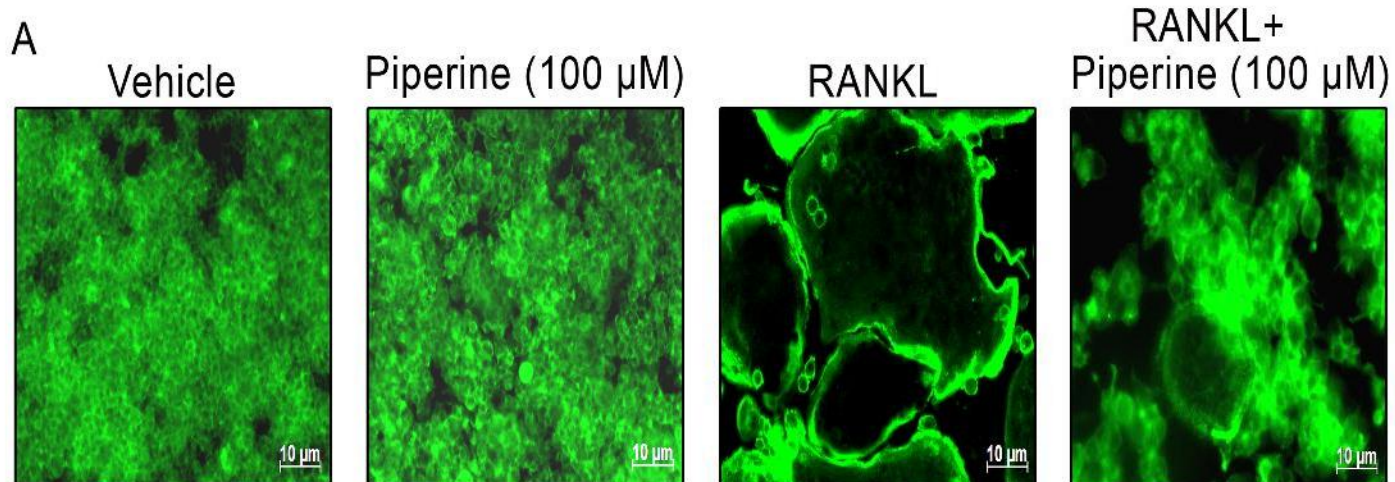


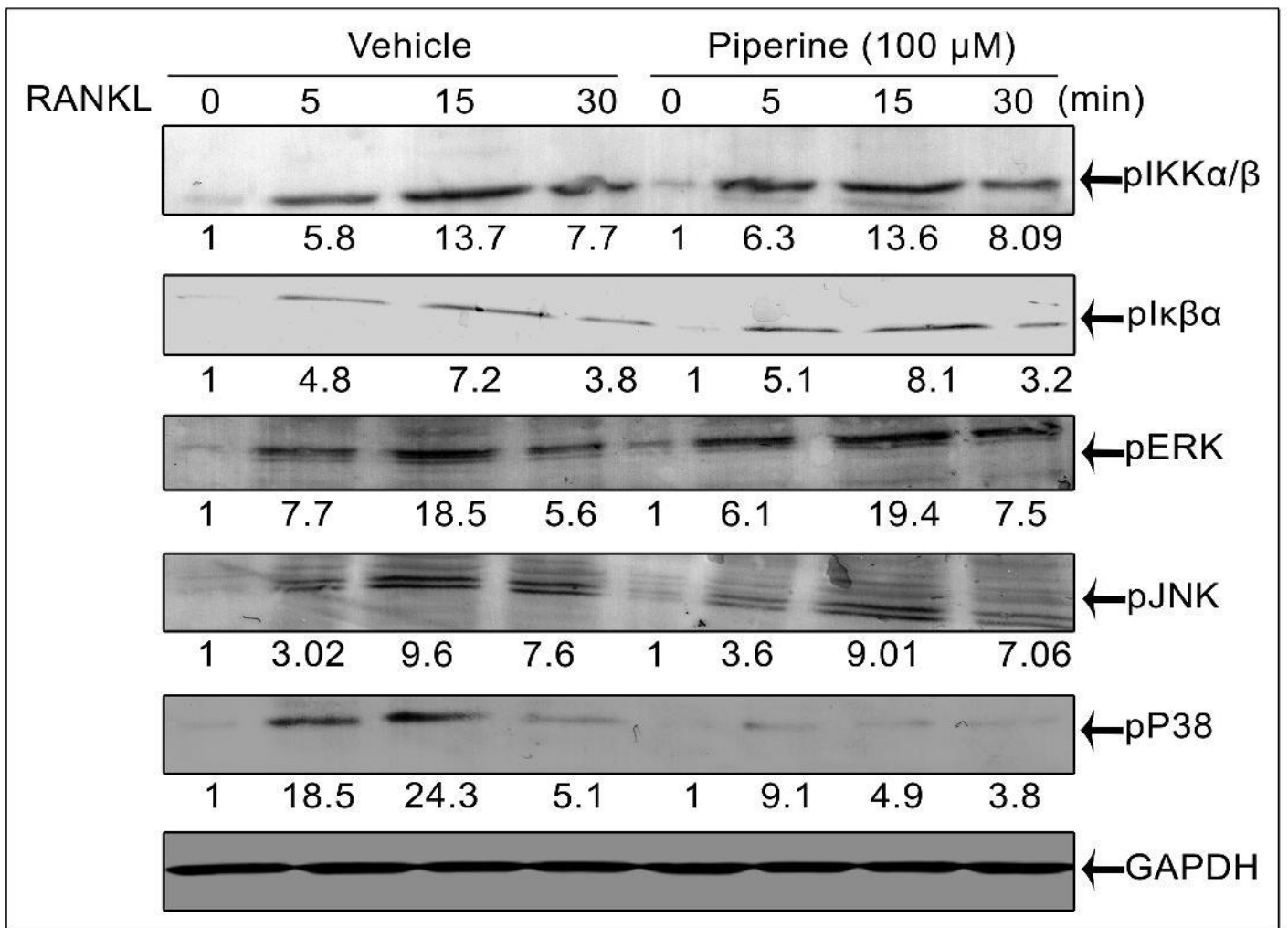
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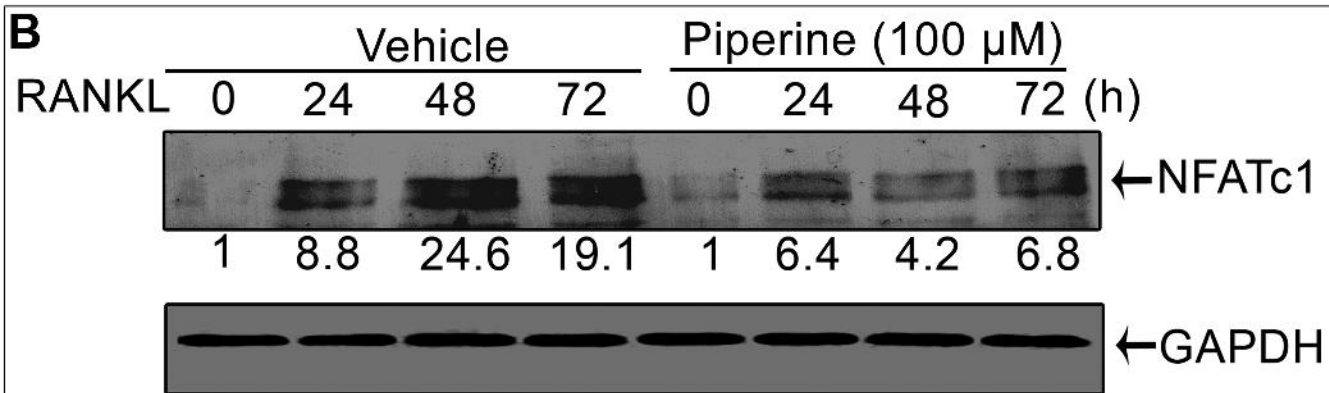
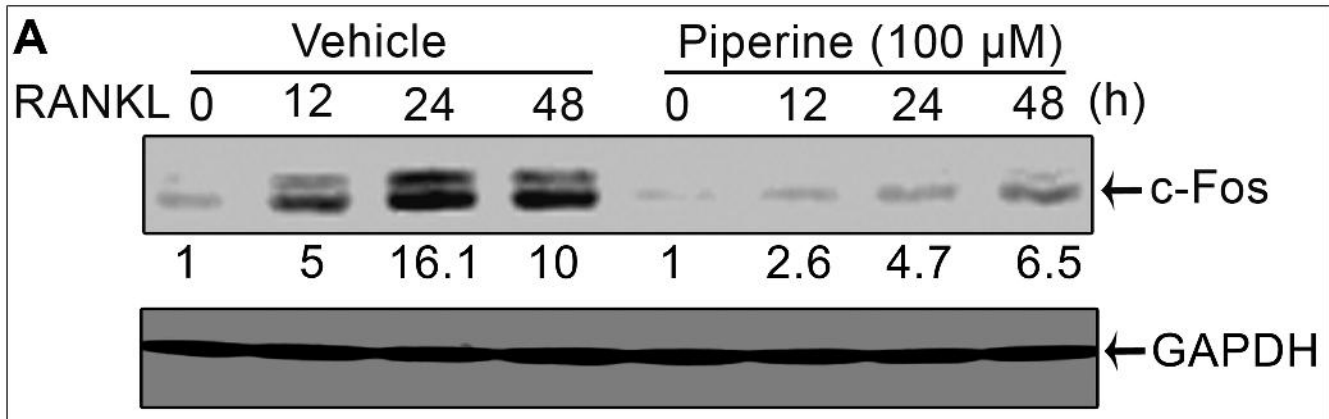


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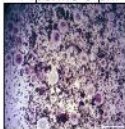
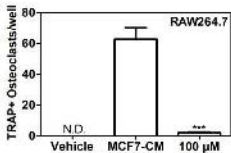




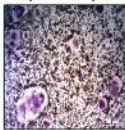


A**+MCF-7 (10% CM)**

Vehicle

Piperine 0 μM Piperine 100 μM **B****+MDA-MB-231 (10% CM)**

Vehicle

Piperine 0 μM Piperine 100 μM 