Research Article

The Coumarin Derivative 5′-Hydroxy Auraptene Suppresses Osteoclast Differentiation via Inhibiting MAPK and c-Fos/NFATc1 Pathways

Basem M. Abdallah,[1,2] **Enas M. Ali**,[1,3] **Hany Elsawy**,[4,5] **Gehan M. Badr**,[1,6] **Ashraf M. Abdel-Moneim**,[1,7] **and Abdullah M. Alzahrani**[1]

[1]*Biological Sciences Department, College of Science, King Faisal University, Hofuf, Saudi Arabia*
[2]*Endocrine Research (KMEB), Department of Endocrinology, Odense University Hospital and University of Southern Denmark, Odense, Denmark*
[3]*Department of Botany and Microbiology, Faculty of Science, Cairo University, Cairo, Egypt*
[4]*Department of Chemistry, Faculty of Science, King Faisal University, Hofuf, Saudi Arabia*
[5]*Department of Chemistry, Faculty of Science, Tanta University, Tanta, Egypt*
[6]*Department of Zoology, Faculty of Science, Ain Shams University, Cairo, Egypt*
[7]*Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt*

Correspondence should be addressed to Basem M. Abdallah; babdallallah@kfu.edu.sa

Received 18 September 2019; Revised 1 December 2019; Accepted 12 December 2019

Academic Editor: Takashi Yazawa

The phytochemical substances, coumarin derivatives, have demonstrated antiresorptive bone effects by suppressing osteoclast differentiation *in vitro* and *in vivo*. Recently, we have identified 5′-hydroxy auraptene (5′-HA), a coumarin derivative isolated from *Lotus lalambensis* Schweinf, as a novel stimulator for osteoblast differentiation. In this study, we investigated the effect of 5′-HA on osteoclast differentiation of mouse bone marrow (BM) cells. The effect of 5′-HA on BM cell proliferation and osteoclast differentiation was determined by measuring cell viability and tartrate-resistant acid phosphatase (TRAP) enzyme activity, quantification of $TRAP^+$ multinucleated cells ($TRAP^+$MNCs), and quantitative real-time PCR (qPCR) of osteoclastic gene expression. Regulation of NF-κB, c-Fos/NFATc1, and MAPK signaling pathways by 5′-HA during osteoclastogenesis was measured by the NF-κB reporter assay and Western blot analysis. 5′-HA significantly suppresses the receptor activator of NF-κB ligand (RANKL) induced osteoclast differentiation of BM cells in a dose-dependent manner. Consistently, treatment of BM cells with 5′-HA significantly inhibited RANKL-induced activation of NF-κB and c-Fos/NFATc1 pathways in a dose-dependent manner. Furthermore, RANKL-induced phosphorylation of ERK1/2, p-38, and JNK was significantly inhibited by 5′-HA in BM cells. In conclusion, we identified 5′-HA as a novel coumarin derivative that suppresses RANKL-induced osteoclastogenesis via inhibiting c-Fos/NFATc1 and MAPK signaling pathways.

1. Introduction

Osteoporosis is an endocrine-metabolic bone disease that is characterized by disorders in the bone remodeling process toward increased bone resorption by osteoclasts over the expenses of bone formation by osteoblasts [1]. Reduced bone mass and strength in osteoporotic patients' resulted in increased susceptibility of bone fractures, which is a major health problem in the elderly population [2, 3].

Osteoclasts are multinucleated cells that are derived from myeloid precursors upon the stimulation with cytokines, macrophage colony stimulating factor (MCS-F), and RANKL that are secreted by osteoblast lineage [4, 5].

The interaction between RANKL and osteoclast precursor cell surface RANK receptors triggers the signals of osteoclast differentiation via NF-κB and MAPK pathways, which in turn recruit tumor necrosis factor (TNF) and receptor-associated factors and activate downstream

signaling of the nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), a master regulator of osteoclast differentiation. NFATc1 plays a vital role in osteoclast maturation and activation via upregulating the gene expression of osteoclastic-related genes including matrix metalloproteinase (*Mmps*), *Trap*, and Cathepsin K (*Ctsk*) [6–9]. Thus, targeting osteoclast formation and differentiation via inhibiting NF-κB and MAPK signaling are an effective strategy toward preventing bone loss related diseases [10, 11].

In this context, several studies have demonstrated biological and pharmacological activities of plant-derived coumarin derivatives including antibacterial, antifungal, anti-inflammatory, antioxidative, and antitumor effects [12–14]. Interestingly, numerous coumarin derivatives demonstrated *in vitro* and *in vivo* antiresorptive effects. Natural coumarin derivatives including daphnetin, psoralen, and wedelolactone were reported to exhibit an inhibitory effect on osteoclastic bone resorption [15, 16]. Furthermore, psoralidin, osthole, and aesculin were protective against bone loss in osteoporotic mouse models [17–19]. Recently, we identified a coumarin derivative, 5′-HA ([7-(5-hydroxy-3,7-dimethylocta-2,6-dienyloxy)-chromen-2-one]), as a novel compound that functions to stimulate osteoblast differentiation from BM-derived stromal stem cells in BMP-dependent mechanism [20]. To provide more detailed information on the effect of 5′-HA on bone metabolism, we aimed in this study to investigate the effect of 5′-HA on osteoclast differentiation of murine BM cells as well as to elucidate its molecular mechanism.

2. Materials and Methods

2.1. Extraction and Purification of 5′-HA. 5′-HA was extracted from *Lotus lalambensis* Schweinf (collected from Saudi Arabia).

The extraction and identification of 5′-HA were performed as described previously by our group [20].

2.2. Osteoclast Culture. Bone marrow (BM) cells were isolated from 8-week-old male C57BL/6J mice as described previously [21]. Mice were bred and housed at the animal housing unit (College of Science, King Faisal University, Saudi Arabia) under standard conditions (21°C, 55% relative humidity) on a 12-hour light/12-hour dark cycle, and ad libitum food (Altromin® Spezialfutter GmbH & Co., Germany) and water were provided in accordance with the ethical clearance of the Standing Committee on Research Ethics. Mice were sacrificed by cervical dislocation and BM was flushed out from tibia and femur. BM was centrifuged for 1 min at 400 g and filtrated through a 70 μm nylon mesh filter. BM cells were then plated in 96-well plates at a density of 1×10^6 cells/well in osteoclast differentiation medium (ODM) containing α-MEM (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco BRL), 100 U/mL of penicillin (Gibco BRL), 100 μg/mL of streptomycin (Gibco BRL), 25 ng/mL of recombinant M-CSF (R&D Systems, Minneapolis, MN, USA), and 25 ng/mL of recombinant RANKL (Pepro-Tech, Rocky Hill, NJ, USA) to induce osteoclast formation. Cells were maintained at 37°C in a 5% CO_2 incubator, and the medium was changed every 3 days.

2.3. Cell Viability Assay. BM cells were cultured in 96-well plates and then treated with different concentrations of 5′-HA for 3 days in the presence or the absence of RANKL and MCS-F. Cell viability was determined using the CellTiter-Blue® cell viability assay according to the manufacturer's instructions (Promega, USA) at OD 579.

2.4. Tartrate-Resistant Acid Phosphatase (TRAP) Staining. BM cells were plated in 96-well plates and induced to osteoclast differentiation as described above. TRAP staining was performed at different time points according to the manufacturer's instruction for using the acid phosphatase, leukocyte (TRAP) kit (Sigma-Aldrich, Germany). $TRACP^+$ MNCs containing more than three nuclei were considered to be osteoclasts and were evaluated using a reflected light microscope.

2.5. Measurement of TRAP Enzyme Activity. BM cells were induced to osteoclasts as mentioned above. At different time points, cells were washed with PBS, lysed in 30 μL 0.1% Triton X-100 for 10 min, and a substrate solution of 100 μL of PNPP (2 g/L *p*-nitro-disodium phenylphosphate, 7.6 g/L sodium L-tartrate, pH 5.2) was added. Cells were then incubated at 37°C for 30 min, and the reaction was stopped by the addition of 1 M NaOH. TRACP enzyme activity was measured at 405 nm absorbance in a microplate reader.

2.6. Osteoclast Bone Resorption Measurement. The activity of differentiated osteoclast was performed by the bone resorption assay kit (Cosmo Bio. Co. Ltd., Japan) according to the manufacturer's instructions. BM cells were cultured on fluoresceinated calcium phosphate-coated 24-well plates in the presence of M-CSF and RANKL without or with 5′-HA for 6 days. Fluorescent released from the calcium phosphate layer into conditioned medium due to osteoclast resorption activity was measured by detecting the fluorescence intensity at an emission wavelength of 535 nm.

2.7. Luciferase Reporter Assay. The modulation of NF-κB pathway was determined by using Cignal™ NF-κB luciferase Reporter Assay Kit (Qiagen Ltd., Manchester, UK). BM cells were cultured in 96-well plates and transfected with a mixture of NF-κB luciferase reporter negative control or positive control, along with Renilla construct (as an internal control) according to the manufacturer's instructions using Lipofectamine 2000 (Thermo Fisher Scientific GmbH). Cells were induced with RANKL in the absence or the presence of different concentrations of 5′-HA and cultured for 24 h. Luciferase activities were determined using the Dual-Luciferase Assay System (Promega, Southampton, UK).

Reporter activities were represented after normalization to the internal Renilla reporter.

2.8. Western Blot Assays. Cells were lysed at different time points in cell lysis buffer [22], supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). 30 μg of protein was separated on 8% to 12% NuPAGE® Novex® Bis-Tris gel systems (Thermo Fisher Scientific GmbH, Dreieich, Germany). Gel was then transferred to PVDF membrane (Millipore, USA), blocked, and probed with antibodies (dil 1 : 1000). Proteins were visualized by ECL chemiluminescence (Thermo Fisher Scientific GmbH). Antibodies (for total or phosphor) ERK1/2 (sc-7383) were purchased from Santa Cruz Biotechnology. Specific antibodies for phosphor p38 MAPK (Thr180/Tyr 182) and JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology, Inc. USA, NFATC1 antibody from Thermo Fisher Scientific GmbH, and anti-TRAF6 antibody and c-Fos antibody from Abcam Biotechnology Company, Cambridge, UK. Quantification of Western blots was performed with ImageJ program.

2.9. RNA Extraction and Real-Time PCR Analysis. Total RNA was extracted from cultured cells using a single-step method of TRIzol (Thermo Fisher Scientific, Inc.) as described [23]. cDNA was synthesized from 1 μg of total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time PCR was performed with Applied Biosystems 7500 Real-Time system using Fast SYBR® Green Master Mix (Applied Biosystems, California, USA) with specific primers (Table S1). The expression of each target gene was normalized to *β-Actin* and *Hprt* mRNA expression as reference genes, using a comparative CT method ((1/(2delta-CT)) formula, where delta-CT is the difference between CT-target and CT-reference) with Microsoft Excel 2007®.

2.10. Statistical Analysis. All values were expressed as mean ± SD (standard deviation) of at least three independent experiments. The power calculation was performed for 2 samples using unpaired Student's *T*-test (2-tailed) assuming equal variation in the two groups. Differences were considered statistically significant at $^{*}P < 0.05$.

3. Results

3.1. Effect of 5′-HA on Cell Viability of RANKL-Induced BM Cells. To examine the effect of 5′-HA on osteoclastogenesis, we first established an osteoclast differentiation time point course for primary isolated murine BM cells. BM cells treated with M-CSF and RANKL displayed the formation of multinucleated osteoclasts (with more than 3 nuclei) in association with increasing TRAP enzyme activity in osteoclasts after 7 days of treatment (Figures 1(a) and 1(b)). We further studied the cytotoxicity of newly isolated 5′-HA compound (Figure 1(c)) on osteoclasts, by measuring cell viability of BM cells in the presence of M-CSF and RANKL with and without different concentrations of 5′-HA (1–100 μM) after 3 days in culture. Figure 1(d) shows that the toxic effect of 5′-HA started at the concentration above 50 μM. Thus, we used 5′-HA between 1 and 50 μM concentrations throughout this study.

3.2. 5′-HA Suppresses Osteoclast Differentiation. We studied the effect of 5′-HA on osteoclast differentiation of murine BM cells. Addition of 5′-HA to RANKL-induced BM cells showed to exert dose-dependent inhibitory effect on a number of $TRAP^{+}$MNCs (Figure 2(a)), as well as on TRAP enzyme activity (Figure 2(b)) during osteoclasts differentiation. Furthermore, 5′-HA exerted a dose-dependent inhibitory effect on osteoclast activity as measured by *in vitro* bone resorption assay in RANKL-induced BM cells (Figure 2(c)). These data demonstrated the inhibitory effect of 5′-HA on osteoclast differentiation and activity.

3.3. 5′-HA Inhibits NF-κB and c-Fos/NFATc1 Activation in RANKL-Induced BM Cells. Since, the binding of RANKL to its receptor RANK stimulates the osteoclast differentiation *in vitro* via the activation of NF-κB signaling pathway, we examined the effect of 5′-HA on RANKL-induced NF-κB reporter activity [24, 25]. As shown in Figure 3(a), 5′-HA significantly suppressed the NF-κB reporter luciferase activity in a dose-dependent manner (Figure 3(a)). We further examined the effect of 5′-HA on the protein expression of c-Fos and NFATc1, two master regulators of osteoclastogenesis. Our results demonstrated the dose-dependent inhibitory effect of 5′-HA on RANKL-induced c-Fos/NFATc1 protein expression in BM cells as assessed by Western blot analysis (Figure 3(b)). In addition, qPCR and Western blot analysis showed a significant inhibitory effect of 5′-HA on the expression TRAF6 (an upstream molecule of c-Fos/NFATc1) at both mRNA and protein expression levels in RANKL-induced BM cells.

3.4. 5′-HA Exerts Dose-Dependent Inhibitory Effect on mRNA Expression of Osteoclast Specific Genes. The activation of c-Fos/NFATc1 by RANKL was shown to upregulate a number of genes involved in osteoclast differentiation including *Ctsk*, *Trap,* and *Mmp9* [25, 26]. Thus, we studied the effect of 5′-HA on the mRNA expression of the above osteoclastic genes in BM cells treated with RANKL. Interestingly, 5′-HA significantly downregulated the mRNA expression of RANKL-induced *Nfatc1* and *c-Fos* genes and their target genes, *Ctsk*, *Trap,* and *Mmp9* in a dose-dependent manner as quantified by qPCR (Figures 4(a)–4(e)).

3.5. 5′-HA Suppresses the RANKL-Induced MAPK Pathway Activation. We further examined the effect of 5′-HA on RANKL-induced MAPK activation [27]. As shown in Figure 5(a), treatment of BM cells with RANKL for 20 min induced ERK, JNK, and p38 phosphorylation levels, the 3 major subfamilies of MAPKs signaling pathway as assessed by Western blot analysis. Interestingly, 5′-HA significantly

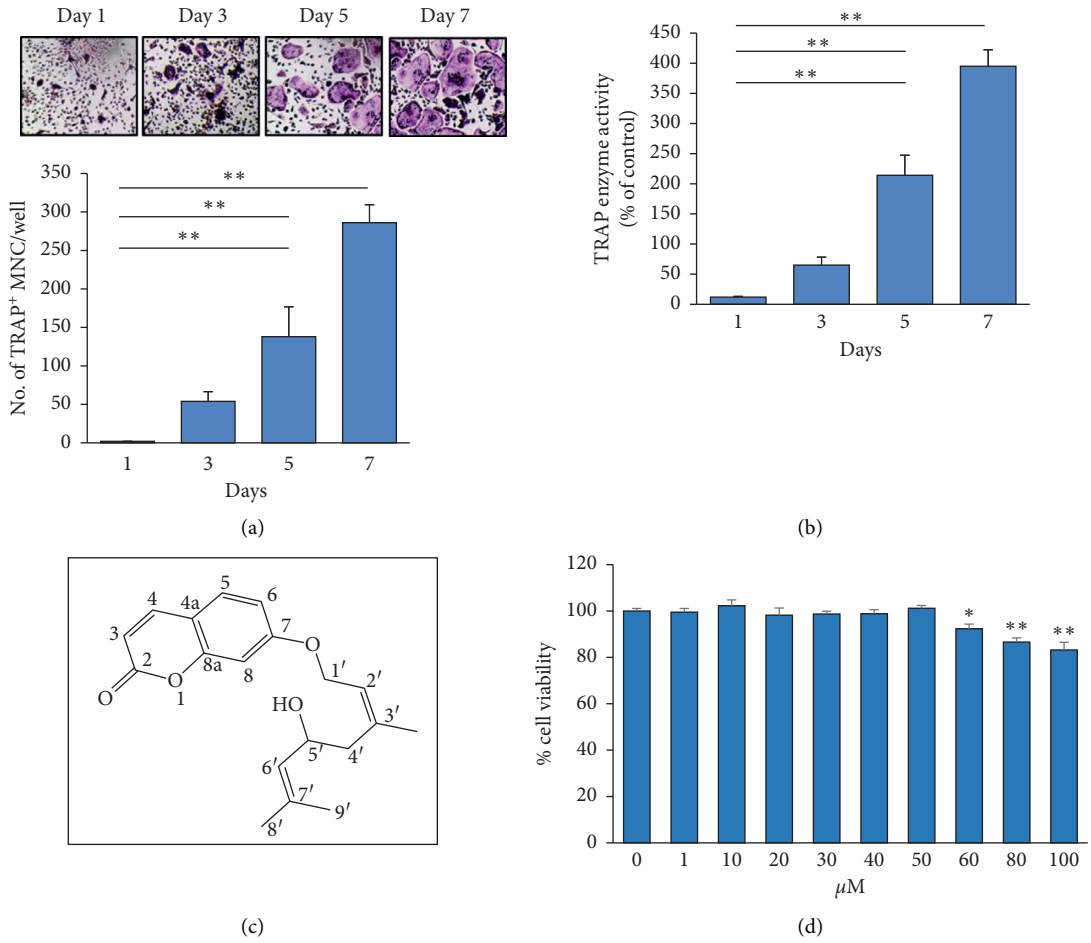


Figure 1: Cytotoxicity of 5′-HA on RANKL-induced osteoclastogenesis of BM cells. (a) Osteoclast differentiation of mouse BM cells. BM cells (mononuclear cells/macrophages) were induced to differentiate into osteoclasts with RANKL and M-CSF as described in Section 2. The TRACP$^+$ cells with more than three nuclei per cell were counted as multinucleated cells (MNCs) at different time points. Images of TRAP staining for MNCs were shown. (b) Quantitative TRAP activity at different time points during osteoclast differentiation of BM cells. (c) Cytotoxicity of 5′-HA on RANKL-induced BM cells. Cells were induced with RANKL and M-CSF in the absence (0) or the presence of different concentrations of 5′-HA and cell viability was measured by CellTiter-Blue® cell viability assay after 3 days in culture. Values are mean ± SD of three independent experiments ($^*P < 0.05$, $^{**}P < 0.005$ compared to day 1 for (a) and (b) and compared to RANKL-induced cells without 5′-HA for (c)).

suppressed the RANKL-induced ERK, JNK, and p38 activation by 65.3%, 43.2%, and 57.1%, respectively. To examine the involvement of MAPK pathway in mediating the inhibitory effect of 5′-HA on osteoclast differentiation, we measured the effect of blocking ERK, JNK, and p38 activation (by the specific inhibitors, U0126, SP600125, and SB203580, respectively) on RANKL-induced osteoclastogenesis in the absence and the presence of 5′-HA using TRAP enzyme activity. As shown in Figure 5(b), blocking of ERK, JNK, and p38 activation in the absence of 5′-HA, inhibited the RANKL-induced osteoclastogenesis by 42.5%, 31.4%, and 24.1%, respectively, while synergized the inhibitory effect of 5′-HA on osteoclastogenesis by 67.5%, 57.8%, and 63.1% respectively (Figure 5(b)). Thus, the inhibitory effect of 5′-HA on osteoclastogenesis is mediated partially via suppressing the MAPK pathway.

4. Discussion

Increased bone resorption by osteoclast is a characteristic feature of bone loss related diseases including osteoporosis, Paget's disease of bone, periodontal disease, rheumatoid arthritis, and cancer-associated bone disease [28, 29]. In this study, we identified coumarin derivative, 5′-HA, as a novel phytochemical inhibitor of RANKL-induced osteoclast differentiation of BM cells via suppressing c-Fos/NFATc1 and MAPKs signaling pathways.

Our data demonstrated the inhibitory effect of 5′-HA on RANKL-induced osteoclast differentiation of BM cells by significantly reducing the number of TRAP$^+$MNCs, TRAP activity, and NF-κB signaling pathway. Consistently, several naturally occurring coumarin compounds showed anti-osteoclastic bone resorption effects *in vitro* and *in vivo*. These

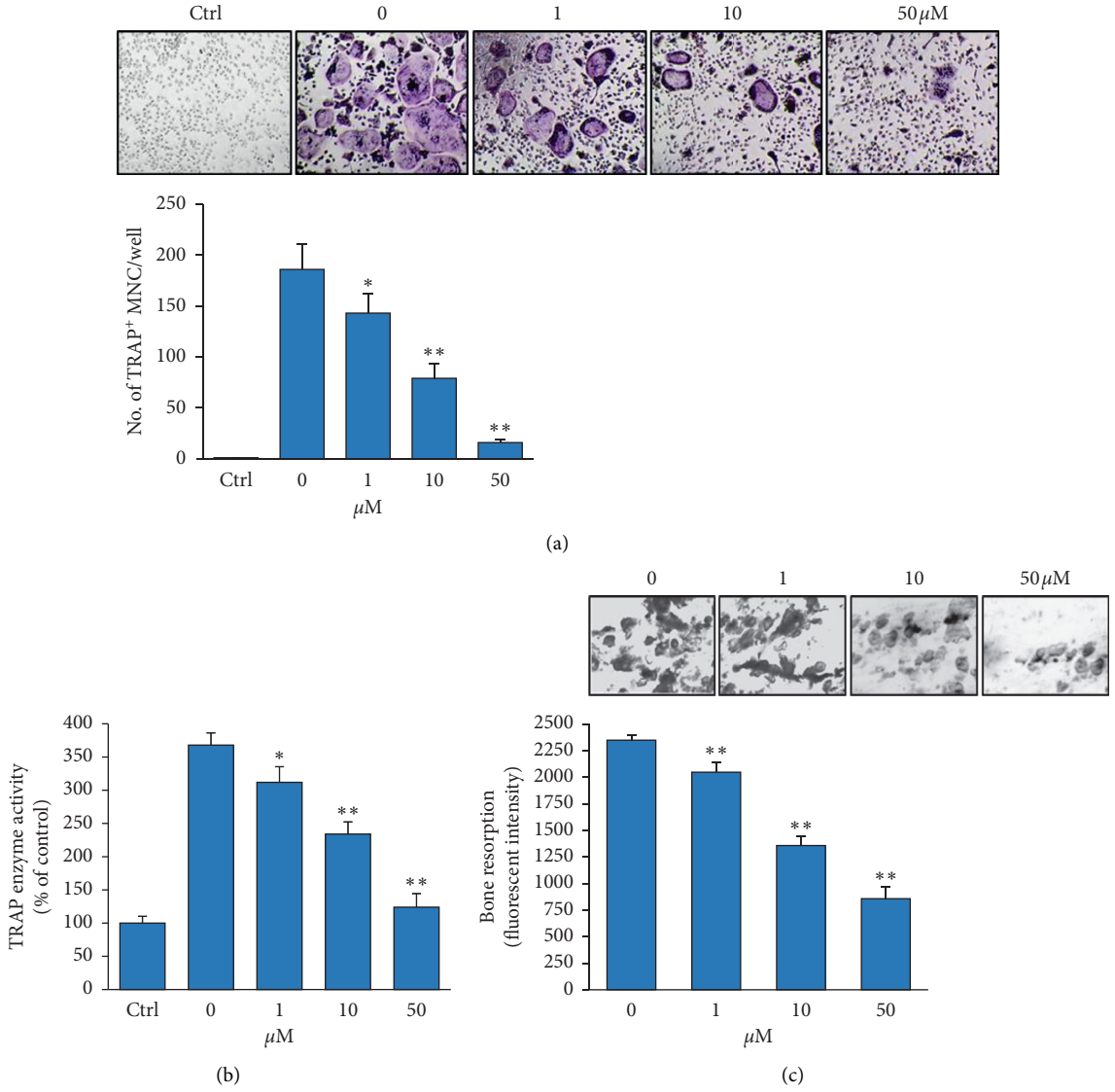


FIGURE 2: Inhibitory effect of 5′-HA on RANKL-induced osteoclastogenesis in BM cells. (a) Dose-dependent inhibitory effect of 5′-HA on osteoclast differentiation of BM cells as measured by quantification of the total number of $TRACP^+$MNCs and (b) quantitative TRAP activity. BM cells were induced to differentiate into osteoclasts without (Ctrl) or with RANKL and M-CSF in the absence (0) or the presence of different concentrations of 5′-HA for 7 days. Images of TRAP staining for MNCs were shown. (c) Dose-dependent inhibitory effect of 5′-HA on osteoclast bone resorption. BM cells were induced with RANKL in the absence or the presence of 5′-HA on fluoresceinated calcium phosphate-coated 24-well plates for 6 days as described in Section 2. Images of pit resorption assay were shown. Values are mean ± SD of three independent experiments ($^*P < 0.05$, $^{**}P < 0.005$ compared to RANKL-induced cells without 5′-HA).

include aesculin, psoralidin, daphnetin, bakuchiol, and esculetin [16, 19, 30, 31]. Thus, coumarin derivatives can be used as promising natural pharmaceutical agents for the treatment of osteoporosis.

After binding of RANKL to its RANK receptor on osteoclast precursors, RANKL transmits its osteoclast differentiation signals through NF-κB pathway and its mediator MAPK proteins, subsequently upregulating the expression of the transcription factors c-Fos and NFATc1 to stimulate the formation and activation of osteoclasts [6, 25]. RANKL was shown to activate all three MAPK family members ERK, JNK, and p38 in association with the stimulation of osteoclast differentiation [27, 28]. In this context, our data demonstrated that 5′-HA inhibits the RANKL-induced activation of NF-kB and MAPK subfamily including p38, JNK, and ERK. Similarly, coumarin derivatives, psoralidin and esculetin, were reported to inhibit RANKL-induced osteoclastogenesis by suppressing the activation of p38, JNK, and ERK [17, 19].

Our data demonstrated that the inhibitory effect of 5′-HA on osteoclastogenesis is mediated via downregulating the expression of both osteoclastic transcription factors, c-Fos and NFATc1. The c-Fos/NFATc1 pathway plays a vital role in osteoclast formation. NFATc1 upregulates the expression of several osteoclast specific genes [32]. c-Fos belongs to the Fos gene family, which is a component of the

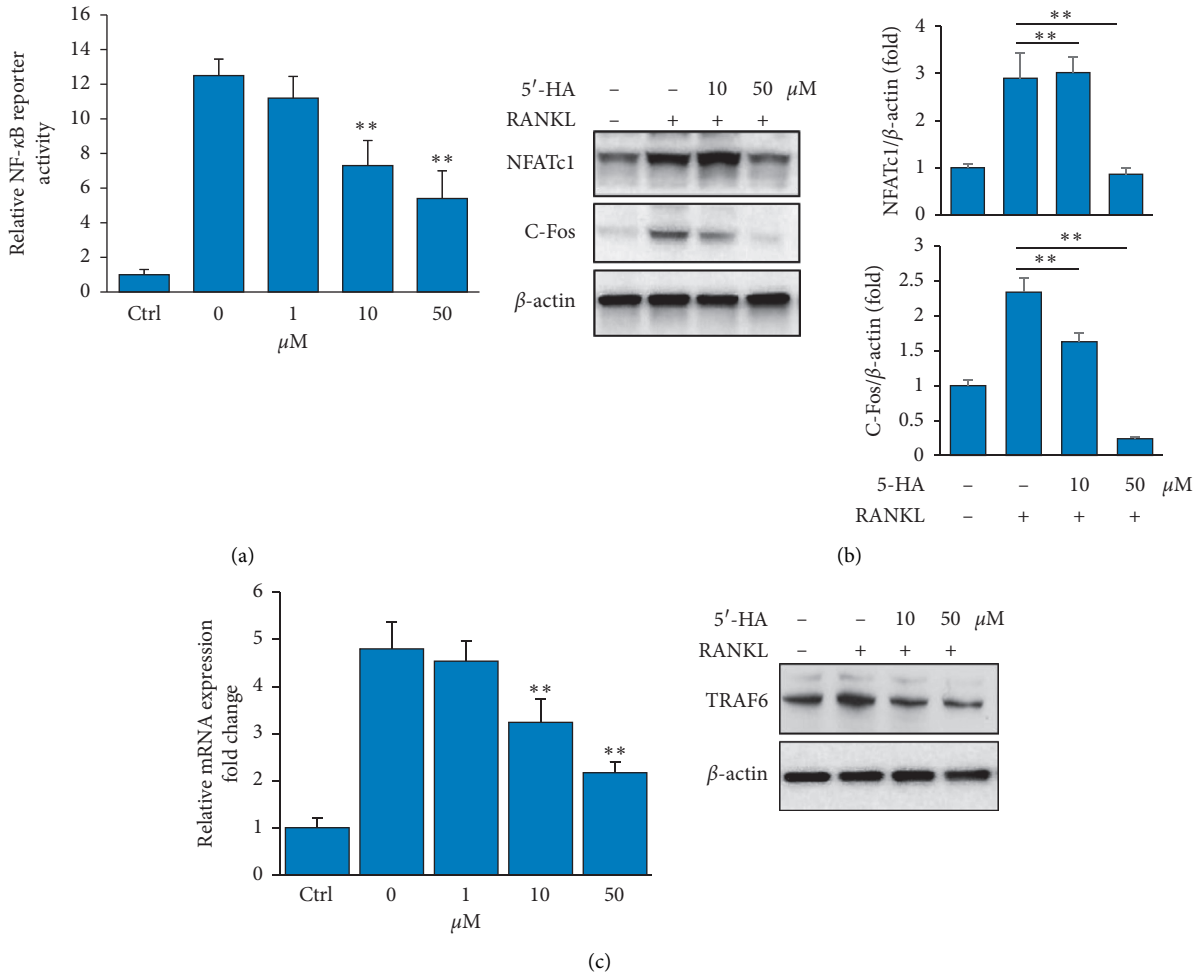


Figure 3: 5′-HA inhibits RANKL-induced NF-kB and c-Fos/NFATc1 pathway. (a) 5′-HA inhibits RANKL-induced NF-κB signaling activity. BM cells were transfected with Cignal NF-κB reporter negative control or positive control and without (Ctrl) or with RANKL and M-CSF in the absence (0) or the presence of different concentrations of 5′-HA for 24 hours. Reporter activity was represented after normalization to the internal Renilla reporter. (b) Western blot analysis of C-Fos and NFATc1 protein expression. Cells were pretreated with 5′-HA (50 μM) for 1 h and then stimulated with RANKL for 20 min. (c) qPCR and Western blot analysis of TRAF6 expression in RANKL-induced BM cells in the absence and the presence of different concentrations of 5′-HA. Values are mean ± SD of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.005$, as compared to RANKL-induced cells without 5′-HA).

transcriptional activator, activator protein-1 (AP-1). BM cells lacking c-Fos were unable to differentiate into mature osteoclast and mice deficient in c-Fos developed osteopetrosis, suggesting the crucial role of c-Fos in osteoclast determination [33, 34]. NFATc1 is another osteoclastic transcription factor that is stimulated and activated by RANKL to provoke the intracellular signal cascades essential for regulating terminal osteoclast differentiation. Ectopic expression of NFATc1 in osteoclast precursor cells enhanced their osteoclastogenesis even in the absence of RANKL stimulation, and mice deficient in NFATc1 expression displayed osteopetrotic defect [35, 36]. In support of our findings, coumarin derivatives including daphnetin, psoralidin, and esculetin were reported to suppress osteoclastogenesis *in vitro* through direct inhibition of c-Fos and NFATc1 expression [16, 17, 30].

Coumarin derivatives have interesting pharmacological properties, and coumarin ring system has been used as a scaffold for developing therapeutic drugs for chronic diseases [37, 38]. There are no specific receptors for natural coumarin and its synthetic analogs. However, coumarin was reported as a potential platform for designing ligands for adenosine receptors. The affinity and binding activity of coumarin toward adenosine receptors were based on the nature of its substituents [39]. Interestingly, coumarin-based selective estrogen receptor modulators (SERMs) with high affinity for estrogen receptor (ER) were synthesized and used to block osteoclastogenesis [40] and to act as an antiestrogen on breast cancer cells [41]. Thus, it is plausible that the regulatory effect of 5′-HA on osteoclast differentiation is mediated at least in part by ER on BM cells; however, this notion needs further extensive experimental work.

Taken together, our data provide 5′-HA as a novel coumarin derivative with antiosteoclastic effect that can be used for the treatment of bone resorption-related disease. However, further *in vivo* study is required to demonstrate

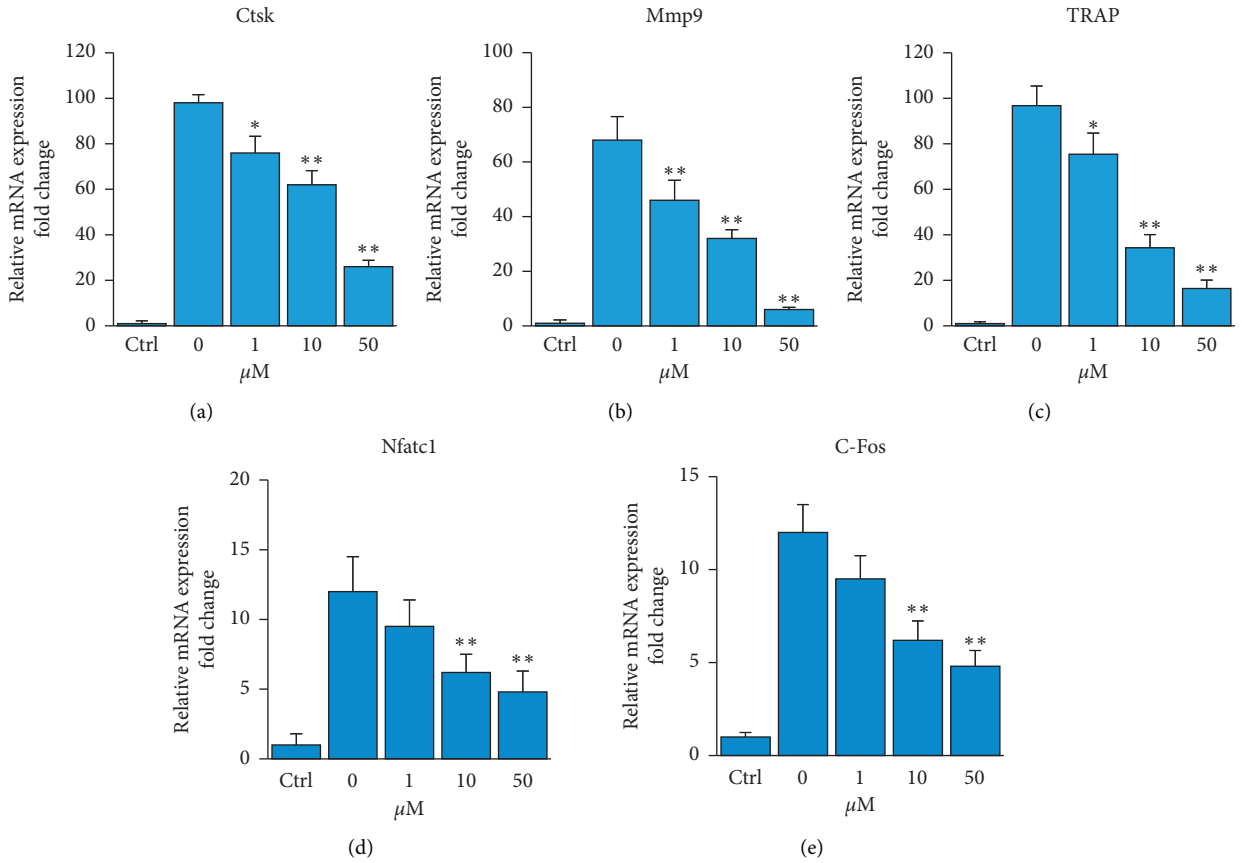
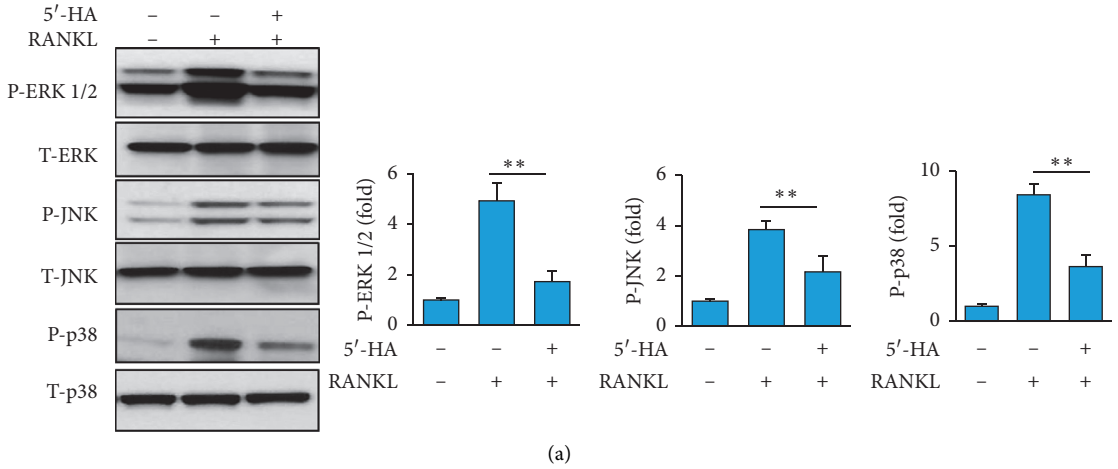
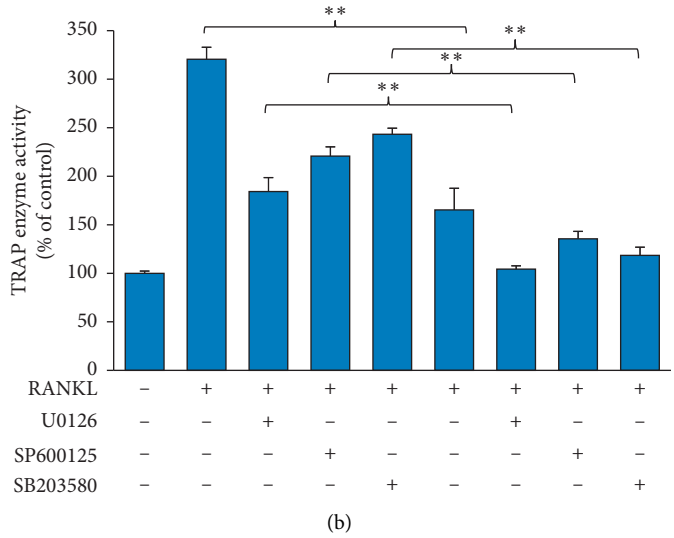


Figure 4: 5′-HA downregulates the RANKL-induced osteoclast specific gene expression. (a–e) qPCR analysis of osteoclastic gene expression in RANKL-induced BM cells. BM cells were induced to differentiate into osteoclasts without (Ctrl) or with RANKL and M-CSF in the absence (0) or the presence of different concentrations of 5′-HA for 7 days. Each target gene was normalized to reference genes and represented as fold change over noninduced control cells. Values are mean ± SD of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.005$, as compared to RANKL-induced cells without 5′-HA).



(a)

Figure 5: Continued.



(b)

Figure 5: 5′-HA inhibits RANKL-induced MAPKs signaling pathway in BM cells. (a) Western blot analysis of ERK1/2, JNK, and p-38 phosphorylation in RANKL-induced BM cells in the absence and the presence of 5′-HA. Cells were pretreated with 5′-HA (50 μM) for 1 h and then stimulated with RANKL for 20 min. (b) Effect of blocking MAPKs on 5′-HA-inhibited osteoclastogenesis. BM cells were pretreated with specific inhibitors for ERK1/2 (U0126, 10 μM), JNK (SP600125, 10 μM), and p38 (SB203580, 10 μM) and then induced with RANKL and M-CSF in the absence or the presence of 5′-HA. Quantitative TRAP activity was measured after 7 days of treatment and values were presented as percentage of control noninduced. Values are mean ± SD of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.005$, as compared to RANKL-induced cells without 5′-HA).

the therapeutic effect of 5′-HA on bone resorption in osteoporotic mouse model.

5. Conclusion

Our data demonstrated the inhibitory effect of 5′-HA on RANKL-induced osteoclast differentiation of BM cells. The inhibitory effect of 5′-HA on osteoclastogenesis was found to be mediated by suppressing MAPK subfamily including p38, JNK, ERK, and osteoclastic transcription factors, c-Fos and NFATc1.

Abbreviations

5′-HA: 5′-Hydroxy auraptene
BM: Bone marrow
TRAP: Tartrate-resistant acid phosphatase
MNCs: Multinucleated cells
RANKL: Receptor activator of nuclear factor kappa-B ligand
NF-κB: Nuclear factor kappa-B
NFATc1: Nuclear factor of activated T cells, cytoplasmic 1
MCS-F: Macrophage colony stimulating factor
TNF: Tumor necrosis factor
Mmps: Matrix metalloproteinase
Ctsk: Cathepsin K.

Data Availability

The data used to support the findings of the study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

BMA conceived the project, designed the study, performed the experiments, analyzed the data, and wrote the manuscript. EMA extracted and purifyied 5′-HA and performed experiments. HE, GMB, AMA, and AMA performed some experiments and data analysis and edited the manuscript.

Acknowledgments

The authors acknowledge the Deanship of Scientific Research at King Faisal University, Saudi Arabia, for the financial support (under Grant No. 17122008).

Supplementary Materials

Table S1: the primer sequences of all genes used by real-time PCR. (*Supplementary Materials*)

References

[1] B. Langdahl, S. Ferrari, and D. W. Dempster, "Bone modeling and remodeling: potential as therapeutic targets for the treatment of osteoporosis," *Therapeutic Advances in Musculoskeletal Disease*, vol. 8, no. 6, pp. 225–235, 2016.

[2] F. Cosman, "Anabolic and antiresorptive therapy for osteoporosis: combination and sequential approaches," *Current Osteoporosis Reports*, vol. 12, no. 4, pp. 385–395, 2014.

[3] M. Lorentzon, "Treating osteoporosis to prevent fractures: current concepts and future developments," *Journal of Internal Medicine*, vol. 285, no. 4, pp. 381–394, 2019.

[4] N. A. Sims and K. W. Ng, "Implications of osteoblast-osteoclast interactions in the management of osteoporosis by antiresorptive agents denosumab and odanacatib," *Current Osteoporosis Reports*, vol. 12, no. 1, pp. 98–106, 2014.

[5] S. L. Teitelbaum, "Therapeutic implications of suppressing osteoclast formation versus function," *Rheumatology*, vol. 55, no. 2, pp. ii61–ii63, 2016.

[6] J.-H. Kang, H. Lim, J.-E. Jeong, and M. Yim, "Attenuation of RANKL-induced osteoclast formation via p38-mediated NFATc1 signaling pathways by extract of euphorbia lathyrisL," *Journal of Bone Metabolism*, vol. 23, no. 4, pp. 207–214, 2016.

[7] K. Matsuo, D. L. Galson, C. Zhao et al., "Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos," *Journal of Biological Chemistry*, vol. 279, no. 25, pp. 26475–26480, 2004.

[8] N. S. Soysa, N. Alles, H. Shimokawa, E. Jimi, K. Aoki, and K. Ohya, "Inhibition of the classical NF-κB pathway prevents osteoclast bone-resorbing activity," *Journal of Bone and Mineral Metabolism*, vol. 27, no. 2, pp. 131–139, 2009.

[9] X. Chen, X. Zhi, J. Wang, and J. Su, "RANKL signaling in bone marrow mesenchymal stem cells negatively regulates osteoblastic bone formation," *Bone Research*, vol. 6, no. 1, p. 34, 2018.

[10] X. Chen, X. Zhi, P. Pan et al., "Matrine prevents bone loss in ovariectomized mice by inhibiting RANKL-induced osteoclastogenesis," *The FASEB Journal*, vol. 31, no. 11, pp. 4855–4865, 2017.

[11] Z. Xin, C. Jin, L. Chao et al., "A matrine derivative M54 suppresses osteoclastogenesis and prevents ovariectomy-induced bone loss by targeting ribosomal protein S5," *Frontiers in Pharmacology*, vol. 9, p. 22, 2018.

[12] A. Detsi, C. Kontogiorgis, and D. Hadjipavlou-Litina, "Coumarin derivatives: an updated patent review (2015-2016)," *Expert Opinion on Therapeutic Patents*, vol. 27, no. 11, pp. 1201–1226, 2017.

[13] C. Kontogiorgis, A. Detsi, and D. Hadjipavlou-Litina, "Coumarin-based drugs: a patent review (2008—present)," *Expert Opinion on Therapeutic Patents*, vol. 22, no. 4, pp. 437–454, 2012.

[14] J. J. Zhu and J. G. Jiang, "Pharmacological and nutritional effects of natural coumarins and their structure-activity relationships," *Molecular Nutrition & Food Research*, vol. 62, no. 14, Article ID e1701073, 2018.

[15] C.-J. Hsieh, P.-L. Kuo, M.-F. Hou et al., "Wedelolactone inhibits breast cancer-induced osteoclastogenesis by decreasing Akt/mTOR signaling," *International Journal of Oncology*, vol. 46, no. 2, pp. 555–562, 2015.

[16] X. Liu, X. Gao, Y. Liu et al., "Daphnetin inhibits RANKL-induced osteoclastogenesis in vitro," *Journal of Cellular Biochemistry*, vol. 120, no. 2, pp. 2304–2312, 2018.

[17] L. Kong, R. Ma, X. Yang et al., "Psoralidin suppresses osteoclastogenesis in BMMs and attenuates LPS-mediated osteolysis by inhibiting inflammatory cytokines," *International Immunopharmacology*, vol. 51, pp. 31–39, 2017.

[18] D. Zhao, Q. Wang, Y. Zhao et al., "The naturally derived small compound Osthole inhibits osteoclastogenesis to prevent ovariectomy-induced bone loss in mice," *Menopause*, vol. 25, no. 12, pp. 1459–1469, 2018.

[19] X.-L. Zhao, L.-F. Chen, and Z. Wang, "Aesculin modulates bone metabolism by suppressing receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis and transduction signals," *Biochemical and Biophysical Research Communications*, vol. 488, no. 1, pp. 15–21, 2017.

[20] B. M. Abdallah and E. M. Ali, "5′-hydroxy auraptene stimulates osteoblast differentiation of bone marrow-derived mesenchymal stem cells via a BMP-dependent mechanism," *Journal of Biomedical Science*, vol. 26, no. 1, p. 51, 2019.

[21] B. M. Abdallah, N. Ditzel, A. Mahmood et al., "DLK1 is a novel regulator of bone mass that mediates estrogen-deficiency induced bone loss in mice," *Journal of Bone and Mineral Research*, vol. 26, no. 7, pp. 1457–1471, 2011.

[22] B. M. Abdallah, "Marrow adipocytes inhibit the differentiation of mesenchymal stem cells into osteoblasts via suppressing BMP-signaling," *Journal of Biomedical Science*, vol. 24, no. 1, p. 11, 2017.

[23] B. M. Abdallah, A. M. Alzahrani, and M. Kassem, "Secreted clusterin protein inhibits osteoblast differentiation of bone marrow mesenchymal stem cells by suppressing ERK1/2 signaling pathway," *Bone*, vol. 110, pp. 221–229, 2018.

[24] J. Mizukami, G. Takaesu, H. Akatsuka et al., "Receptor activator of NF-κB ligand (RANKL) activates TAK1 mitogen-activated protein kinase kinase kinase through a signaling complex containing RANK, TAB2, and TRAF6," *Molecular and Cellular Biology*, vol. 22, no. 4, pp. 992–1000, 2002.

[25] T. Wada, T. Nakashima, N. Hiroshi, and J. M. Penninger, "RANKL-RANK signaling in osteoclastogenesis and bone disease," *Trends in Molecular Medicine*, vol. 12, no. 1, pp. 17–25, 2006.

[26] B. F. Boyce, Y. Xiu, J. Li, L. Xing, and Z. Yao, "NF-κB-mediated regulation of osteoclastogenesis," *Endocrinology and Metabolism*, vol. 30, no. 1, pp. 35–44, 2015.

[27] S. E. Lee, K. M. Woo, S. Y. Kim et al., "The phosphatidylinositol 3-kinase, p38, and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation," *Bone*, vol. 30, no. 1, pp. 71–77, 2002.

[28] J. F. Charles and A. O. Aliprantis, "Osteoclasts: more than "bone eaters"," *Trends in Molecular Medicine*, vol. 20, no. 8, pp. 449–459, 2014.

[29] A. Maurizi and N. Rucci, "The osteoclast in bone metastasis: player and target," *Cancers*, vol. 10, no. 7, p. 218, 2018.

[30] J. M. Baek, S.-H. Park, Y.-H. Cheon et al., "Esculetin attenuates receptor activator of nuclear factor kappa-B ligand-mediated osteoclast differentiation through c-Fos/nuclear factor of activated T-cells c1 signaling pathway," *Biochemical and Biophysical Research Communications*, vol. 461, no. 2, pp. 334–341, 2015.

[31] L. Chai, K. Zhou, S. Wang et al., "Psoralen and Bakuchiol ameliorate M-CSF plus RANKL-induced osteoclast differentiation and bone resorption via inhibition of AKT and AP-1 pathways in vitro," *Cellular Physiology and Biochemistry*, vol. 48, no. 5, pp. 2123–2133, 2018.

[32] T. N. Crotti, M. Flannery, N. C. Walsh, J. D. Fleming, S. R. Goldring, and K. P. McHugh, "NFATc1 regulation of the human β3 integrin promoter in osteoclast differentiation," *Gene*, vol. 372, pp. 92–102, 2006.

[33] A. Grigoriadis, Z. Wang, M. Cecchini et al., "c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling," *Science*, vol. 266, no. 5184, pp. 443–448, 1994.

[34] K. Matsuo, J. M. Owens, M. Tonko, C. Elliott, T. J. Chambers, and E. F. Wagner, "Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation," *Nature Genetics*, vol. 24, no. 2, pp. 184–187, 2000.

[35] H. Takayanagi, "The role of NFAT in osteoclast formation," *Annals of the New York Academy of Sciences*, vol. 1116, no. 1, pp. 227–237, 2007.

[36] H. Takayanagi, S. Kim, T. Koga et al., "Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts," *Developmental Cell*, vol. 3, no. 6, pp. 889–901, 2002.

[37] M. Musa, J. Cooperwood, and M. O. Khan, "A review of coumarin derivatives in pharmacotherapy of breast cancer," *Current Medicinal Chemistry*, vol. 15, no. 26, pp. 2664–2679, 2008.

[38] G. Derosa, P. Maffioli, and A. Sahebkar, "Auraptene and its role in chronic diseases," *Advances in Experimental Medicine and Biology*, vol. 929, pp. 399–407, 2016.

[39] A. Gaspar, J. Reis, S. Kachler et al., "Discovery of novel A3 adenosine receptor ligands based on chromone scaffold," *Biochemical Pharmacology*, vol. 84, no. 1, pp. 21–29, 2012.

[40] M. Sutherland, S. G. Lipps, N. Patnaik et al., "SP500263, a novel SERM, blocks osteoclastogenesis in a human bone cell model: role of IL-6 and GM-CSF," *Cytokine*, vol. 23, no. 1-2, pp. 1–14, 2003.

[41] H. Brady, S. Desai, L. M. Gayo-Fung et al., "Effects of SP500263, a novel, potent antiestrogen, on breast cancer cells and in xenograft models," *Cancer Research*, vol. 62, no. 5, pp. 1439–1442, 2002.