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

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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 precursors cell surface RANK receptors triggers the signals of osteoclast differentiation via NF-xB 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 osteoclast-related genes including matrix metalloproteinase (Mmps), Trap, and Cathepsin K (Ctks) [6–9]. Thus, targeting osteoclast formation and differentiation via inhibiting NF-kB 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 ascelin 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 × 10⁶ 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% CO₂ 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).
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 Minsus 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 manufacturer’s instructions. Quantitative real-time PCR was performed with Applied Biosystems 7500 Real-Time system. 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 Minsus 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 manufacturer’s instructions. Quantitative real-time PCR was performed with Applied Biosystems 7500 Real-Time system. 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 Minsus 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 manufacturer’s instructions. Quantitative real-time PCR was performed with Applied Biosystems 7500 Real-Time system.

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 osteoclast 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 RANK 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 osteoclast genes in BM cells treated with RANKL. Interestingly, 5′-HA significantly downregulated the mRNA expression of RANKL-induced Ctsk and Trap 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...
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
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].

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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
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 (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).

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 3](image-url)

Figure 3: 5′-HA inhibits RANKL-induced NF-κB 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).
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).

Figure 5: Continued.
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.

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Supplementary Materials

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

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