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Original Article

Columbianetin Inhibits RANKL-Induced Osteoclast Differentiation in RAW264.7 Cells by Inhibiting Autophagy through the SGK1/FOXO3a Signaling Pathway

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SUMMARY

Background: Osteoporosis is a common skeletal disorder characterized by increased bone fragility and fracture risk. Current treatments are limited, necessitating the development of novel therapeutic agents.
Methods: RAW264.7 cells were treated with CBT (0, 10, 20, 40 μg/mL) and stimulated with RANKL to induce osteoclast differentiation. Cell viability was assessed by CCK-8 assay, while osteoclast differentiation was evaluated through TRAP staining. Immunoblot analysis was conducted to confirm the underlying mechanism.
Results: Columbianetin significantly inhibited the differentiation and activity of osteoclasts. Furthermore, Columbianetin was found to suppress autophagy in osteoclasts. Mechanically, Columbianetin exerted its effects by inhibiting the SGK1/FOXO3a signaling pathway.
Conclusion: Columbianetin effectively inhibits osteoclast differentiation and autophagy by modulating the SGK1/FOXO3a pathway, highlighting its potential as a therapeutic agent for osteoporosis treatment.

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1. Introduction

Osteoporosis is a systemic skeletal disorder characterized by reduced bone strength, leading to an increased risk of fractures.[1] This condition arises primarily due to an imbalance between bone resorption and bone formation, processes mediated by osteoclasts and osteoblasts respectively. An excessive rate of bone resorption or insufficient bone formation disrupts bone homeostasis and contributes to osteoporosis.[2] Bone remodeling is a critical process that maintains mineral homeostasis and skeletal integrity.[3] However, when the balance between bone resorption and formation is disrupted, it can lead to bone mineral disorders.[4] As a major global public health concern, osteoporosis necessitates the need for innovative therapeutic strategies. Despite advancements in treatment, there remains a critical demand for the development of novel drugs and treatment approaches to combat this condition effectively. Traditional Chinese Medicine (TCM) has a long-standing history of treating bone diseases, providing a potentially safe and alternative approach for osteoporosis management. *Angelica pubescens*, a widely used herb in TCM, is particularly recognized for its efficacy in strengthening muscles and bones.[5] Columbianetin (CBT), a major active compound extracted from the roots of *Angelica pubescens*, has been shown to exhibit various pharmacological and biological activities, including antioxidant, antiproliferative, and anti-nitric oxide generation properties.[6–8] Previous studies have demonstrated that CBT has significant anti-inflammatory effects in mast cells, suggesting its potential to regulate allergic inflammatory responses mediated by these cells.[9] Additionally, CBT has been reported to exert neuroprotective effects, protect keratinocytes from damage, and inhibit platelet aggregation.[10] Recent studies have further indicated that CBT alleviates lipopolysaccharide (LPS)-induced inflammation and apoptosis in chondrocytes by activating autophagy through the SGK1 inhibition.[11] However, the precise role and underlying mechanisms of CBT in osteoclast differentiation remain unclear and warrant further investigation.

In this study, we investigated the effects of CBT on the RANKL-induced osteoclast differentiation in RAW264.7 cells and sought to elucidate its potential mechanisms of action.

2. Materials and methods

2.1. Cell culture and reagents

RAW264.7 cells (mouse macrophage cell line) were purchased from ATCC (TIB-71) and cultured in DMEM (Gibco, #11965092) supplemented with 10% FBS (26140079) at 37 °C in a humidified atmosphere with 5% CO_2. For osteoclast differentiation, RAW264.7 cells were stimulated with 100 ng/mL RANKL (Receptor Activator for Nuclear Factor-κB Ligand; Beyotime, #P3430). CBT was prepared at 0, 10, 20, and 40 μg/mL for cell treatment.

2.2. Cell viability assay

Cell viability was assessed using the cell counting kit-8 (CCK-8; Beyotime, #C0038). RAW264.7 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and treated with different concentrations of CBT. Absorbance was measured at 450 nm using a

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microplate reader (BioTek, ELX800).

2.3. Osteoclast differentiation and TRAP staining

TRAP staining was performed using a TRAP Staining Kit (Beyotime, #C186). TRAP-positive multinucleated cells (≥ 3 nuclei) were counted under a light microscope (Zeiss, Axio Observer A1).

2.4. Immunoblot analysis

Samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, #IPVH00010). Membranes were blocked with 5% BSA (Beyotime, #ST023) and incubated overnight at 4 °C with primary antibodies against NFATc1 (1:1000, Abcam, ab272106), MMP-9 (1:1000, Abcam, ab38898), c-FOS (1:1000, Abcam, ab222699), CTSK (1:1000, Abcam, ab19027), LC3 (1:1000, Abcam, ab192890), p62 (1:1000, Abcam, ab155686), SGK1 (1:1000, Abcam, ab59337), p-SGK1 (1:1000, Abcam, ab192874), FOXO3a (1:1000, Abcam, ab23683), and p-FOXO3a (1:1000, Abcam, ab131339). After washing, membranes were further incubated with HRP-conjugated secondary antibodies (1:5000, Beyotime, #A0208) and detected using an ECL detection kit (Beyotime, #P0018).

2.5. Statistical analysis

Data were presented as mean ± SD. Statistical analysis was performed using GraphPad Prism 8 software. Differences between groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Columbianetin inhibited the activity and differentiation of osteoclasts

To assess the effect of CBT on osteoclast differentiation, RAW264.7 cells were stimulated with RANKL and treated with various concentrations of CBT (0, 10, 20, and 40 μg/mL). The molecular structure of CBT is shown in Figure 1A. CCK-8 assays showed that CBT had no significant effect on the viability of RAW264.7 cells at any

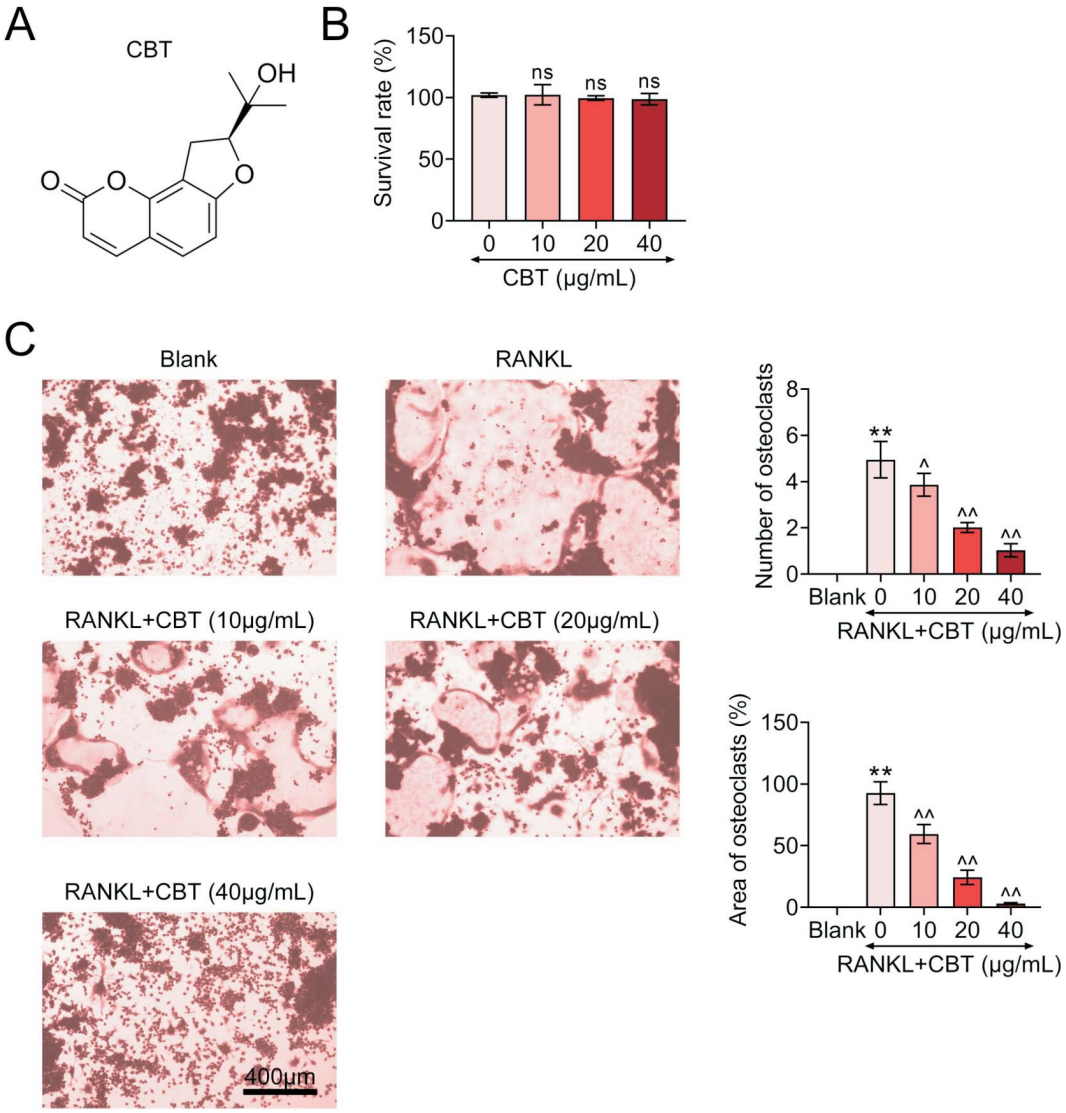

Figure 1. Columbianetin (CBT) inhibits the activity and differentiation of osteoclasts. (A) The chemical structure of CBT. (B) Cell viability of RAW264.7 cells treated with various concentrations of CBT (0, 10, 20, and 40 μg/mL), assessed using the CCK-8 assay for 48 h. The OD450 value was measured. (C) Representative images of TRAP-stained RAW264.7 cells treated with different concentrations of CBT (0, 10, 20, 40 μg/mL) and stimulated with RANKL to induce osteoclast differentiation, showing TRAP-positive multinucleated cells. The number of osteoclasts and percentage of TRAP-positive multinucleated cells were quantified. Scale bar = 400 μm. Data are presented as mean ± SD (n = 3). ns, not significant.

concentration, indicating that CBT is not cytotoxic at the tested concentrations (Figure 1B). However, TRAP staining showed a concentration-dependent decrease in the number of TRAP-positive multinucleated osteoclasts. Additionally, the percentage of TRAP-positive multinucleated cells reduced with increasing concentrations of CBT (Figure 1C). These findings suggest that CBT effectively inhibits RANKL-induced osteoclast differentiation and activity.

3.2. Columbianetin disrupted the bone resorption activity of osteoclasts

To further evaluate the effect of CBT on osteoclast function, we detected its effects on the bone resorption activity of osteoclasts. Immunoblot analysis was used to measure the expression levels of osteoclast differentiation markers, such as NFATc1, MMP-9, c-FOS, and CTSK (Figure 2A). The results showed a significant downregulation of these markers after CBT treatment, suggesting the suppression of bone resorption (Figure 2A). Additionally, immunofluorescence staining of F-actin ring formation, a hallmark of active osteoclasts, showed a marked decrease in F-actin ring structures in cells treated with CBT (Figure 2B). This further supports the inhibitory effect of CBT on osteoclast bone resorption activity.

3.3. Columbianetin suppressed autophagy in osteoclasts

The potential role of autophagy in osteoclast differentiation and function was further investigated by examining the expression levels of autophagy markers LC3 and p62. Immunoblot analysis revealed that CBT treatment led to a decrease in the LC3-II/LC3-I ratio and an increase in P62 levels in RANKL-stimulated RAW264.7 cells (Figure 3). These results suggest that CBT suppresses autophagy in osteoclasts.

3.4. Columbianetin modulated the SGK1/FOXO3a pathway in osteoclasts

To elucidate the molecular mechanism underlying the inhibitory effects of CBT on osteoclasts, we analyzed the SGK1/FOXO3a signaling pathway, which is known to regulate cell survival and autophagy. Immunoblot analysis showed that CBT treatment significantly reduced the phosphorylation levels of SGK1 and FOXO3a (Figure 4). These findings suggest that CBT suppresses the activation of the SGK1/FOXO3a pathway.

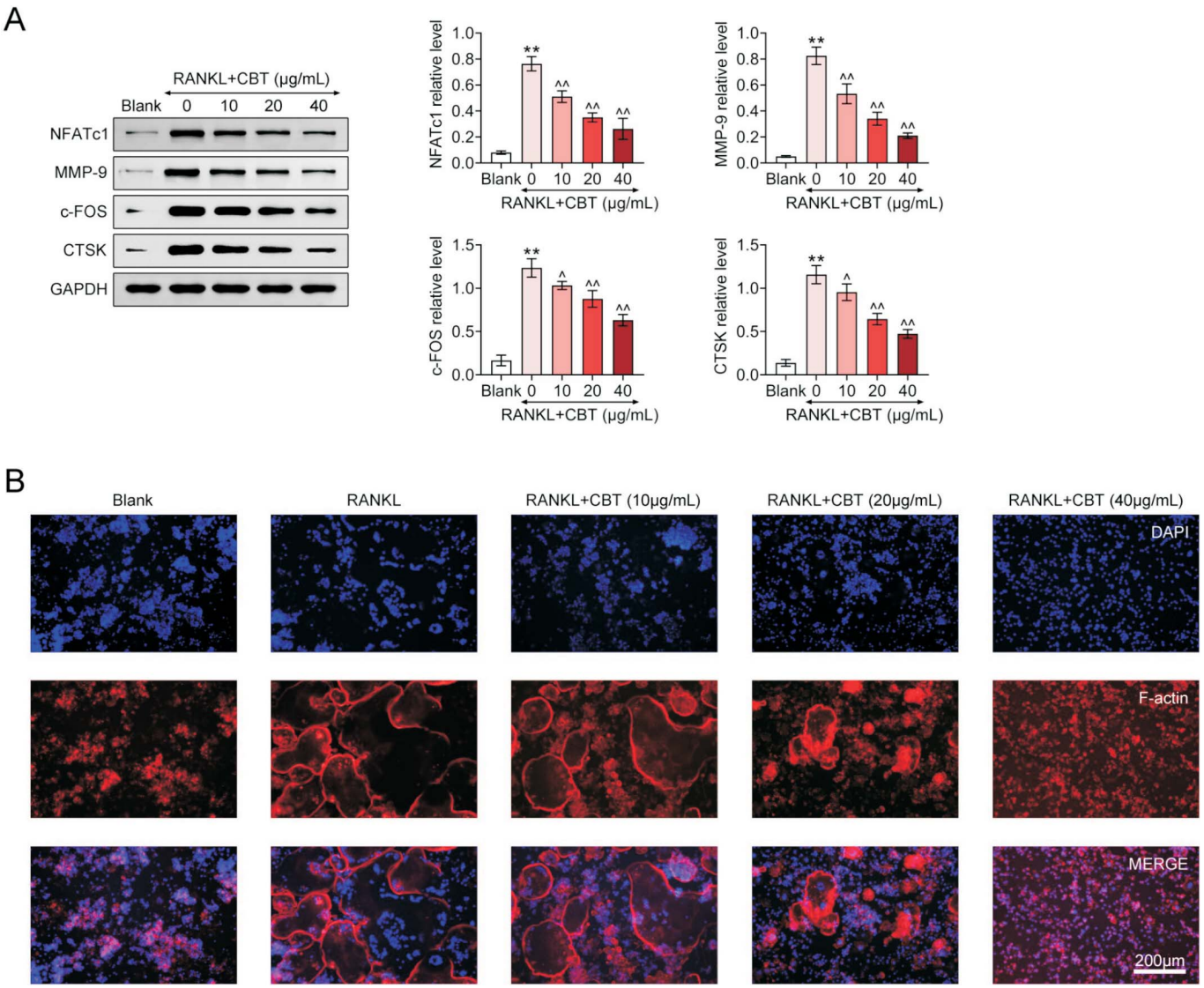

Figure 2. Columbianetin (CBT) inhibits the activity of osteoclasts. (A) Immunoblot analysis of osteoclast differentiation markers (NFATc1, MMP-9, c-FOS, and CTSK) in RAW264.7 cells treated with various concentrations of CBT (0, 10, 20, and 40 μg/mL) and stimulated with RANKL. (B) Immunofluorescence staining of RAW264.7 cells treated with different concentrations of CBT (0, 10, 20, 40 μg/mL) and stimulated with RANKL, showing DAPI-stained nuclei (blue) and F-actin (red). Scale bar = 200 μm. Data are presented as mean ± SD (n = 3). ** $p < 0.01$ compared to the control group (0 μg/mL); ns: not significant.

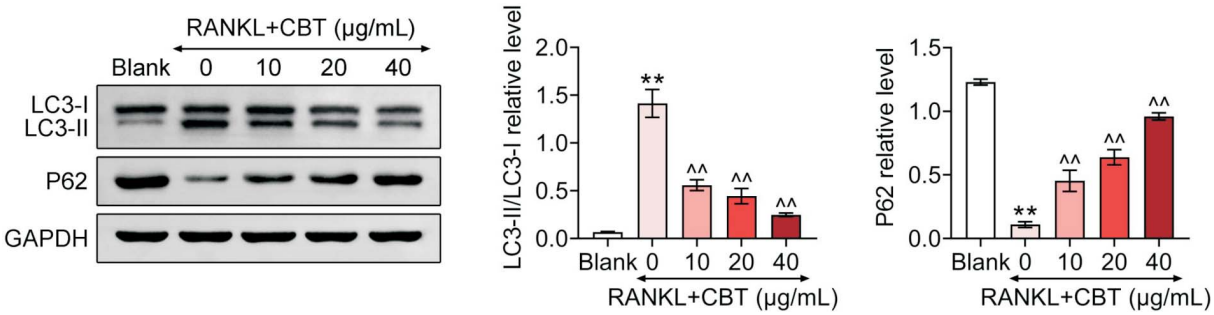

Figure 3. Columbianetin (CBT) inhibits autophagy in osteoclasts. Immunoblot analysis of autophagy markers (LC3 and P62) in RAW264.7 cells treated with various concentrations of CBT (0, 10, 20, and 40 μg/mL) and stimulated with RANKL. The ratio of LC3II/LC3I and P62 levels were compared. Data are presented as mean ± SD (n = 3). ** $p < 0.01$ compared to the control group (0 μg/mL); ns: not significant.

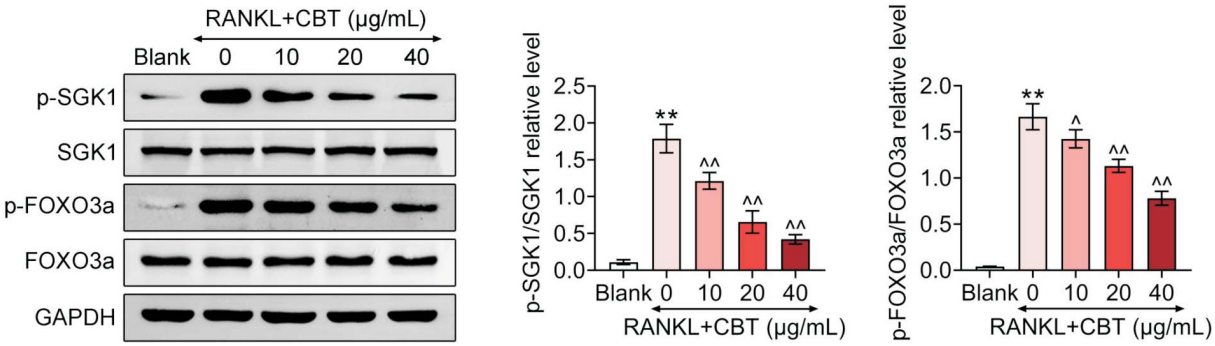

Figure 4. Columbianetin (CBT) inhibits the SGK1/FOXO3a pathway in osteoclasts. Immunoblot analysis of expression and phosphorylation of SGK1 and FOXO3a in RAW264.7 cells treated with various concentrations of CBT (0, 10, 20, and 40 μg/mL) and stimulated with RANKL. The relative phosphorylation levels of SGK1 and FOXO3a levels were compared. Data are presented as mean ± SD (n = 3). ** $p < 0.01$ compared to the control group (0 μg/mL); ns: not significant.

4. Discussion

Osteoporosis is a prevalent metabolic bone disease which reduces bone mass and increases its susceptibility to fractures.[12] The disease is most commonly associated with aging, hormonal changes, and a deficiency in calcium or vitamin D, all of which contribute to weakened bone structure.[13,14] Traditional treatments for osteoporosis focus mainly on inhibiting bone resorption or stimulating bone formation.[12] However, these treatments often have limited efficacy and can cause significant side effects. As a result, there is growing interest in exploring natural compounds for osteoporosis treatment, owing to their potentially lower side effect profile and ability to target multiple pathways.[13,15,16] Our study has identified CBT's significant role in inhibiting osteoclast differentiation and activity, highlighting its potential as a therapeutic agent for osteoporosis. This discovery opens avenues for the development of CBT-based treatments that target the fundamental mechanisms underlying bone resorption and formation.

RANKL is a crucial mediator of osteoclast differentiation and activation, playing a pivotal role in bone remodeling and the pathogenesis of osteoporosis.[17] RANKL binds to its receptor RANK on the surface of osteoclast precursors, initiating a cascade of signaling events that lead to the differentiation, activation, and survival of osteoclasts.[18] This process is vital for bone resorption, which, when unregulated, contributes to osteoporosis. Recent studies have highlighted the interplay between RANKL signaling and autophagy in osteoclasts, suggesting that autophagy may support osteoclast differentiation and survival by providing cellular energy and maintaining homeostasis.[18] Our findings demonstrate that CBT effectively inhibits RANKL-induced osteoclast differentiation and reduces autophagic activity in these cells, suggesting a dual mechanism by which CBT can modulate both osteoclast function and autophagy. This dual inhibition may enhance the therapeutic efficacy of CBT in managing osteoporosis by simultaneously targeting key pathways involved in bone resorption and cellular metabolism.

The relationship between RANKL-induced osteoclast differentiation and osteoporosis has been extensively studied in both cellular and animal models.[18] *In vitro* models using RAW264.7 cells and primary macrophages provide valuable insights into the molecular mechanisms of osteoclastogenesis and the effects of various compounds on this process.[17] Similarly, animal models of osteoporosis, such as ovariectomized mice or rats, mimic the postmenopausal state in humans and are used to evaluate the efficacy of potential therapeutic agents.[17] Our study utilized the RAW264.7 cell model to investigate the effects of CBT on osteoclast differentiation, providing evidence that CBT significantly inhibits RANKL-induced osteoclastogenesis. This finding is consistent with previous studies showing the role of autophagy in osteoclast differentiation and highlights the potential of CBT to alter disease progression by modulating these cellular pathways. Future studies should explore the effects of CBT in animal models of osteoporosis to validate its therapeutic potential *in vivo*.

CBT is a bioactive compound with a wide range of pharmacological activities.[8,9,19] It has been studied for its potential therapeutic applications in various diseases, such as arthritis, cardiovascular diseases, and neurodegenerative disorders.[9,19] For instance, CBT has been shown to reduce inflammation in models of rheumatoid arthritis and to protect against oxidative stress in models of neurodegenerative diseases.[7,20] These properties suggest that CBT may exert beneficial effects in diseases characterized by chronic inflammation and oxidative damage. Our research extends these findings to bone health, demonstrating that CBT can inhibit osteoclast differentiation and activity, which are critical processes in osteoporosis. This suggests that CBT could be a multifunctional agent with applications beyond its currently known therapeutic effects.

CBT stands out among natural compounds due to its unique

dual mechanism of action, inhibiting both osteoclast differentiation and autophagy via the SGK1/FOXO3a pathway.[8] While other natural compounds, such as berberine and curcumin, have demonstrated osteoprotective effects mainly through pathways like NF-κB and MAPK, CBT's targeted modulation of the SGK1/FOXO3a pathway represents a distinctive and less explored approach.[19] This highlights CBT's potential as a novel agent for osteoporosis treatment, meriting further investigation to compare its efficacy with existing natural therapies and standard pharmacological treatments.

The role of autophagy in bone metabolism, particularly in osteoclast differentiation and function, is an emerging area of interest.[21,22] Autophagy is a cellular process that degrades and recycles cellular components, maintaining cellular homeostasis and energy balance, especially under stress conditions.[22] In osteoclasts, autophagy appears to support cell survival and differentiation by providing essential building blocks and energy required for bone resorption.[21] Our study shows that CBT inhibits autophagy in RANKL-induced osteoclasts, as evidenced by reduced levels of autophagy-related proteins. This inhibition of autophagy by CBT may contribute to its ability to suppress osteoclast differentiation and activity, providing a novel mechanism of action that could enhance its therapeutic potential in osteoporosis management. Further research is needed to fully elucidate the pathways through which CBT modulates autophagy and to determine whether this mechanism is central to its effects on bone metabolism.

The SGK1/FOXO3a pathway plays a critical role in regulating cell survival, proliferation, and autophagy, influencing both osteoclast and osteoblast activity.[23] SGK1 affects ion transport, cell growth, and survival.[24] FOXO3a, a transcription factor regulated by SGK1, is known to promote autophagy and apoptosis under stress conditions.[23] Dysregulation of the SGK1/FOXO3a pathway has been implicated in osteoporosis, as it affects the balance between bone resorption and formation.[24] Our findings indicate that CBT inhibits the SGK1/FOXO3a pathway in osteoclasts, thereby reducing both osteoclast activity and autophagy. This suggests that CBT may exert its anti-osteoporotic effects by targeting this pathway, providing a mechanistic basis for its potential therapeutic use. Further studies are warranted to explore the broader implications of SGK1/FOXO3a pathway inhibition in bone health and its potential as a therapeutic target.

Dysregulation of the SGK1/FOXO3a axis has been shown to exacerbate osteoclast activity, contributing to the progression of osteoporosis. By inhibiting SGK1 phosphorylation and the downstream activation of FOXO3a, CBT may reduce osteoclast survival and autophagic processes that support osteoclast function, contributing to its anti-osteoporotic effects. In addition to the SGK1/FOXO3a pathway, osteoclast differentiation is regulated by various other pathways, including NF-κB, MAPK, and PI3K/Akt.[24] While our study primarily focused on the SGK1/FOXO3a pathway, future research could explore whether CBT has broader effects on these critical signaling cascades. Investigating its influence on inflammatory and metabolic pathways may provide a more comprehensive understanding of CBT's role in osteoclast regulation. This approach could also include advanced tools such as RNA interference (RNAi) or CRISPR-based gene editing to validate the molecular targets influenced by CBT and further elucidate its mechanism of action.

The potential dual role of CBT in regulating autophagy across different cellular models underscores its multifaceted pharmacological properties. In osteoclasts, where the SGK1/FOXO3a pathway plays a significant role in cell survival and differentiation, the inhibitory effect of CBT on this pathway and autophagy is crucial for reducing bone resorption and osteoclastogenesis. This pathway-specific inhibition sets CBT apart from general autophagy inhibitors like chloroquine (CQ) and hydroxychloroquine HCQ, contributing to the compound's uniqueness in osteoporosis research. Future research should aim to further elucidate the cell-type specific responses and explore CBT's potential interactions with other signaling pathways involved in bone metabolism.

Despite these promising findings, our study has several limitations to be addressed. First, our experiments were conducted *in vitro* using RAW264.7 cells. While this is a widely used model for studying osteoclast differentiation, it may not fully replicate the complexity of osteoclastogenesis *in vivo*. Second, the study did not explore the effects of CBT in animal models of osteoporosis, which would provide a more comprehensive understanding of its therapeutic potential. Further, these findings suggest that CBT's dual action on osteoclast differentiation and autophagy positions it as a promising candidate for further development into a therapeutic agent for osteoporosis. Future research should explore the clinical applicability of CBT, including its safety profile and efficacy in animal models and human trials, to better assess its potential as an alternative or complementary treatment for osteoporosis.

In conclusion, our study demonstrates that Columbianetin holds significant potential as a therapeutic agent for osteoporosis by inhibiting osteoclast differentiation and autophagy through the SGK1/FOXO3a pathway.

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Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

Contribution of authors

Li Zhang, Yan Li — designed the study and carried them out; Li Zhang, Yan Li — supervised the data collection, Li Zhang, Yan Li — analyzed the data, Li Zhang, Yan Li — interpreted the data, Li Zhang, Yan Li — prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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