Alpinetin Inhibits RANKL-Induced Osteoclastogenesis and Ovariectomy-Induced Bone Loss by Modulating NFATc1 Transcription and Lysosomal Function

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Abstract

Background

Postmenopausal osteoporosis is a chronic metabolic bone disease caused by excessive osteoclast activation, and osteoclasts are considered to be the sole participants in the degeneration and resorption of bone matrix for controlling bone integrity and continuity. The biological functions of osteoclasts depend critically on the number and activity of fused polykaryon. Hence, targeting osteoclast differentiation and activity can modulate bone resorption and alleviate osteoporosis. Alpinetin is widely used for excellent anti-inflammatory activities and little side-effect, but its role in osteoporosis remains unknown.

Results

In this study, we investigated for the first time the ability of alpinetin to inhibit estrogen deficiency-induced bone loss. Alpinetin significantly reduced the expression levels of NFATc1 and its downstream genes, thereby inhibiting osteoclast differentiation in a concentration- and time-dependent manner. Additionally, alpinetin inhibited F-actin ring formation and bone resorption, as well as reduced the activation levels of NF-κB, ERK, and AKT signaling cascades. In mature osteoclasts, alpinetin remarkably inhibited integrin-mediated migration and lysosomal biogenesis and trafficking by modulating the PKCβ/TFEB and ATG5/LC3 axes. Importantly, alpinetin treatment in mice alleviated ovariectomy-induced bone volume loss.

Conclusion

Our findings strongly suggest that alpinetin plays a significant role in the regulation of NFATc1 production for the differentiation of osteoclasts and inhibits integrin-mediated cell migration and lysosomal function in mature osteoclasts, thus weaken the increased osteolytic ability due to estrogen deficiency. Alpinetin may represent a promising agent for the treatment of osteoporosis and other metabolic bone diseases.

1. Introduction

Postmenopausal osteoporosis is a relatively common chronic condition resulting in decreased bone mass [1–3]. Abnormal bone metabolism, which is associated with the imbalance between bone resorption and bone formation [4], plays a significant role in osteoporosis [5] and thus contributes to a series of complications, including systemic pain, muscle weakness, and increased risk of fractures [6, 7]. Osteoporosis-induced bone fractures pose significant challenges in individuals, families, and national medical financial systems [8], and total expenditure on osteoporosis-related health care is still rising year by year [9].

Osteoclasts are giant multinucleated cells derived from monocytes or macrophages in response to receptor activator of nuclear factor-kappa B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) signaling [10, 11]. Osteoclasts play a critical role in regulating osteolysis [12]. M-CSF stimulation of macrophage precursors induces the expression of RANK, which upon binding to RANKL, triggers the activation of osteoclast-associated genes and osteoclast differentiation by activating mitogen-activated protein kinases (MAPKs), protein kinase B (AKT), and nuclear factor (NF)-κβ signaling cascades [13]. Excessive bone resorption by osteoclasts has been implicated in various bone metabolism-associated diseases. Lysosomes are key regulators of bone metabolism, facilitating the degradation and dissolution of the bone extracellular matrix (ECM) by promoting environmental acidification [14]. Lysosomes are also involved in the accumulation of proteases at the ruffled border membrane of the resorption lacuna, the primary site of active bone resorption [15].

Various anti-osteoporosis drugs have been approved for clinical use by the US Food and Drug Administration (FDA). However, most of these agents suffer from limited efficacy or cause severe side effects, including hepatorenal damage and gastrointestinal complications [16–18]. Hence, the development of novel, safer, and more effective agents targeting pathological osteolysis are needed.

Alpinetin C16H14O4, ([2S]-7-hydroxy-5-methoxy-2-phenyl-2,3-dihydrochromen-4-one) is a flavonoid isolated from the herbaceous plant *Alpinia katsumadai* Hayata [19]. Numerous studies have shown that alpinetin exerts anti-inflammatory [20, 21], anti-tumor [22], antioxidant [23], and gastrointestinal protective effects [24]. Recently, alpinetin has also proved effective in alleviating osteoarthritis by protecting chondrocytes and maintaining lipid homeostasis [25]. Furthermore, preclinical studies have shown that alpinetin not only does not induce severe side effects but also improves the function of the liver, kidneys, and colon [26–28]. Therefore, alpinetin has emerged as a promising and safe approach to treat numerous diseases.

Chronic inflammation in the bone microenvironment results in osteoclast differentiation and activation, thereby promoting excessive bone resorption [29]. Although alpinetin exerts strong anti-inflammatory effects, its potential usefulness in the treatment of osteoporosis remains unknown. Herein, we report that alpinetin inhibits RANKL-induced osteoclast formation and prevents ovariectomy (OVX)-induced bone mass loss *in vivo*. We also show that alpinetin modulates osteoclast activity by regulating integrin-mediated migration and lysosome function.

2. Materials And Methods

2.1 Materials

Alpinetin (> 98% purity; Fig. 1A) was purchased from Tongtian Biotechnology Co. (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO). Modified minimal essential medium (α-MEM) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Scoresby, Vic, Australia). Recombinant mouse RANKL and M-CSF proteins were purchased from Novoprotein Scientific Inc. (Shanghai, China). Rabbit

primary antibodies against JNK, ERK, p38, P-JNK, P-ERK, p-p38, AKT, P-AKT, IKβα, p-IKβα, p65, p-p65, ATG7, and LC3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies against NFATc1, c-Fos, c-Src, cathepsin K (CTSK), and lysosomal-associated membrane protein 1 (LAMP1) were purchased from Abcam (Cambridge, MA, USA), and primary antibodies against integrin β_3, PKC-β, and TFEB were obtained from Proteintech (Wuhan, Hubei, China). Reagents for tartrate-resistant acid phosphatase (TRAP) staining, western blotting, and quantitative real-time PCR (qRT-PCR) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cell isolation and cell viability assay

Long bones were obtained from 8-week-old C57BL/6 mice, and bone macrophage precursors were isolated by rinsing the bone medullary cavity. Bone marrow macrophages (BMMs) were obtained by treating macrophage precursors with 40 ng/mL M-CSF for at least 3 days. Subsequently, adherent cells were used for the cell viability assay and osteoclast differentiation.

BMMs and MC3T3-E1 cells were seeded in 96-well plates and treated with increasing concentrations of alpinetin (0–50 μM) for 4 days. Subsequently, CCK-8 solution (10 μL/well) was added, and cells were incubated for an additional 4 h to assess cell viability. The optical density was measured at 450 mm using the ELX808 absorbance microplate reader (BioTek, Winooski, VT, USA).

2.3 Osteoclast differentiation and TRAP staining

To determine the inhibitory effect of alpinetin on osteoclast differentiation, BMMs were seeded in 48-well plates (2×10^4 cells/well) and treated with 40 ng/mL M-CSF and 75 ng/mL RANKL for 4 days. Additionally, cells were exposed to different concentrations of alpinetin (0, 5, 10, and 20 μM). After incubation, cells were carefully washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for at least 30 min. The number and size of mature osteoclasts were determined using ImageJ (National Institutes of Health, Bethesda, MD, USA); only cells containing at least three nuclei were considered differentiated osteoclasts.

2.4 F-actin ring and immunofluorescence staining

BMMs were incubated with RANKL and different concentrations of alpinetin. After treatment, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 30 min. After a 1 h incubation with rhodamine-phalloidin, cells were carefully washed three times and stained with DAPI for 5 min. Stained cells were observed under a confocal microscope.

BMMs treated with different concentrations of alpinetin in osteoclastogenic medium were also used for immunofluorescence staining. Fixed and permeabilized cells were blocked with 2% bovine serum albumin for 20 min and incubated with fluorescently labeled primary antibodies overnight. Subsequently, cells were incubated with secondary antibodies for 1 h at 37°C and stained with DAPI for 5 min. Cells were observed under a confocal microscope, and fluorescent signals were analyzed using ImageJ.

2.5 Bone resorption assay

BMMs were seeded onto bone discs (2×10^4 cells/well) and grown in complete α-MEM containing 40 ng/mL M-CSF overnight. Subsequently, cells were stimulated with 75 ng/mL RANKL and increasing concentrations of alpinetin (0, 5, 10, and 20 μM) for 7–8 days. Adherent cells were thoroughly removed from the bone discs, and the resorption pits were imaged using a Hitachi S-3700N scanning electron microscope (Chiyoda, Tokyo, Japan). Three random fields were used to determine the bone absorption area.

2.6 qRT-PCR

To assess the effects of alpinetin on osteoclast-associated gene expression, BMMs were seeded in 12-well plates and cultured with or without different concentrations of alpinetin. Total RNA was isolated using TRIzol reagent (Takara, Dalian, China) and reverse-transcribed into cDNA, which was used for qRT-PCR. The expression levels of target genes were normalized to those of *Gapdh*. qRT-PCR assays were repeated at least three times. The murine primer sequences of osteoclast-specific markers were presented in Table 1.

Table 1
Primer sequences for RT-PCR

Gene	Forward (5'-3')	Reverse (3'-5')
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
TRAP	CACTCCCACCCTGAGATTTGT	CCCCAGAGACATGATGAAGTCA
V-ATPase-a3	GCCTCAGGGGAAGGCCAGATCG	GGCCACCTCTTCACTCCGGAA
NFATc1	CCGTTGCTTCCAGAAAATAACA	TGTGGGATGTGAACTCGGAA
Cathepsin K	CTTCCAATACGTGCAGCAGA	TCTTCAGGGCTTTCTCGTTC
DC-STAMP	AAAACCCTTGGGCTGTTCTT	AATCATGGACGACTCCTTGG
MMP-9	CAAAGACCTGAAAACCTCCAA	GGTACAAGTATGCCTCTGCCA
TFEB	CCACCCCAGCCATCAACAC	CAGACAGATACTCCCGAACCTT
Clcn7	GACTGGCTGTGGGAAAGGAA	TCTCGCTTGAGTGATGTTGACC
Tcirg1	GAGACCTCAACGAATCCGTGA	CATTTCAGGGGTGTGCCTTCA
PKCβ	TCCCTGATCCCAAAAGTGAG	AACTTGAACCAGCCATCCAC
Acp5	ACACAGTGATGCTGTGTGGCAACTC	CCAGAGGCTTCCACATATATGATGG
Atp6v0d2	AAGAGCTGCGTGGAGACAAA	CCACCTTCTTCAGCCTTCGT
Integrin-β_3	TGACATCGAGCAGGTGAAAG	GAGTAGCAAGGCCAATGAGC
c-Src	CCAGGCTGAGGAGTGGTACT	CAGCTTGCGGATCTTGTAGT

2.7 Western blotting

Cell lysates were obtained by high-speed centrifugation (14,000 rpm) for 15 min, and proteins were collected from the supernatants. Equal amounts of proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes for 2 h. After blocking with 10% milk, PVDF membranes were incubated with primary antibodies overnight at 4℃ followed by secondary antibodies for 2 h at 4°C. Protein signals were detected using the Bio-Rad XRS chemiluminescence detection system (Hercules, CA, USA) and analyzed using ImageJ.

2.8 Establishment of an osteoporosis mouse model and bone histological analysis

To confirm the inhibitory effects of alpinetin on estrogen deficiency-induced bone mass loss and osteoclast activity, we established an osteoporosis mouse model. Twenty 8-week-old female C57BL/6 mice were randomly divided into four different groups: sham-surgery, OVX, low dose (LD), and high dose (HD) groups. One week after surgery, mice in the LD and HD groups were intraperitoneally injected with 5 mg/kg or 25 mg/kg alpinetin, respectively, every 2 days. Mice in the sham-surgery and OVX groups received an equal volume of PBS. After 4 weeks of treatment, mice were euthanized by anesthetic overdose. Bilateral femurs were isolated, fixed in 4% paraformaldehyde solution for 3 days, and evaluated by microcomputed tomography (micro-CT). Subsequently, bones were decalcified with 10% EDTA for half a month and cut into 4-μm sections, which were subjected to histological analysis.

2.9 Alkaline phosphatase (ALP) and alizarin red staining (ARS)

To determine the effects of alpinetin on osteogenesis *in vitro*, we seeded MC3T3-E1 cells in 12-well plates, and after overnight incubation with complete α-MEM, cells were treated with alpinetin (0, 20, and 50 μM) in osteogenic medium for 3 or 14 days. Cells treated for 3 days were subjected to ALP staining, and those treated for 14 days were used for ARS.

2.10 Measurement of reactive oxygen species (ROS)

To assess the effects of alpinetin on cellular ROS, we seeded RAW 264.7 cells in 12-well plates (5×10^4 cells/well) and pretreated them with alpinetin for 12 h. Subsequently, alpinetin-pretreated cells were incubated with 10 mM 2',7'-dichlorodihydro-fluorescein diacetate (H_2DCFDA) for 30 min in the dark, and then stimulated with 75 ng/mL RANKL for another 30 min. Cellular ROS levels were measured on a multimodal microplate reader (SpectraMaxM5; Molecular Devices, Sunnyvale, CA, USA).

2.11 Statistical analysis

All results are presented as means ± standard deviation (SD). Statistical significance was determined by Student's *t*-test or one-way ANOVA. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1 Alpinetin suppresses RANKL-induced osteoclast differentiation but does not affect osteogenesis *in vitro*

Cell viability results revealed that alpinetin (0–50 μM) did not significantly affect the viability of BMMs or MC3T3-E1 cells (Fig. 1B), although it suppressed RANKL-induced osteoclast differentiation in a concentration- and time-dependent manner (Fig. 1C–G). Next, we assessed the effects of alpinetin on the early (0–2 days) and late stages (2–4 days) of osteoclast formation; we found that the number and size of TRAP-positive cells were reduced both at the early and late stages, although the inhibitory effects were more profound during late-stage osteoclast formation (Fig. 1H-J). These data suggest that alpinetin strongly suppresses RANKL-induced osteoclast formation. However, ARS and ALP staining results indicated that alpinetin had no effect on osteogenesis *in vitro* (Fig. 1L-N).

3.2 Alpinetin inhibits bone resorption and RANKL-induced ROS production

The F-actin ring is a characteristic cytoskeletal structure essential for the osteolytic function of osteoclasts [30]. To determine the effects of alpinetin on F-actin ring formation, we treated BMMs with different concentrations of alpinetin during osteoclastogenesis. Then, we stained alpinetin-treated BMMs with rhodamine-phalloidin and measured the number and area of F-actin rings; we found that alpinetin inhibited actin ring formation in a dose-dependent manner (Fig. 2A–C). Additionally, we found that alpinetin treatment remarkably decreased the bone resorption area (Fig. 2D, E), suggesting that alpinetin suppresses bone resorption by osteoclasts.

Aberrant oxygen metabolism leads to the accumulation of ROS, thereby promoting the development of osteoporosis and other inflammatory diseases [31]. Interestingly, we found that alpinetin treatment significantly reduced intracellular ROS levels in RANKL-treated cells (Fig. 2F, G).

3.3 Alpinetin blocks RANKL-mediated activation of ERK/AKT/NF-κβ signaling

To elucidate the molecular mechanism by which alpinetin affects osteoclast differentiation, we pretreated RAW 264.7 cells with 20 μM alpinetin and investigated activation of the osteoclast-related signaling pathways MAPK, AKT, and NF-κβ in response to RANKL stimulation (75 ng/mL; 0–60 min). Alpinetin-treated RAW 264.7 cells exhibited profoundly lower p-ERK levels than control cells. However, activated p38 and JNK levels, downstream components of the MAPK pathway, were not affected by alpinetin treatment.

AKT is a master regulator of osteoclast differentiation and survival [32]. Notably, AKT signaling has been shown to promote osteoclast fusion and subsequent osteoclast formation [33]. In this study, we found that alpinetin significantly decreased p-AKT levels. NF-κβ signaling also plays a pivotal role in osteoclast differentiation and function [34]. We found that alpinetin (20 μM) significantly reduced the phosphorylation levels of p65. Consistent with the decrease in p-p65 levels, IκBα pathway activation was

inhibited by alpinetin treatment (Fig. 3A, B). To further confirm the effects of alpinetin on the transcriptional activity of NF-κB, we performed immunofluorescence analysis and found that alpinetin suppressed the nuclear translocation of p65 (Fig. 3C, D).

3.4 Alpinetin inhibits NFATc1 induction and osteoclast-related gene expression

The transcription factors NFATc1 and c-Fos are crucial for osteoclast differentiation [35]. Interestingly, we found that alpinetin suppressed the expression of NFATc1 and c-Fos in a time-dependent manner; when used at 20 μM, the inhibitory effects of alpinetin on NFATc1 and c-Fos expression lasted for 4 days. Additionally, the expression of CTSK, which also plays a key role in osteoclast formation and function, was also suppressed by alpinetin (Fig. 3A–E). Immunofluorescence staining confirmed that alpinetin reduced the levels of mature NFATc1 in a dose-dependent manner (Fig. 3F, G). Moreover, alpinetin significantly reduced the mRNA levels of the osteoclast-related genes *Nfatc1*, *Trap*, *Dc-stamp*, *Ctsk*, *V-ATPase-a3*, and *Mmp9* in a concentration and time-dependent manner (Fig. 3H, I), further confirming the ability of alpinetin to suppress osteoclastogenesis.

3.5 Alpinetin inhibits osteoclast activity by modulating integrin-mediated cell migration

Cell migration is critical for osteoclast activity, and is required for osteoclast migration and resorption ability [36]. We found that alpinetin markedly impaired the migration ability of differentiated osteoclasts and BMMs (Fig. 5A, B). Integrin β_3 and c-Src are highly expressed during osteoclast fusion and are required for osteoclast migration [37]. Here, we show that although integrin β_3 and c-Src expression levels gradually increase during osteoclast fusion, alpinetin treatment remarkably suppressed their expression (Fig. 5C, D). Consistently, alpinetin treatment (20 μM for 1 or 2 days) in mature osteoclasts derived from RANKL-stimulated BMMs significantly reduced integrin β_3 and c-Src levels both at the mRNA (Fig. 5E, F) and protein level (Fig. 5G–J). Collectively, these results suggest that alpinetin inhibits osteoclast activity by modulating integrin-mediated osteoclast migration.

3.6 Alpinetin inhibits lysosomal biogenesis and trafficking by modulating the PKCβ-TFEB and ATG5-LC3 axes

The ability of mature osteoclasts to resorb bones is strongly dependent on lysosomes, which are enriched at the osteoclast ruffled border and mediate degradation of the extracellular bone matrix [38]. To assess the effects of alpinetin on lysosomal biogenesis, we investigated the expression levels of TFEB, a transcription factor required for lysosomal biogenesis [39]. We found that although RANKL stimulation increased TFEB levels, alpinetin suppressed the ability of RANKL to induce TFEB expression. Additionally, alpinetin inhibited the expression of PKCβ (which is upstream of TFEB activation) and the lysosomal marker LAMP1 (Fig. 6A-D). In mature osteoclasts, alpinetin treatment reduced the protein levels of PKCβ, TFEB, and LAMP1, as well as the mRNA levels of the TFEB target genes [40] *Acp5*, *Aatp6v0d2*, *Clcn7*, and

Tcirg1 (Fig. 6E). To further confirm the effects of alpinetin on lysosomal biogenesis, we performed immunofluorescence staining for LAMP1 and found that alpinetin reduced the levels of LAMP1 in osteoclasts (Fig. 6F, G). These results suggest that alpinetin suppresses lysosomal biogenesis by modulating the PKCβ-TFEB axis.

Lysosomal trafficking plays a critical role in osteoclast-mediated osteolysis [41]. We found that although ATG7, CTSK, and LC3 protein levels were gradually increased during RANKL-induced osteoclastogenesis, CTSK is considered one of the most effective protease, of which main function is to regulate lysosome secretion, thereby promoting bone resorption ability [42]. Alpinetin (20 μM) significantly decreased the levels of all these proteins (Fig. 7A, B). Similarly, alpinetin treatment profoundly decreased the levels of ATG7, CTSK, and LC3 in mature osteoclasts (Fig. 7C, D). Consistently, BMMs treated with alpinetin exhibited significantly lower *Atg5* and *Ctsk* mRNA levels than control BMMs (Fig. 7E). Collectively, these data indicate that alpinetin inhibits lysosomal trafficking by modulating the ATG5-LC3 axis.

3.7 Alpinetin alleviates OVX-induced bone mass loss and inhibits osteoclast formation in an osteoporosis mouse model

To assess the therapeutic potential of alpinetin *in vivo*, we investigated the effects of alpinetin on bone volume loss and osteoclast activity in an OVX-induced osteoporosis mouse model. Micro-CT findings revealed profound bone loss in the distal femurs of mice in the OVX group, confirming the establishment of an osteoporosis mouse model. Compared with mice in the OVX group, the femurs of alpinetin-treated mice exhibited a significantly increased bone volume, indicating the protective effect of alpinetin against bone mass loss (Fig. 8A–G). Additionally, we found that the protective effects of alpinetin against OVX-induced bone mass loss were significantly stronger in mice treated with 25 mg/kg alpinetin (HD group) than those treated with 5 mg/kg alpinetin (LD group), suggesting that the protective effects of alpinetin are dose-dependent.

Histological analyses (hematoxylin and eosin, Masson, and TRAP staining) of decalcified femurs indicated that alpinetin alleviated the OVX-induced bone mass loss and osteoclast formation, especially when used at 25 mg/kg (Fig. 8H–K). Taken together, these data demonstrate that alpinetin prevents OVX-induced bone mass loss and osteoclast formation in mice with osteoporosis.

4. Discussion

Bone metabolism homeostasis disruption due to imbalances in bone formation and bone resorption has been associated with various bone metabolism disorders, including rheumatoid arthritis [43], Paget's disease [44], and osteoporosis [45], imposing a serious public health challenge. Osteoporosis is a prevalent disease, affecting millions of individuals around the world. Given the importance of osteoclasts in osteolysis, targeting osteoclast activity is considered a promising approach for the management of osteolysis-related bone diseases. Although numerous drugs have gained regulatory approval for use in

patients with osteoporosis, they suffer from poor efficacy and high toxicity. In this study, we confirmed that alpinetin reduced osteoclast formation *in vitro* and inhibited estrogen deficiency-induced bone loss by suppressing osteoclast activity, without affecting osteoblast formation *in vivo*.

The RANKL/RANK axis plays a critical role in osteoclast formation and function [46]. Binding of RANKL to RANK promotes the recruitment of TNF receptor-associated factor 6 (TRAF6), which activates the TGF-β-activated kinase 1 (TAK1) [47], IKK/Ikβα, AKT, and MAPKs. IKKα/β phosphorylation enhances the degradation of IκB, thereby activating NF-κβ and AP-1 [48]. Subsequently, AP-1 and NF-κB induce the expression of NFATc1, which, in turn, activates the expression of osteoclast-related genes and promotes osteoclast differentiation. In this study, we found that alpinetin inhibited RANKL-mediated activation of NF-κβ, ERK, and AKT signaling, without affecting activation of p38 and JNK.

Excessive amounts of ROS disrupt reduction-oxidation homeostasis and physiological cell metabolism [31]. Activation of RANKL-RANK signaling promotes ROS production during osteoclast formation [49], which further enhances osteoclast differentiation and bone resorption. All results demonstrated that alpinetin remarkably attenuated ROS production in RAW 264.7 cells.

Cell adhesion and migration are essential for osteoclast-mediated osteolysis, with integrin regulating both of these processes [50]. Integrin is highly expressed in differentiated osteoclasts, promoting transport of acidifying vesicles to the bone matrix surface. In this study, we found that alpinetin inhibited osteoclastogenesis and the migration of osteoclasts and osteoclast precursors by inhibiting integrin β3 and c-Src.

In addition to cell migration, lysosomal biogenesis and trafficking are also required for bone degradation and resorption. TFEB, a newly identified transcription factor involved in lysosomal biogenesis, has been shown to regulate the expression of various genes promoting ECM acidification and degradation [39], including *Acp5*, *Atp6v0d2*, *Clcn7*, and *Tcirg1*. Upon RANKL-RANK interaction and subsequent PKCβ activation, TFEB translocates into the cell nucleus, activating expression of its target genes [51]. PKCβ inhibition has been demonstrated to suppress TFEB stabilization, impairing lysosome biogenesis and increasing bone mass [52]. In line with these findings, we found that alpinetin destabilized TFEB via inhibition of PKCβ expression, thereby suppressing lysosomal biogenesis. Autophagy and autophagy-related proteins, including ATG5 and LC3, have been shown to regulate lysosomal trafficking and secretion. In the absence of these proteins, lysosomal trafficking in osteoclasts and subsequent bone degradation is significantly impaired [53]. Importantly, alpinetin inhibited lysosomal trafficking and secretion of lysosomal enzymes by modulating the ATG5/LC3 axis. However, the underlying mechanisms remain to be elucidated.

In conclusion, our findings suggest that alpinetin inhibits NFATc1 expression and subsequent osteoclast differentiation by modulating the NF-κB, AKT, and ERK signaling cascades. Our results also indicate that alpinetin inhibits lysosomal biogenesis and trafficking by modulating the PKCβ-TFEB and ATG5-LC3 axes, respectively. Importantly, alpinetin alleviated OVX-induced bone mass loss in an osteoporosis mouse model, suggesting that alpinetin may represent a promising anti-osteoporosis agent.

Declarations

Ethical Approval and Consent to participate

This work has been approved for animal ethics by the Second Affiliated Hospital, School of Medicine, Zhejiang University.

Consent for publication

Not applicable.

Data availability statement

All the data that support the findings of this study are included in the manuscript.

Competing Interest

The authors declare that they have no competing interests

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Author contributions

JL and RH: conception and design; JL, YC, TC, CY, WH, LT, CW, ZK, SX XC and SJ: experiments and/or data analysis; JL, YC and TC: intellectual input and supervision; JL and RH: article writing with contributions from other authors.

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Figures

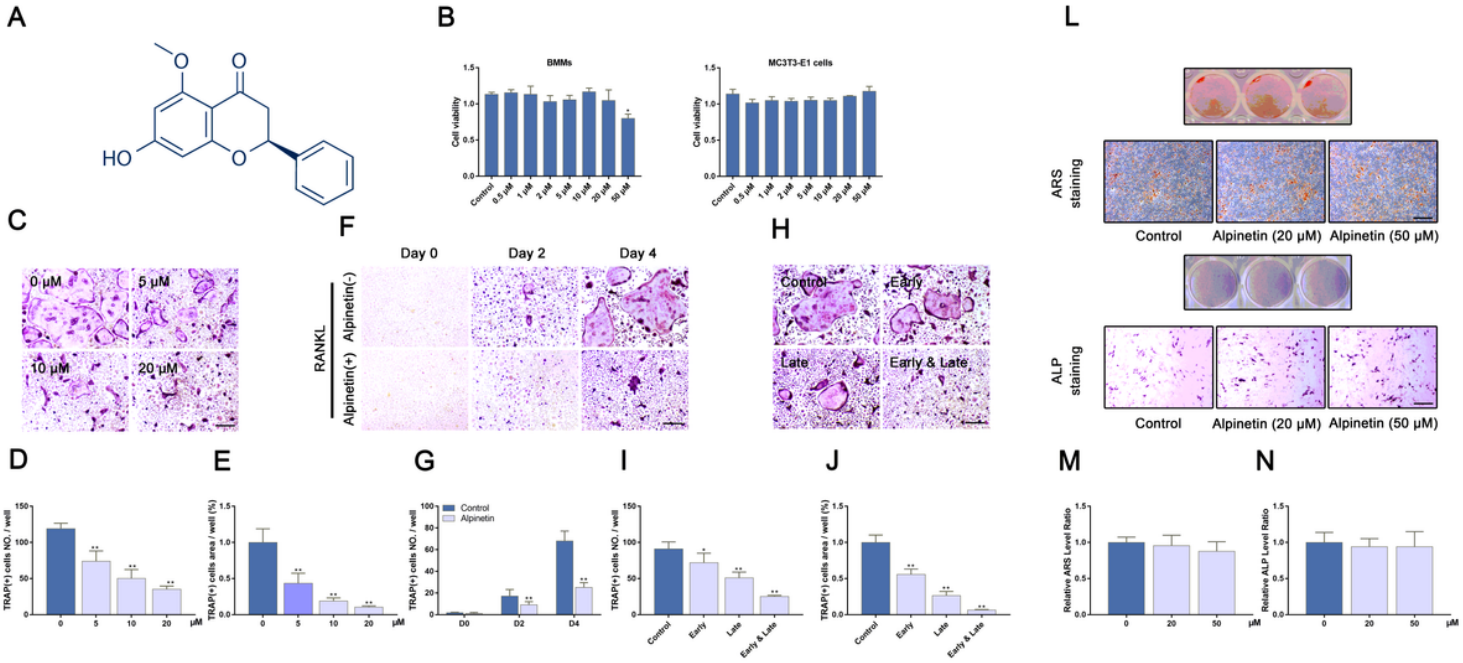

Figure 1

Alpinetin inhibits RANKL-induced osteoclast differentiation but does not affect osteogenesis in vitro. A) The chemical structure of alpinetin. B) The cell viability of alpinetin-treated osteoclast precursors and MC3T3-E1 cells were measured from the relative absorbance value at 450 nm. C-J) BMMs were stimulated with 40 ng/ml M-CSF and 75 ng/ml RANKL in the presence of different concentrations of alpinetin for the indicated times, then all cells were fixed and subjected to TRAP staining. The number and area of mature osteoclasts significantly decreased in concentration and time-dependent manner. Scale bar = 500 μm. L-N) MC3T3-E1 cells were treated with indicated concentrations of alpinetin for 3 days or 14 days, respectively. The results obtained from ARS and ALP staining images confirmed that alpinetin had no obvious effect on osteogenesis. Scale bar = 500 μm. *P < 0.05, **P < 0.01 vs. the control group.

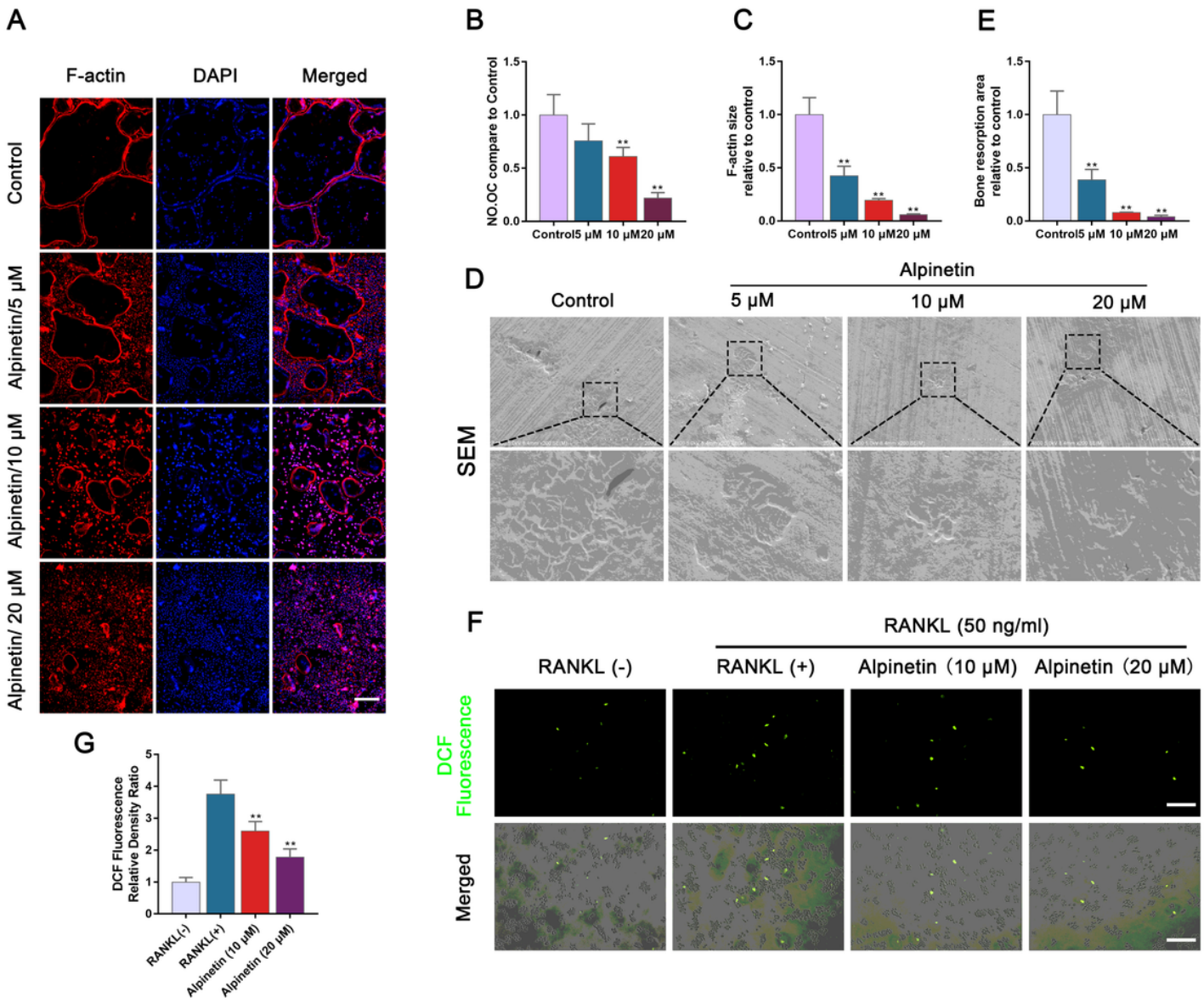

Figure 2

Inhibition of bone resorption function and RANKL-induced ROS production by alpinetin. A-C) BMMs were seeded in 48-well plates and then treated with alpinetin (0-20 μM) for 4 days. After fixed and performed with rhodamine-phalloidin staining, the number and size of F-actin rings were analyzed under a confocal microscope, and the results showed that F-actin formation were remarkably suppressed. Scale bar = 200 μm. D, E) RANKL stimulation of macrophage precursors induced bone resorption on bone discs. After treated with indicated concentrations of alpinetin for 7-8 days, the resorption pits were visualized and quantified by SEM. Scale bar = 200 μm. F, G) Cellular ROS levels also increased upon RANKL incubation and decreased in the presence of alpinetin. Scale bar = 200 μm. *P < 0.05, **P < 0.01 vs. the control group.

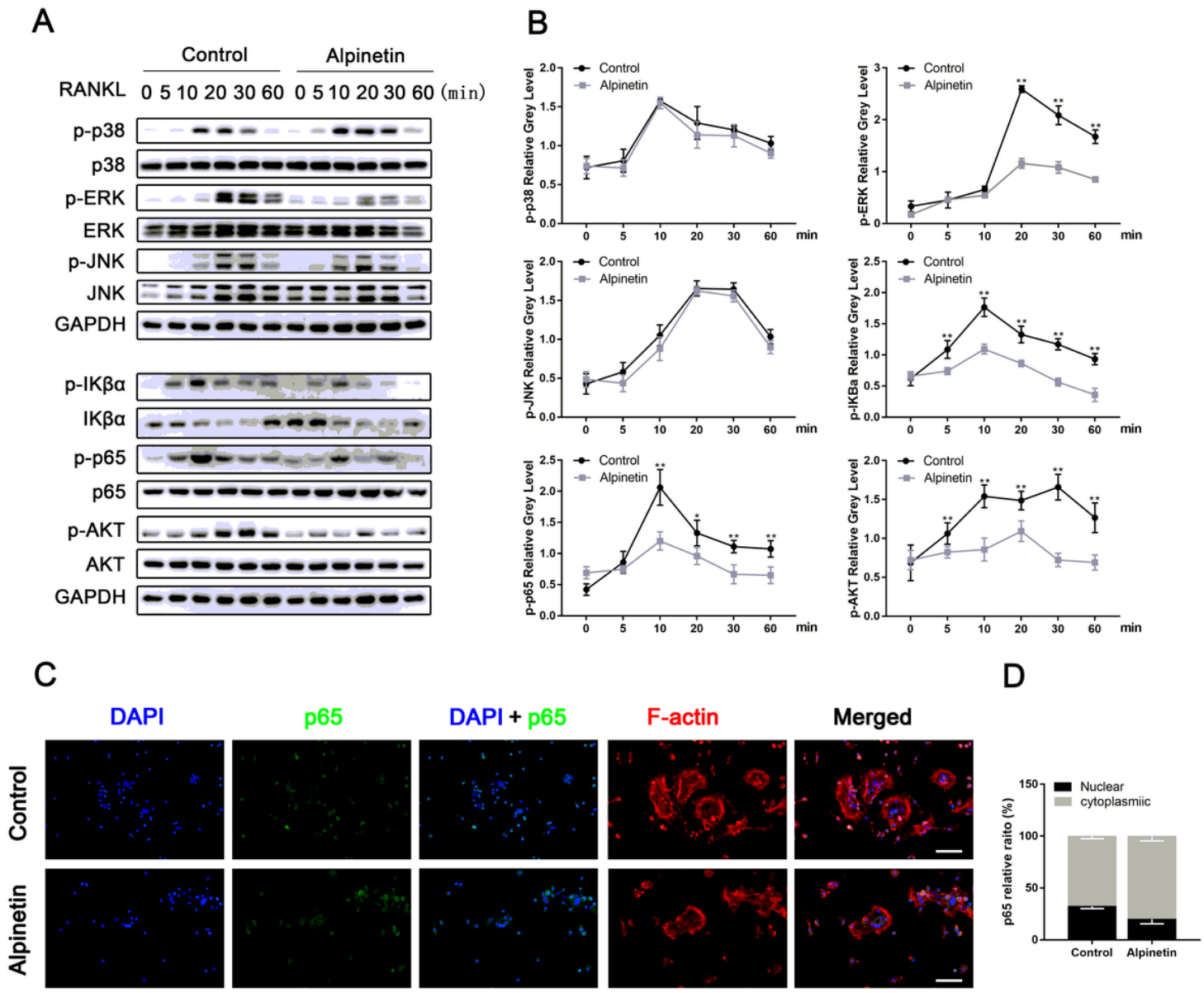

Figure 3

Alpinetin inhibits osteoclast differentiation via the ERK, AKT and NF-κβ signaling cascades. A, B) RAW264.7 cells were pretreated with or without 20 μM alpinetin for 4-6 h and then stimulated with 75 ng/ml RANKL for 0, 5, 10, 20, 30, 60 min, respectively. All cells were lysed and cell proteins were subjected to immunoblotting analysis. C, D) BMMs treated with or without indicated concentrations of alpinetin were used to detect the transcriptional activity of NF-κB, and the results suggested that alpinetin attenuated the nuclear translocation of p65. Scale bar = 200 μm. *P < 0.05, **P < 0.01 vs. the control group.

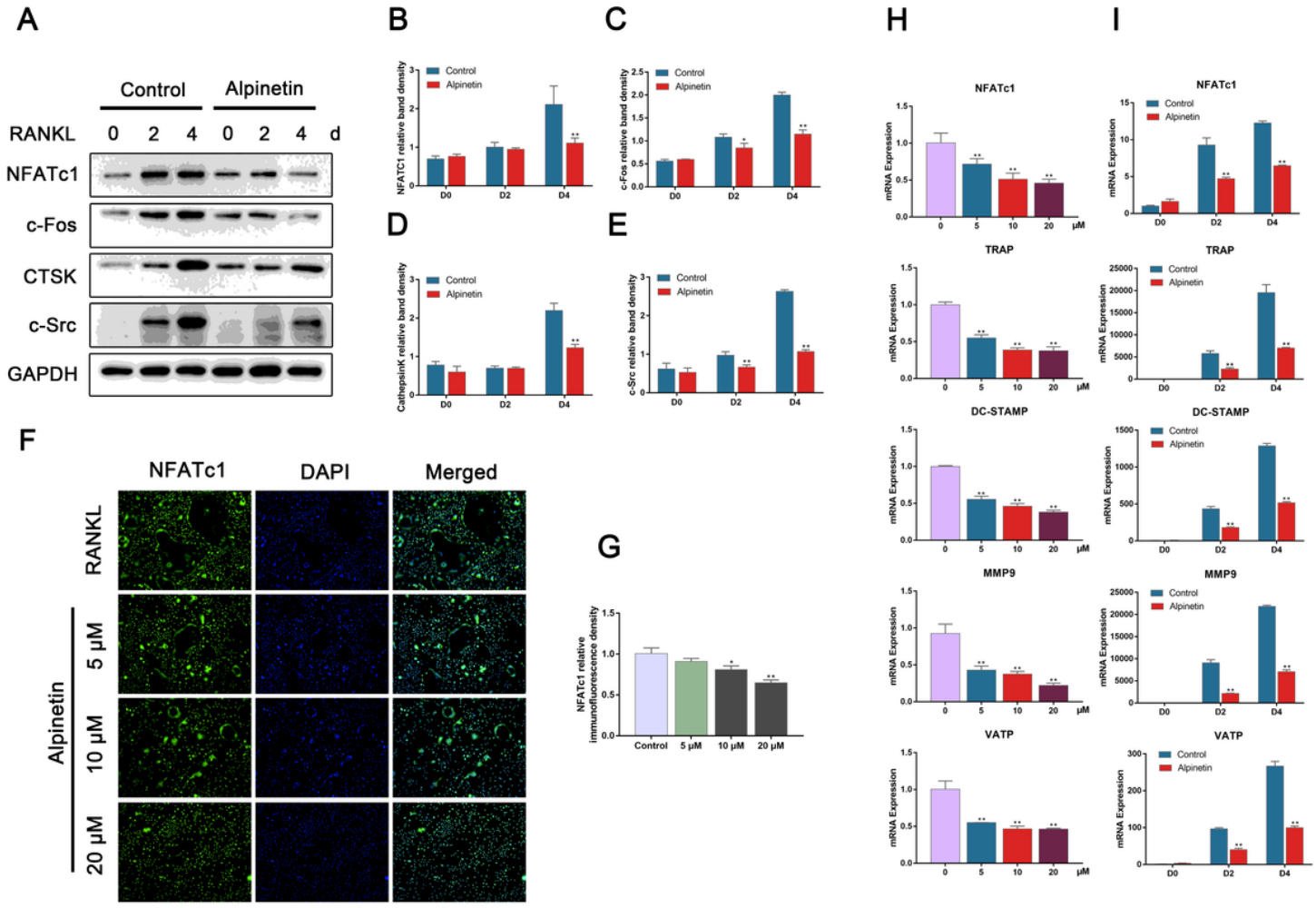

Figure 4

Alpinetin inhibits protein and mRNA expression of osteoclast-specific markers in vitro. A-E) BMMs were cultured with osteoclastogenic medium in the presence of 20 μM alpinetin for 4 days, and the protein expression of osteoclast-specific markers including NFATc1, c-Fos, CTSK and c-Src were detected by western blot analysis. F, G) The expression of osteoclastogenesis-specific transcription factor NFATc1 was measured by immunofluorescence staining after BMMs were treated with different concentrations of alpinetin. Scale bar = 200 μm. H, I) qRT-PCR was performed to quantity the mRNA expression of osteoclast-related genes including Nfatc1, Trap, Dc-stamp, Ctsk, V-ATPase-a3, and Mmp9. *P < 0.05, **P < 0.01 vs. the control group.

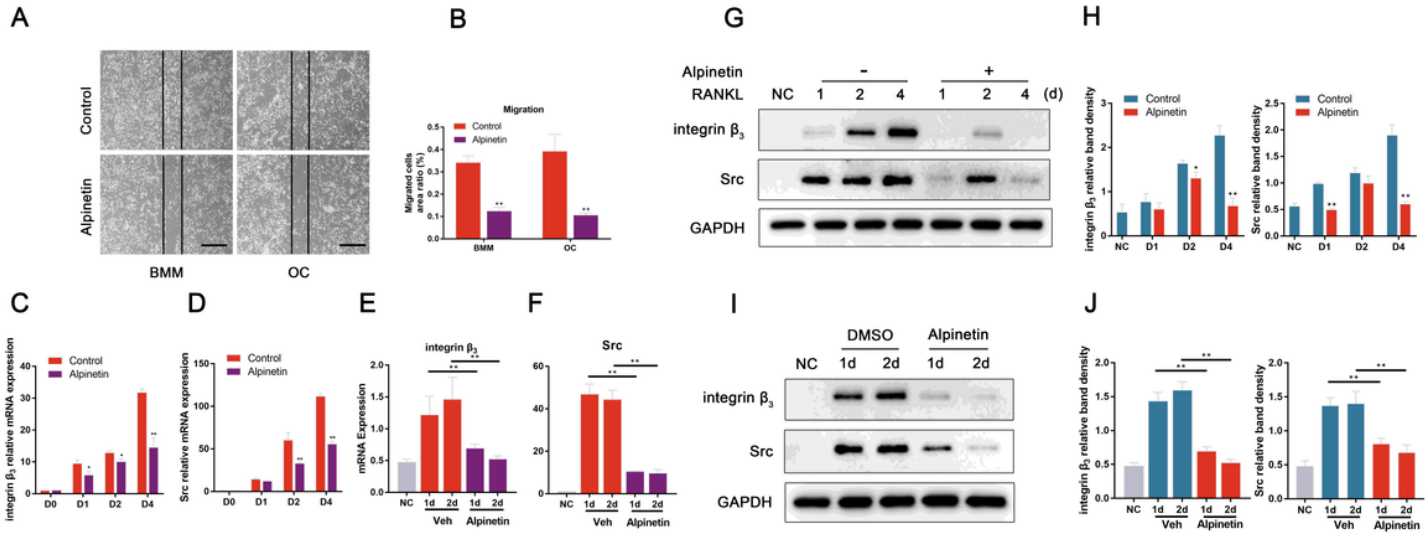

Figure 5

Inhibition of osteoclast activity via down-regulation of integrin-mediated migration by alpinetin. A, B) BMMs were incubated with or without RANKL for 3 days, and then cultured in the presence of 20 μM alpinetin or not for another 2 days after gentle scratching. The proportion of migrated cells area over the scratching area was counted by ImageJ software. Scale bar = 500 μm. C-J) BMMs were treated with or without 20 μM alpinetin for the indicated times, and the protein and mRNA expression of integrin β3 and Src were detected by immunoblotting analysis and qRT-PCR. BMMs were cultured with osteoclastogenic medium for 4 days until mature osteoclasts formed, after which the cells were incubated with 20 μM alpinetin for 1 day or 2 days, respectively. The levels of protein and mRNA relative to BMM were measured as described above. *P < 0.05, **P < 0.01 vs. the control group.

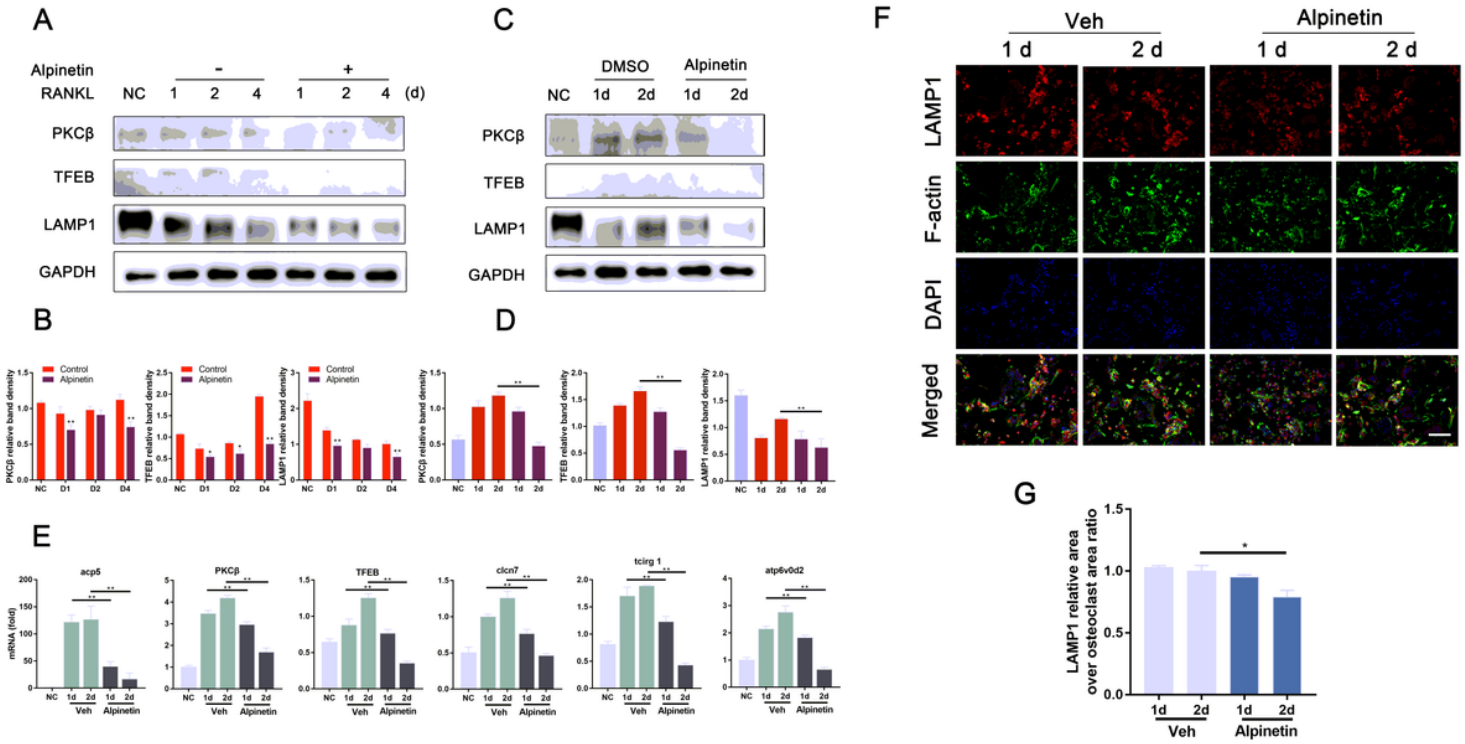

Figure 6

Alpinetin abrogates suppressing lysosomal biogenesis via the PKCβ-TFEB axes. A, B) BMMs were incubated with osteoclastogenic medium in the presence of 20 μM alpinetin for 4 days. Cell lysates were collected and used for western blotting analysis. C, D) Alpinetin (20 μM) was added in differentiated osteoclasts for the indicated times, and the protein signals relative to BMM were detected using chemiluminescent immunoassay. E) The levels of lysosome-specific genes including TFEB, PKCβ, Acp5, Aatp6v0d2, Clcn7, and Tcirg1, were also determined by qRT-PCR. F, G) BMMs were treated with or without alpinetin as previously described, then all cells were blocked and stained with LAMP1 primary antibody. The percentage of the LAMP1 relative area over osteoclast area was quantified. Scale bar = 200 μm. *P < 0.05, **P < 0.01 vs. the control group.

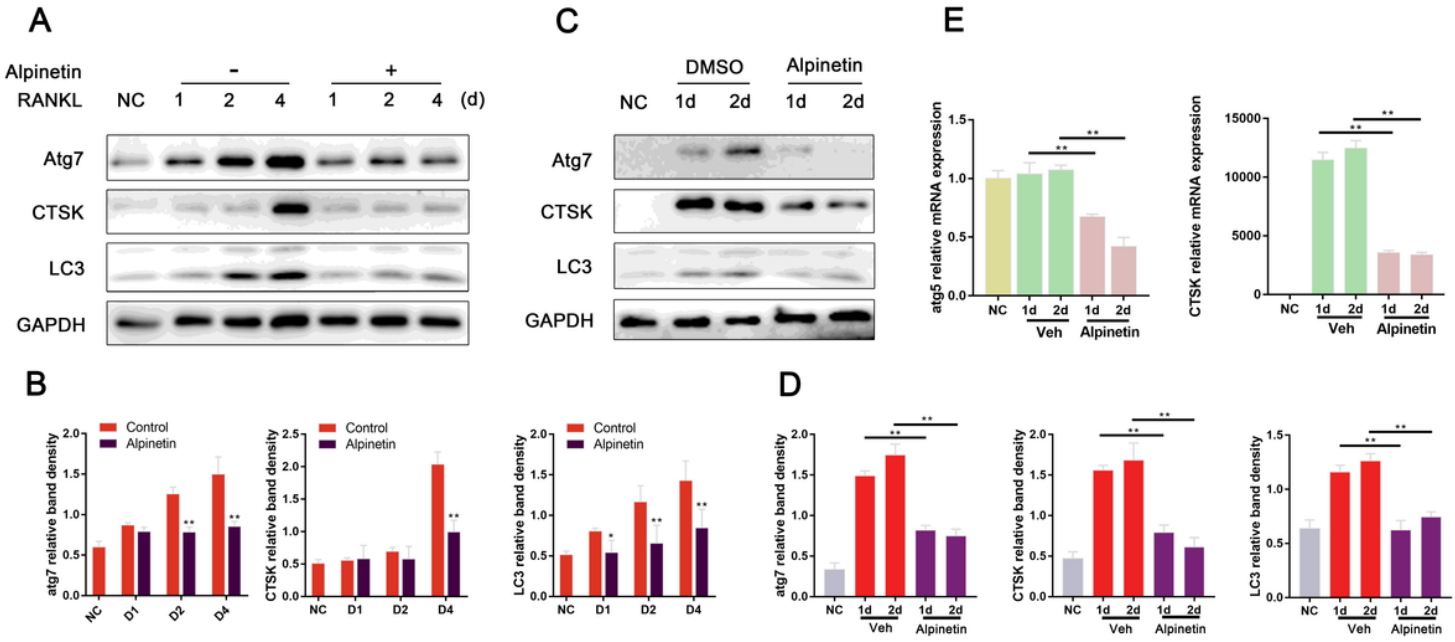

Figure 7

Inhibition of lysosome secretion via the down-regulation of ATG5-LC3 axes by alpinetin. A-E) BMMs were cultured with osteoclastogenic medium in the presence of 20 μM alpinetin for 4 days. Western blotting assay was performed to detect the levels of ATG5, CTSK and LC3 proteins. BMMs were also cultured with RANKL for the indicated times until mature osteoclasts formed, then the cells were exposed to alpinetin (20 μM) for 1 day or 2 days, respectively. The protein amounts and individual mRNA levels relative to BMM were subjected to chemiluminescence analysis and qRT-PCR. *P < 0.05, **P < 0.01 vs. the control group.

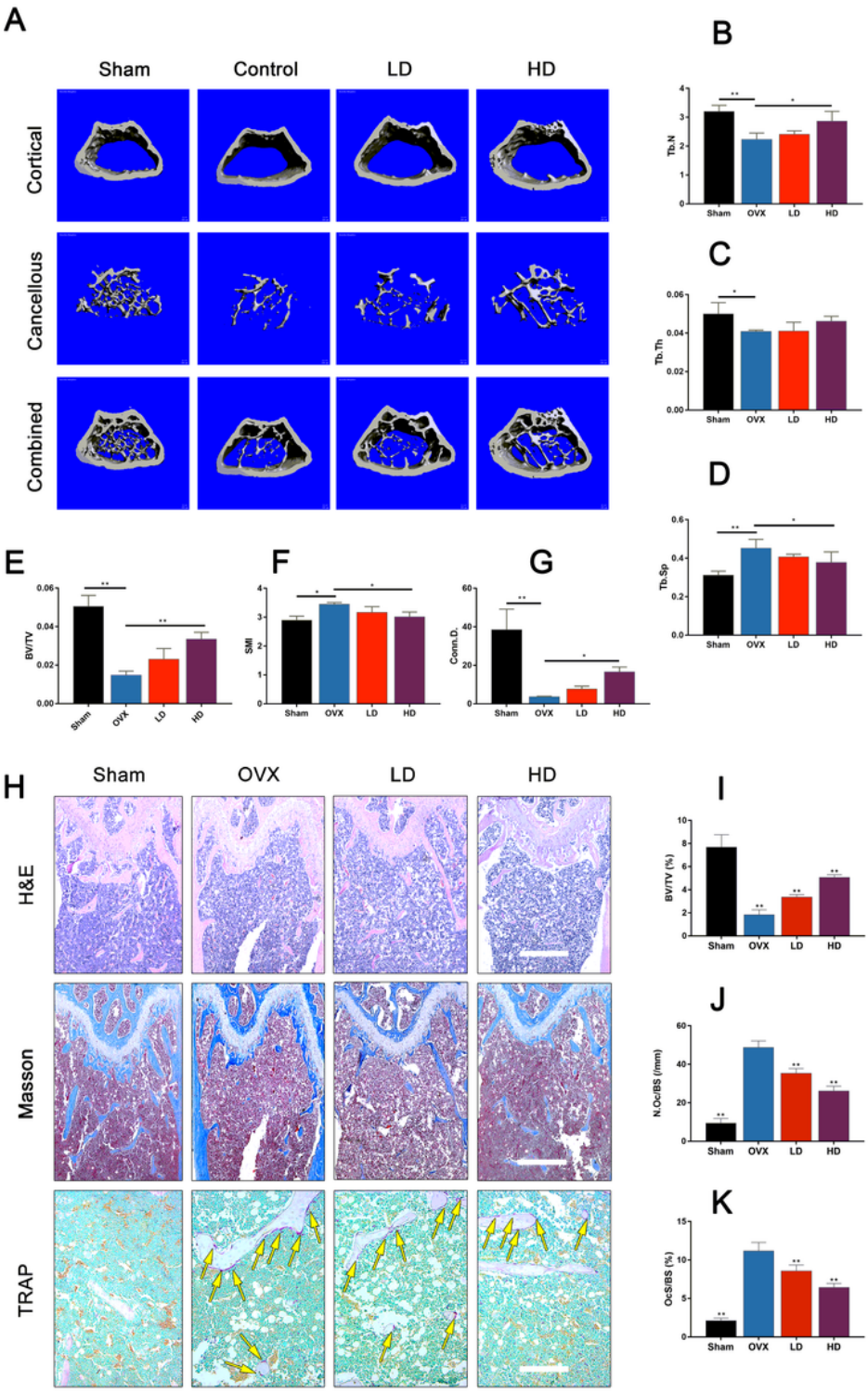

Figure 8

Alpinetin prevents OVX-induced bone mass loss in vivo. A-G) Representative micro-CT images of trabecular bone of distal femurs were obtained from four different groups: sham-surgery, OVX, low dose (LD), and high dose (HD) groups. Scale bar = 500 μm. The statistical results of Tb.N, Tb.Th, Tb.Sp, BV/TV, Conn.D and SMI form micro-CT analysis were evaluated. H-K) H&E and Masson's trichrome staining were used for bone histological analysis. Scale bar = 500 μm. Representative images of TRAP staining were

obtained for evaluating osteoclasts activity. Scale bar = 200 μm. The values of histomorphology staining images were quantified and analyzed by ImageJ software. BV/TV: bone volume/tissue volume; N.Oc/BS: the number of osteoclasts normalized to the bone surface; OcS/BS: the surface area of osteoclasts normalized to bone surface area. *P < 0.05, **P < 0.01 vs. the control group.

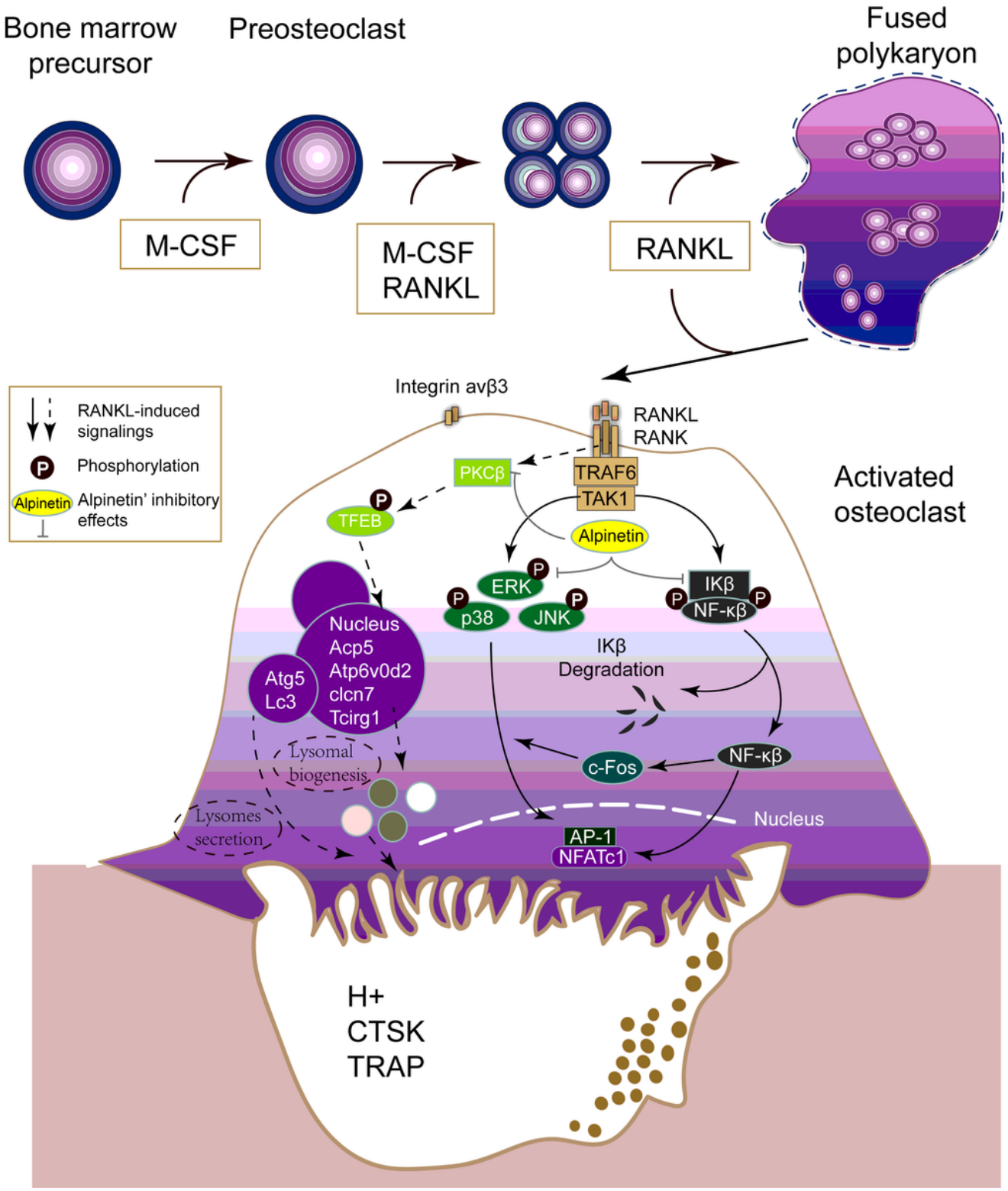

Figure 9

A proposed mechanism diagram of alpinetin inhibiting osteoclast differentiation and function has been presented. Alpinetin attenuates NFATc1 production via inhibiting RANKL-induced downstream signaling cascades, thereby suppressing osteoclast formation. Alpinetin inhibits osteoclast function by regulating integrin-mediated cell migration and lysosomal biogenesis via the PKCβ-TFEB axes. Alpinetin also abrogates bone resorption by suppressing lysosome trafficking via the down-regulation of Atg5 expression in differentiated osteoclasts.