Cilengitide inhibits osteoclast adhesion through blocking the αvβ3-mediated FAK/Src signaling pathway

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ABSTRACT

The remodeling of actin cytoskeleton of osteoclasts on the bone matrix is essential for osteoclastic resorption activity. A specific regulator of the osteoclast cytoskeleton, integrin αvβ3, is known to provide a key role in the degradation of mineralized bone matrixes. Cilengitide is a potent inhibitor of integrins and is capable of affecting αvβ3 receptors, and has anti-tumor and anti-angiogenic and apoptosis-inducing effects. However, its function on osteoclasts is not fully understood. Here, the cilengitide role on nuclear factor κB ligand-receptor activator (RANKL)-induced osteoclasts was explored. Cells were cultured with varying concentrations of cilengitide (0,0.002,0.2 and 20 μM) for 7 days, followed by detected *via* Cell Counting Kit-8, staining for tartrate resistant acid phosphatase (TRAP), F-actin ring formation, bone resorption assays, adhesion assays, immunoblotting assays, and real-time fluorescent quantitative PCR. Results demonstrated that cilengitide effectively restrained the functionality and formation of osteoclasts in a concentration-dependent manner, without causing any cytotoxic effects. Mechanistically, cilengitide inhibited osteoclast-relevant genes expression; meanwhile, cilengitide downregulated the expression of key signaling molecules associated with the osteoclast cytoskeleton, including focal adhesion kinase (FAK), integrin αvβ3 and c-Src. Therefore, this results have confirmed that cilengitide regulates osteoclast activity by blocking the integrin αvβ3 signal pathway resulting in diminished adhesion and bone resorption of osteoclasts.

1. Introduction

Osteoporosis, characterized by disruption of the homeostasis between bone formation and resorption, manifests as bone loss and elevated risks of fracture [1–5]. Currently, bisphosphonates are the most widely used anti-osteoporosis drugs in clinical practice. However, there are a handful of adverse effects due to the prolonged use of bisphosphonates, including femoral osteonecrosis, jaw osteonecrosis, and gastrointestinal side effects [6], thereby limiting their therapeutic efficacy. Thus, new classes of antiresorptive drugs primarily targeting osteoclasts involving a different mechanism of action are urgently needed.

Osteoclasts, originated from hematopoietic progenitors, are specialized multinucleated cells that can adhere to and resorb bone matrix [7,8]. The process of osteoclast differentiation and maturation relies on signaling pathways mediated by macrophage

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colony-stimulating factor (M-CSF) and RANKL [9,10]. When osteoclasts resorb bone mineral and matrix, they adhere to the bone matrix and undergo cytoskeletal changes to form a characteristic adhesion structure, the sealing zone which is composed of actin rings. Here, protons and proteases are released through its ruffled border causing localized bone degradation [11–13]. At the molecular level, the sealing zone is composed of adhesion molecules and adhesion-induced signaling proteins [14]. It is believed that osteoclast function is initiated when extracellular matrix components are recognized, presumably mediated by integrin $\alpha_v\beta_3$, which is a crucial functional adhesion receptor [15,16]. Because osteoclast adhesion is crucial for bone resorption, one available potential therapeutic option would be to block adhesion molecule formation thus preventing a sealing zone from forming.

Cilengitide, an arginine-glycine-aspartic acid (RGD) peptide that targets integrins, can selectively inhibit integrin subunit oligomerization, including alpha v, beta3 ($\alpha_v\beta_3$) and beta5 ($\alpha_v\beta_5$) [17,18]. Previous studies have demonstrated that cilengitide inhibited angiogenesis and induced endothelial cell apoptosis *via* the inhibition of integrins and the extracellular matrix [17,19]. Moreover, cilengitide has also been demonstrated to have antitumor activity by blocking integrin function [20,21]. However, the efficacy of cilengitide as an inhibitor of osteoclast adhesion remains to be elucidated.

In this study, the effects and potential mechanism of action of cilengitide on osteoclast formation and adhesion *in vitro* was explored. This investigation showed that treatment with cilengitide effectively impeded osteoclast formation and adhesion. These effects were attributed to the suppression of NFATc1 activation and the FAK/c-Src signaling pathways in primary bone marrow monocytes (BMMs) providing insight into the precise molecular mechanisms by which cilengitide modulates osteoclast behavior.

2. Materials and methods

2.1. Reagents and antibodies

Fetal bovine serum (FBS) and the alpha modification of Eagle's medium (α-MEM) were sourced from GibcoBRL (Gaithersburg, MD, USA). Life Tein (Beijing, China) supplies recombinant M-CSF and recombinant mouse RANKL. Cilengitide (purity>98%) was purchased from AbMole (Shanghai, China). Sigma-Aldrich (St. Louis, MO, USA) provided a TRAP staining assay kit. Following is a list of the primary antibodies and their dilutions: *anti*-GAPDH (1:10000; Abcam), *anti*-NFATc1 (1 μg/ml; Abcam), *anti*-Integrin $\alpha_v\beta_3$ (1:1000; Absin), *anti*-CTSK (1:10000; Abcam), FAK (1:2000; Abcam), *p*-FAK (1:1000; Abcam); c-Src (1:1000; Abcam); *p*-Src (1:500; Santa Cruz Biotechnology) and *anti*-β-actin (1:10000; Abcam). Beyotime Biotechnology (Shanghai, China) provided the CCK-8 assay kit, streptomycin/penicillin, QuickBlock™ Blocking Buffer, Actin-Tracker Red-Rhodamine, and DAPI.

2.2. Cell culture and osteoclast differentiation

The femur and tibia of C57BL/6 mice (age:7±1weeks, weight:22 ± 2 g, purchased from Kawensi, Changzhou, China) were utilized to harvest total bone marrow cells [22]. These cells were subsequently cultured in culture medium supplemented with 10% FBS, 1% penicillin and streptomycin, and 30 ng/ml M-CSF at 37 °C and 5% CO_2 [23]. Osteoclast induction was performed when cells reached 90%–100% confluence. A 96-well plate was seeded with BMMs (8×10^3/well) and incubated overnight to ensure complete cell adhesion. Subsequently, cells were cultured in culture medium consisting of 30 ng/ml M-CSF, 100 ng/ml RANKL, and varying doses of cilengitide (0, 0.002, 0.02, 0.2, 2, 20, and 200 μM) [24]. A medium change was performed every two days during osteoclast formation. Following the completion of osteoclast formation, TRAP staining was performed according to the kit instructions. An osteoclast was defined as a TRAP-positive cell with greater than or equal to three nuclei.

2.3. Cytotoxicity test

The cytotoxicity of cilengitide to BMMs was determined by CCK8 method [25]. BMMs were incubated overnight, subsequently, the cells were treated with medium containing various doses of cilengitide at different time points. The culture supernatant was then removed and a culture medium containing 10% CCK8 solution was added and incubated for 2 h. The absorbance values were measured at 450 nm. The above experiment was repeated three times and after obtaining the average value for each triplicate.

2.4. Actin ring formation assay

A 96-well plate was seeded with BMMs (8×10^3/well) and the cells were cultured for 7 days with M-CSF, RANKL, and different doses of cilengitide (0, 0.002, 0.2, and 20 μM). The cells were next fixed with Immunol Staining Fix Solution for 10−20 min, and subsequently, cells were incubated with a stain solution containing Actin-Tracker Red (rhodamine-conjugated phalloidin) for 60 min. Three washing steps were performed using PBS containing 0.1% Triton X-100. After this, DAPI stained cells for 5 min and cells were washed three times with PBS. It is worth noting that the staining procedure was conducted at room temperature and shielded from light. Finally, the results were visualized and captured using a fluorescence microscope.

2.5. Bone resorption assay

In a 96-well plate pre-coated with bone slices, BMMs were cultured for 7 days with M-CSF, RANKL, and various doses of cilengitide (0, 0.002, 0.2, and 20 μM) to facilitate the formation of mature osteoclasts. Once mature osteoclasts were observed, the culture was continued to allow the osteoclasts to complete the process of bone resorption. After this, the bone slices were exposed to sonication to

remove the cells, followed by gradient dehydration and drying, gold coating, and scanning electron microscopy examination [26].

2.6. RNA isolation and quantitative RT-PCR

A 6-well plate was seeded with BMMs (8×10^5/well) and osteoclasts were cultured and treated according to the mentioned above. Then, RNA was extracted using a cell/tissue total RNA extraction kit. To detect differential gene expression, a real-time quantitative RT-PCR system (QuantStudio 3, ThermoFisher, Waltham, USA) was employed. The sequences of the primers employed are presented in Table 1 [27,28].

2.7. Western blot analysis

Osteoclasts were cultured and treated as described above. Osteoclasts were lysed with a cell lysis solution on ice for 15 min. The components of cell lysis solution were radio immunoprecipitation assay (RIPA) lysis buffer, $100 \times$ phenylmethylsulfonyl fluoride (PMSF), $50 \times$ phosphatase inhibitor, and $100 \times$ protease inhibitor. The lysed cell suspension was centrifuged at 10,000 g for 20 min and boiled for 10 min to obtain the protein solution. The protein samples were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with primary antibodies overnight at 4 °C after blocking with QuickBlock™ Blocking Buffer. Subsequently, the secondary antibody was incubated for 1 h after washing with PBST. The protein bands were visualized using an enhanced chemiluminescence (ECL) solution (Tanon, Shanghai, China). Finally, quantification of the proteins was performed using Image J software. (National Institutes of Health, Bethesda, MD, USA).

2.8. Adhesion assay

Osteoclast precursors (1×10^5 cells/well) were inoculated into 96-well plates coated with osteopontin (Abcam, Shanghai, China) and poly-D-lysine (PDL, Absin, Shanghai, China), respectively, to evaluate the impact of cilengitide on integrins and its effect on osteoclast adhesion [24]. Osteopontin is a ligand that can bind to $\alpha_v\beta_3$. And PDL, is less cytotoxic than poly-L-lysine (PLL), and provides a poly-cationic capability for integration-independent cell adhesion to this synthetic matrix molecule. After 60 min of incubation, PBS was added, and non-adherent cells were gently washed away. Subsequently, 4% paraformaldehyde (200μL/well) was used to fix the cells for 20 min. Following washed by PBS, the cells were stained for 30 min with 0.5% crystal violet. After three washes with deionized water and removal of excess liquid, the cells were dissolved by DMSO at 100 μL/well, and finally, the absorbance value at 570 nm was measured. It is worth noting that the experiment was performed at room temperature.

2.9. Data and statistical analyses

The following statistical methodologies were employed in this study: The Shapiro-Wilk test and the Levene test (Levene, 1960) were used to assess the normal distribution and the homogeneity of variances of the data, respectively. For comparing two groups with normally distributed continuous variables, the *t*-test was utilized. In cases where there were multiple groups (n > 2) with normal distributions, one-way analysis of variance (ANOVA) was employed, followed by Tukey's test. To assess the cytotoxic effect of cilengitide on BMM, a 2-way ANOVA was performed to correct for the effect of time. $p < 0.05$ was considered statistically different. Statistical analyses were performed utilizing GraphPad Prism 8.2.1. (GraphPad Software, Inc., La Jolla, CA, United States).

Table 1
Primer sequences.

gene	Primer sequence
NFATc1	F:5′-CCGTTGCTTCCAGAAAATAACA-3′
	R:5′-TGTGGGATGTGAACTCGGAA-3′
CTSK	F:5′-CTTCCAATACGTGCAGCAGA-3′
	R:5′-TCTTCAGGGCTTTCTCGTTC-3′
integrin α_v	F:5′-TTGATTCAACAGGCAATCGAGA-3′
	R5′-AGCATACTCAACGGTCTTTGTG-3′
integrin β_3	F: 5′-CCCCGATGTAACCTGAAGGAG-3′
	R: 5′-GAAGGGCAATCCTCTGAGGG-3′
GAPDH	F: 5′-ACCCAGAAGACTGTGGATGG-3′
	R: 5′-CACATTGGGGGTAGGAACAC-3′

NFATc1- nuclear factor of activated T cells c1; CTSK- cathepsin K; integrin α_v-integrin alpha v; integrinβ_3-integrin beta 3; GAPDH- glyceraldehyde-3-phosphate dehydrogenase.
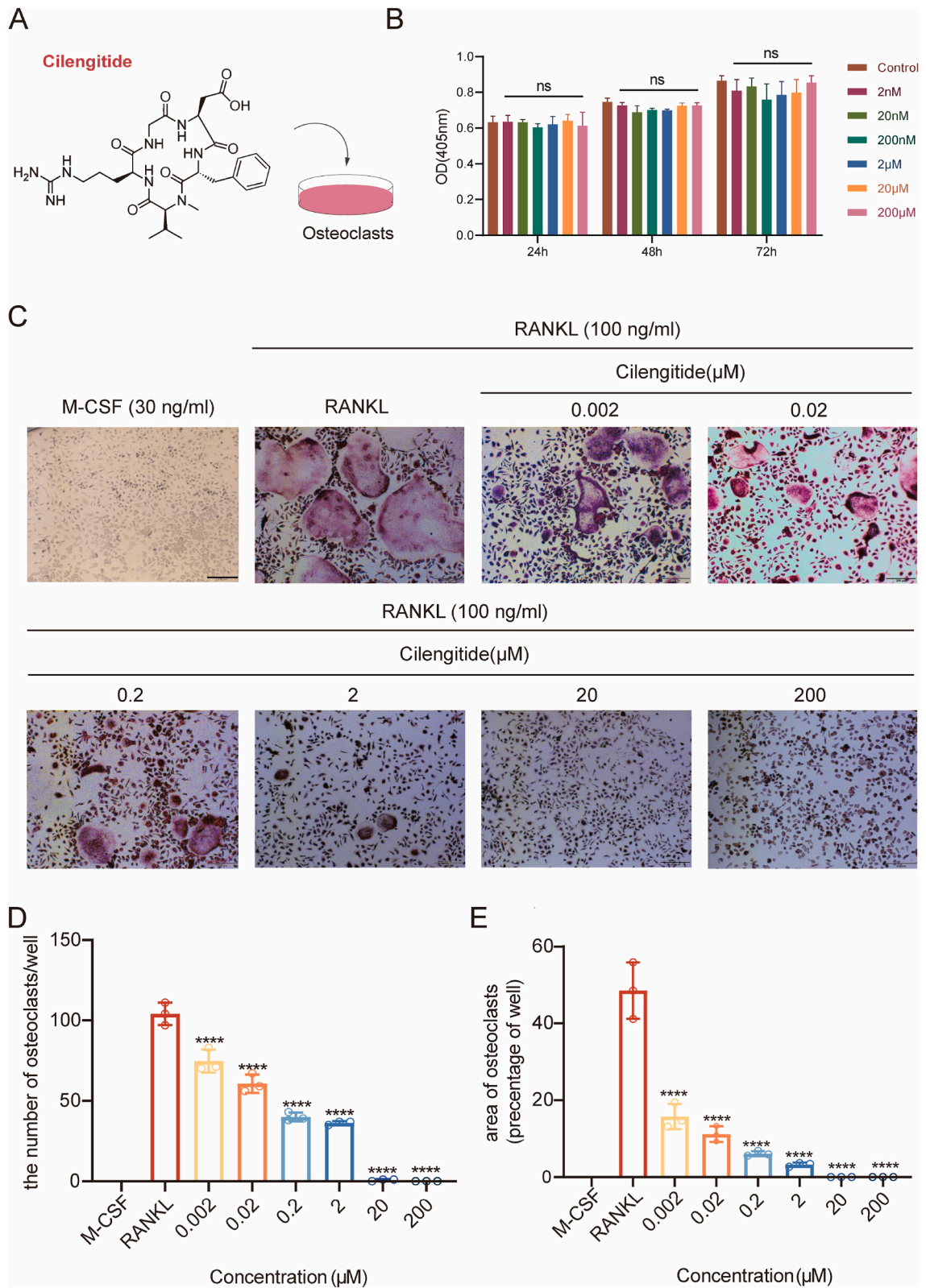
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Fig. 1. Cilengitide inhibits RANKL-induced osteoclastogenesis *in vitro*. (A) Chemical structure of cilengitide. (B) Cytotoxic effects of cilengitide on BMMs at 24 h, 48 h, and 72 h using the CCK8 assay. (C) BMMs were treated with 30 ng/ml M-CSF and 100 ng/ml RANKL and various concentrations of cilengitide for 7 days, then stained for TRAP. Scale bar: 200 μm. (D–E) Number and area of TRAP$^+$ osteoclasts, defined by $\geq$ 3 nuclei. The data are expressed as the means $\pm$ SD; ****p < 0.0001 relative to the RANKL-induced group.

3. Results

3.1. Cilengitide inhibits osteoclastogenesis in vitro

Fig. 1B revealed that cilengitide, depicted by its chemical structure in Fig. 1A, did not exhibit any cytotoxicity compared to the control group (p > 0.05). As illustrated in Fig. 1C, upon administration of M-CSF and RANKL, the BMMs matured to TRAP-positive multinuclear osteoclasts. Conversely, after cilengitide treatment, osteoclast formation was inhibited, with the greatest effect on inhibition at 20 μM and 200 μM. Cilengitide significantly reduced the number and area of TRAP-positive osteoblasts (p < 0.0001), and this inhibition was concentration dependent (Fig. 1D–E). Together, these findings indicated that cilengitide reduced the number and
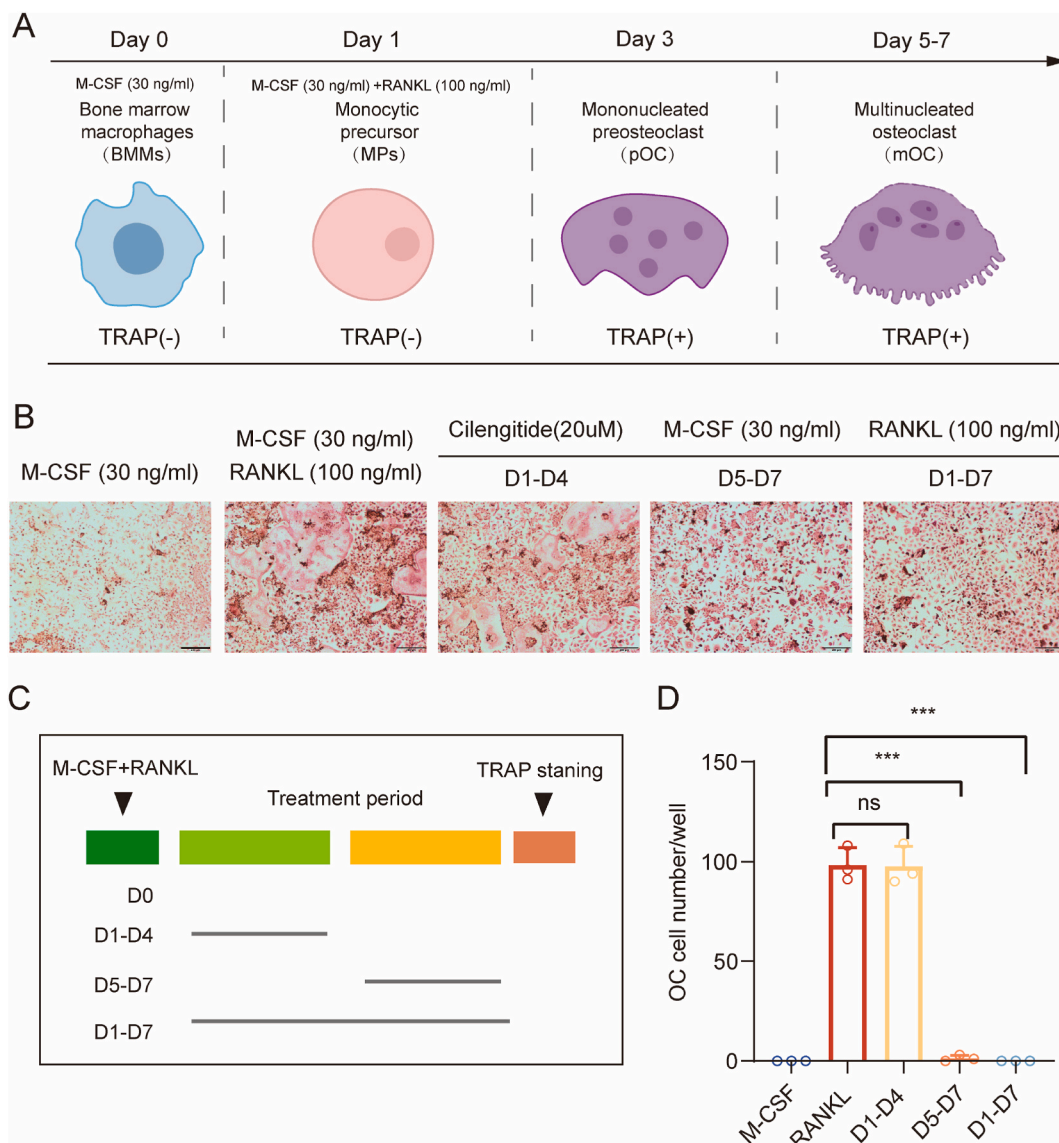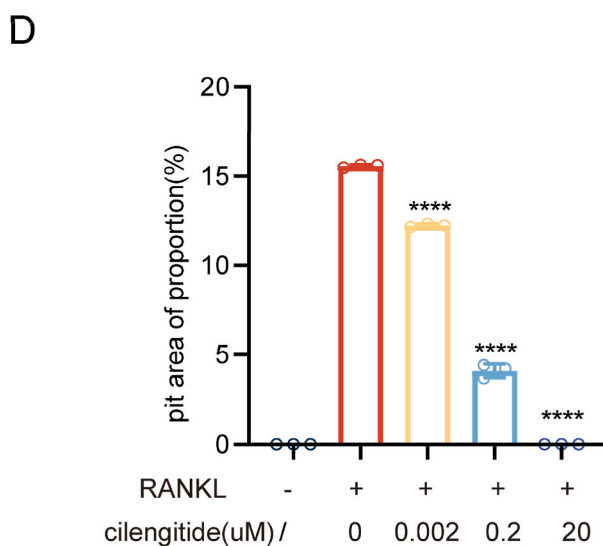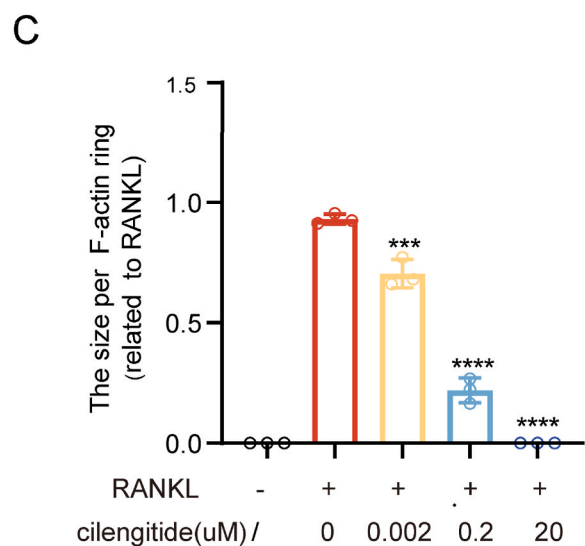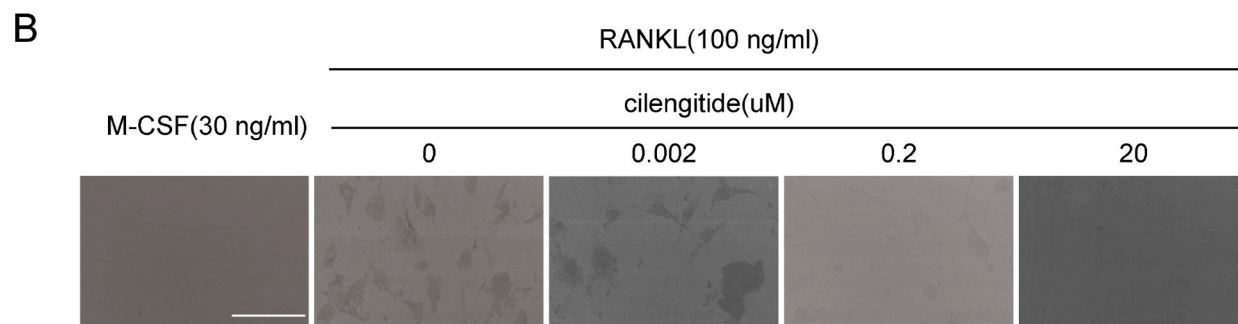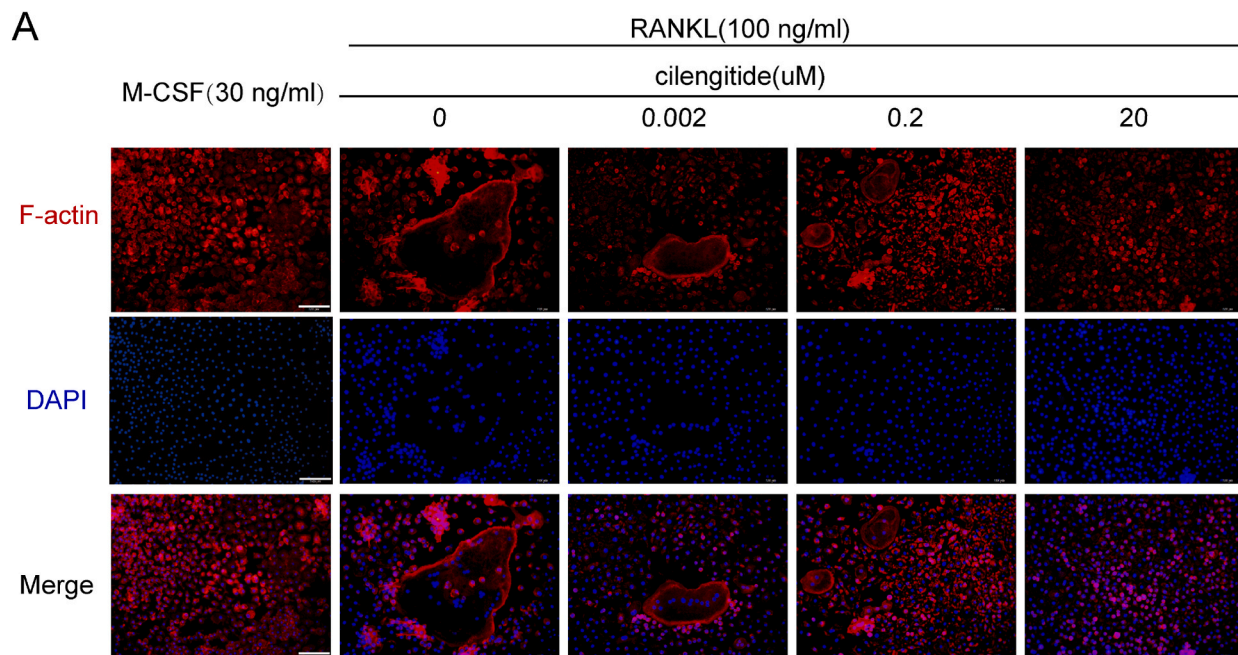

Fig. 2. Cilengitide disrupts osteoclast activation but did not affect the early stages of osteoclastogenesis. (A) Schematic diagram of osteoclast formation. (B) BMMs were treated with 20 μM cilengitide for the indicated times and were stained for TRAP. Scale bar: 200 μm. (C) Schematic diagram of BMMs processed with cilengitide at different treatment periods. (D) Number of TRAP$^+$ osteoclasts with $\geq$3 nuclei. The data are expressed as the means $\pm$ SD; ***p < 0.001 relative to the RANKL-induced group.

A

RANKL(100 ng/ml)

M-CSF(30 ng/ml)

cilengitide(uM)

| | 0 | 0.002 | 0.2 | 20 |

F-actin

DAPI

Merge

B

RANKL(100 ng/ml)

M-CSF(30 ng/ml)

cilengitide(uM)

| | 0 | 0.002 | 0.2 | 20 |

C

D

Fig. 3. Cilengitide blocks F-actin ring formation and bone-resorption of osteoclasts *in vitro*. (A) BMMs were treated with different concentrations of cilengitide (0, 0.002, 0.2, and 20 μM) for 7 days and stained for F-actin rings and DAPI staining. Scale bar: 100 μm. (B) Quantification of F-actin ring area per well. (C) Representative images of bone resorption by osteoclasts treated with different concentrations of cilengitide on bovine bone fragments. Scale bar:100 μm. (D) Quantification of bone resorption area per well. The data are expressed as the means $\pm$ SD; ***$p < 0.001$, ****$p < 0.0001$ relative to the RANKL-induced group.

area of multinucleated osteoclasts, demonstrating cilengitide-mediated impairment of mononuclear fusion.

3.2. Cilengitide disrupts osteoclast activation but did not affect the early stage of osteoclastogenesis

To determine the specific period during which cilengitide suppresses osteoclast differentiation, time-dependent investigations were conducted on RANKL-induced osteoclasts treated with 20 μM cilengitide (Fig. 2A and C). The results depicted in Fig. 2B and D reveal that cilengitide had the most significant inhibitory effect during the latter stage (D5$-$7) of osteoclast differentiation ($p < 0.0001$), but little effect during the early stage (D1$-$4, $p = 0.9998$). These findings suggested that cilengitide may primarily affect the late stage of osteoclastogenesis, rather than the early stage.

3.3. Cilengitide blocks F-actin ring formation and bone-resorption of osteoclasts in vitro

The RANKL-induced group developed distinctive F-actin rings, as seen in Fig. 3A. However, compared to the RANKL-induced group, cilengitide significantly reduced the size of the F-actin rings ($p < 0.001$), suggesting that cilengitide suppressed F-actin formation in mature osteoclasts (Fig. 3C). Additionally, Fig. 3B and D demonstrates that there were a considerable numbers of resorption pits in the RANKL-induced group , but in the cilengitide-treated group, there was a significantly reduced area of bone resorption (p < 0.0001), with virtually no bone resorption pits in the 20 μM cilengitide group. Together, these indicated that cilengitide efficiently diminished the formation of osteoclast F-actin rings and bone resorption.

3.4. Cilengitide inhibits cell adhesion and the expression of osteoclast activation related markers in osteoclasts

OPN and PDL were chosen to study the potential effect of cilengitide on the binding of integrins to relevant RGD ligands in osteoclasts. As depicted in Fig. 4A and B, cilengitide demonstrated a significant inhibition of the binding between BMMs and OPN ($p < 0.05$). However, it had no notable effect on the binding between BMMs and PDL ($p > 0.05$). These results suggested that cilengitide exhibits specific inhibitory activity against the binding of osteoclasts to bone matrix components.

To elucidate the underlying mechanism involved in the inhibitory effect of cilengitide on osteoclasts, the mRNA levels of osteoclast activation related markers, namely NFATc1, CTSK, integrin α_v, and integrin β_3 were measured. As illustrated in Fig. 4C–F, the expression of these markers was significantly diminished upon cilengitide treatment (**$p < 0.01$, ***$p < 0.001$).

Furthermore, protein levels of the above molecules were examined by immunoblot analysis to better determine the inhibition of cilengitide on osteoblast differentiation. As depicted in Fig. 4G-J, compared with the RANKL-induced group, cilengitide significantly suppressed the protein expression of integrin $\alpha_v\beta_3$, NFATc1 and CTSK (**$p < 0.01$,***p < 0.001, ****p < 0.0001). The present results provide additional evidence that cilengitide effectively inhibits the expression of key marker genes and proteins involved in osteoclast activation and adhesion.

3.5. Cilengitide inhibits RANKL-induced FAK/Src signaling

As seen in Fig. 5A–C, in contrast to the RANKL-induce group, the phosphorylation levels of FAK were considerably decreased by cilengitide administration at 60 min ($p = 0.0017$), similarly, cilengitide also significantly reduced *p*-Src expression at 60 min ($p = 0.0046$). These findings demonstrated that cilengitide inhibited osteoclast bone resorption by inhibiting downstream signaling by integrin $\alpha_v\beta_3$.

4. Discussion

Newly formed bone is in a state of flux, whereby new bone continuously replaces old bone at the same location, even at skeletal maturity [29]. This process of bone remodeling consists of two phases: bone formation and resorption. Bone homeostasis is sustained by a dynamic balance between osteoblasts and osteoclasts, whose roles are bone formation and bone resorption, respectively [30]. The present study confirmed that cilengitide suppressed osteoclast formation and bone resorption activity. Furthermore, these results confirmed that cilengitide regulated RANKL-mediated osteoclast activity by inhibiting NFATc1 and blocking the signaling pathway of integrin $\alpha_v\beta_3$ to reduce osteoclast adhesion and bone resorption.

It is known that M-CSF is well-established for its role in promoting the proliferation of osteoclastic precursor cells, known as BMMs, and their subsequent differentiation into mature osteoclasts. Conversely, RANKL is responsible for driving the differentiation of BMMs into fully mature osteoclasts. This study provide evidence that cilengitide significantly decreased osteoclast differentiation and was not cytotoxic [24]. Furthermore, we found that cilengitide principally affected the late stage of osteoclast formation. Considering that obtaining a higher number of BMM cells, these cells were collected from the femur and tibia [22].
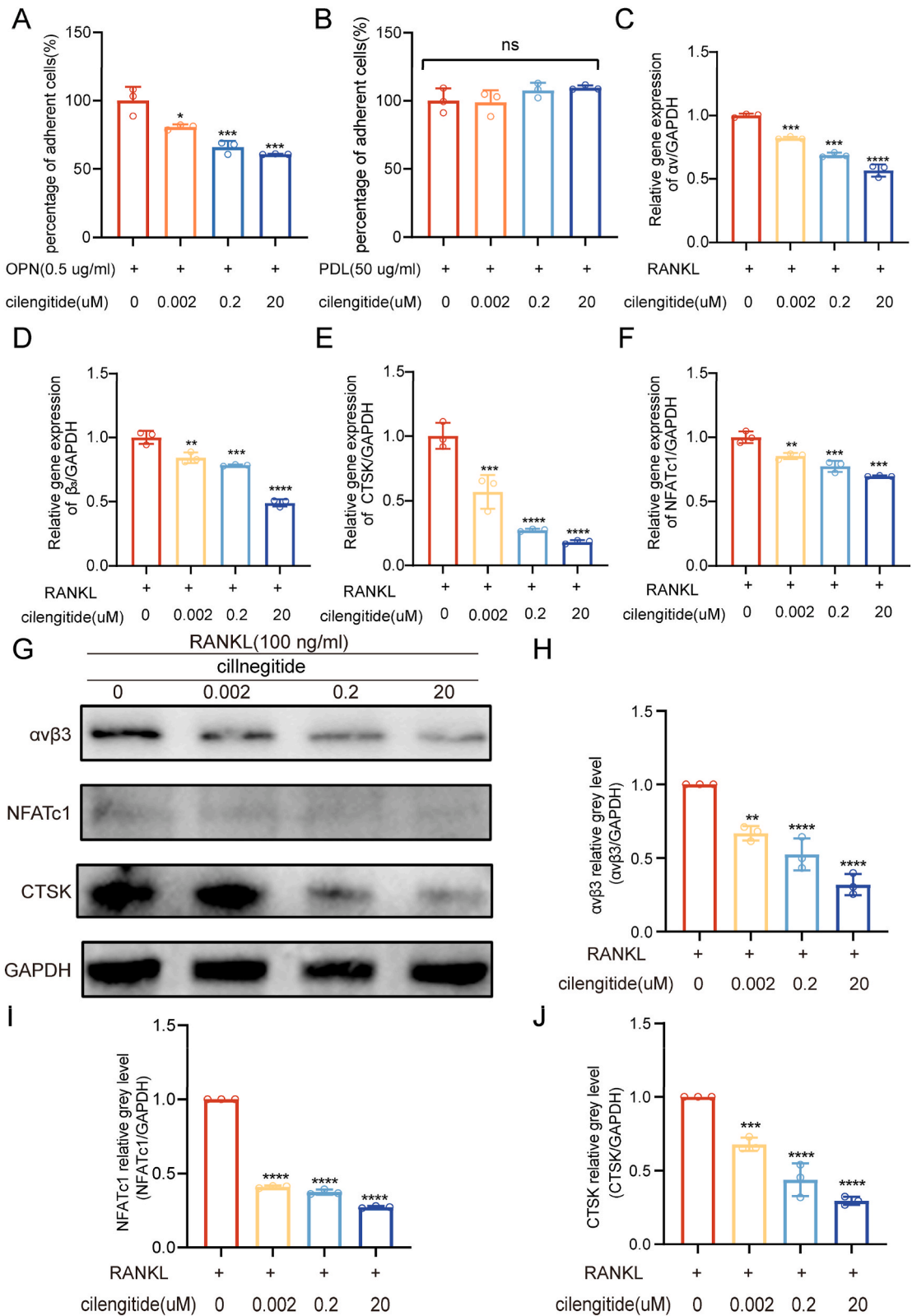
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Fig. 4. Cilengitide inhibits cell adhesion and the expression of osteoclast activation related markers in osteoclasts. (A–B) BMMs were seeded into 96-well plates coated with osteopontin (0.5 μg/ml) and PDL (50 μg/ml), respectively, in the presence of different concentrations of cilengitide (0, 0.002, 2, and 20 μM). Cell adhesion was quantified by crystalline violet staining and enzymatic labeling. (C–F) The expression of the osteoclast-related genes NFATc1, CTSK, and integrin genes (α_v, β_3) were analyzed by real-time PCR, and their expression levels were normalized to that of GAPDH. (G) Representative immunoblot images of integrin $\alpha_v\beta_3$, NFATc1, CTSK and GAPDH. (H–J) Analysis of the intensity of differentially expressed protein bands at different concentrations of cilengitide. The data are expressed as the means ± SD; **$p < 0.01$,***$p < 0.001$, ****$p < 0.0001$ relative to the RANKL-induced group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
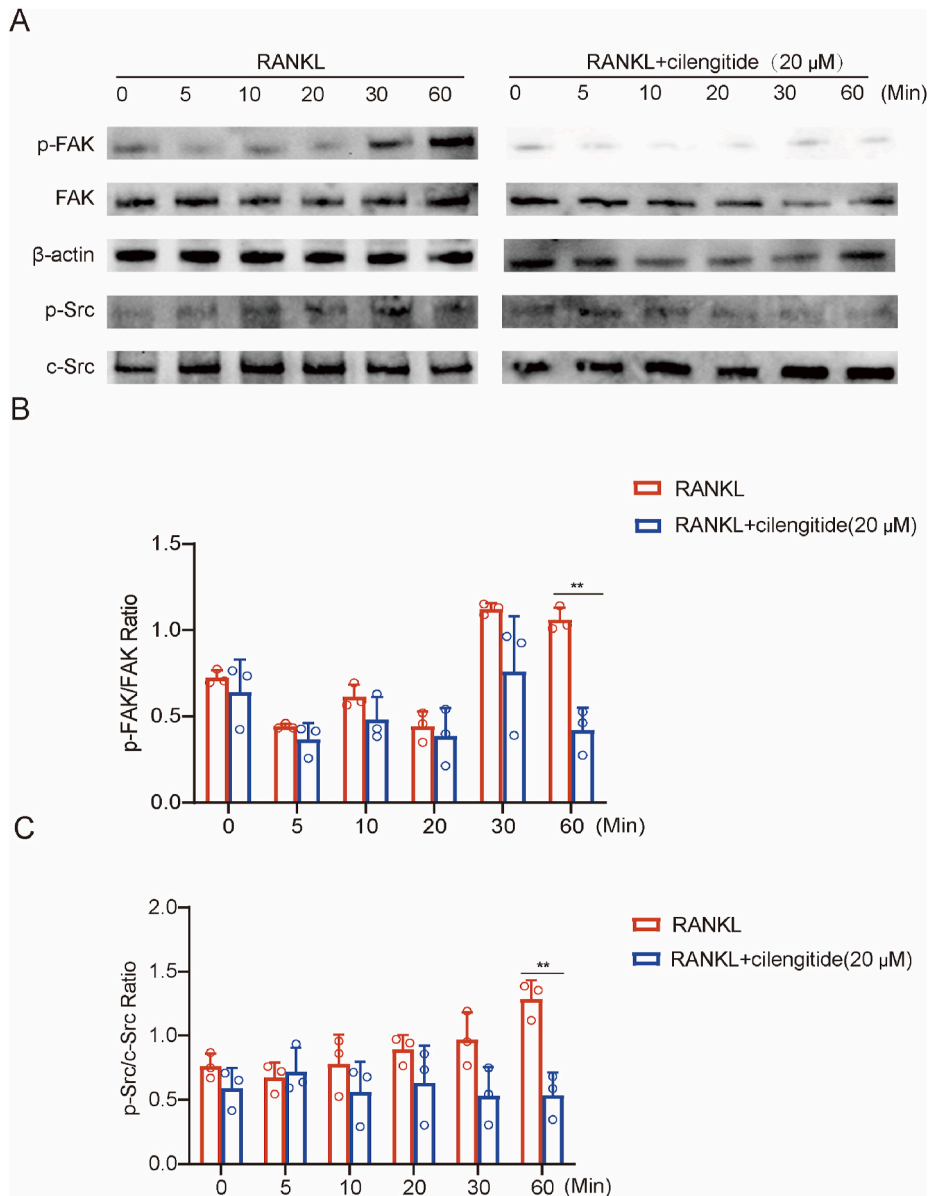

Fig. 5. Cilengitide inhibits FAK/Src signaling. (A) Representative immunoblot images of FAK, *p*-FAK, c-Src, *p*-Src and β-actin. (B–C) Analysis of the intensity of differentially expressed protein bands in the different groups. The data are expressed as the means ± SD; **$p < 0.01$ relative to the RANKL-induced group.

In addition, F-actin rings represent a crucial structure for osteoclast resorption, and the F-actin rich sealing zone formed by these rings represents the main structure responsible for bone resorption in osteoclasts [31]. It also distinguishes mature osteoclasts during osteoclastogenesis [32]. Previous research has demonstrated that the inhibition of F-actin ring formation can impact the

bone-resorbing function of osteoclasts [33]. Consistently, results from this study also determined that RANKL-induced osteoclasts generated a greater number of F-actin ringsand had a greater potential for bone resorption, and these features are suppressed by cilengitide. Thus, the findings support a view the regulation of cilengitide is crucial in the late stage of osteoclastogenesis.

M-CSF and c-fms receptor binding promotes osteoclast survival, differentiation, and proliferation [34–36]; RANKL combines with RANK, a new element of the TNF receptor family. Their interaction triggers the recruitment of the TRAF family of proteins which is vital for osteoclast differentiation [37]. NFATc1 is identified as a pivotal regulator of differentiation of osteoclast in previous studies [38]. CTSK is a momentous protein involved in osteoclastic resorption of bone matrix and its expression can be regulated by NFATc1 [39–41]. In agreement with previous reports, the present study found that both genes and proteins levels of the above factors were expressed during osteoclast formation. The tendency of this upregulation was reversed by cilengitide, which further confirmed our hypothesis that cilengitide inhibits bone resorption by osteoclasts.

Osteoclasts express a variety of integrins, the major integrin is $\alpha_v\beta_3$, which is required for osteoclast attachment to bone [42]. Osteopontin, an arginine-glycine-aspartic acid (RGD) ligand in the bone matrix, can be recognized by integrin $\alpha_v\beta_3$ through its extracellular domain, to polarize mature osteoclasts onto the bone surface [43,44]. Poly-D-lysine (PDL) and its polycationic properties enable integrin-independent cells to adhere to this artificial matrix molecule. Previous studies have shown that inhibiting RANKL-induced osteoclast differentiation can be achieved by blocking the interaction between the bone surface and integrin $\alpha_v\beta_3$ [45]. In this study, cell adhesion was regulated downward in a dose-dependent manner when cilengitide was present. Besides, the experimental results also confirmed the ability of cilengitide to inhibit the expression of integrin $\alpha_v\beta_3$ at the gene and protein levels.

The cytoskeleton of osteoclasts, which is made up of an actin core and an actin cloud, plays a crucial role in their proper functioning [46]. Adhesion-related proteins including integrins, FAK family kinases, and others surround the actin core. Among these, integrin $\alpha v\beta3$ in osteoclasts has been shown to activate FAK and c-Src kinases. Phosphorylated c-Src further triggers downstream signaling events, leading to cytoskeletal reorganization and bone resorption [47,48]. Consistent with previous studies [24], the results indicated that treatment with cilengitide significantly reduces the levels of phosphorylated FAK and c-Src proteins compared to the RANKL-induced group. Notably, the expression of total FAK and Src proteins remains unaffected by cilengitide, indicating that its inhibitory effect is specific to phosphorylation events. Therefore, this study has revealed that cilengitide inhibited the resorption function of osteoclasts by inhibiting the integrin signaling pathway.

In summary, this study clearly demonstrated the potent inhibition of RANKL-induced osteoclast adhesion by cilengitide and showed that cilengitide suppressed FAK/Src signaling. In conclusion, this research offers new insights into the possibility of cilengitide as a therapy for osteolytic bone disorders.

Author contribution statement

Danyang Guo: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Zhonghua Chen, Yifei Fu, Junjie Wu, Zhengdong Yuan: Contributed reagents, materials, analysis tools or data.

Yueyue Li, Mengnan Chen: Performed the experiments.

Fenglai yuan: Conceived and designed the experiments.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17841.

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