INTRODUCTION

In the adults, bone undergoes constant remodelling through the balance of osteoblast-mediated bone formation and osteoclast-induced bone resorption.1,2 Disruption of the balance may result in various bone-related diseases, including osteoporosis, which is characterized by reduction of bone mass or bone mineral density (BMD), as well as deterioration of the bone structure, leading to fragility fracture.3,4 Osteoporosis has become a major social health problem as ageing population increased rapidly all over the world despite currently available treatments.5

Asperpyrone A attenuates RANKL-induced osteoclast formation through inhibiting NFATc1, Ca\(^{2+}\) signalling and oxidative stress

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Abstract

Imbalance of osteoblast and osteoclast in adult leads to a variety of bone-related diseases, including osteoporosis. Thus, suppressing the activity of osteoclastic bone resorption becomes the main therapeutic strategy for osteoporosis. Asperpyrone A is a natural compound isolated from Aspergillus niger with various biological activities of antitumour, antimicrobial and antioxidant. The present study was designed to investigate the effects of Asperpyrone A on osteoclastogenesis and to explore its underlining mechanism. We found that Asperpyrone A inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner when the concentration reached 1 µm, and with no cytotoxicity until the concentration reached to 10 µm. In addition, Asperpyrone A down-regulated the mRNA and protein expression of NFATc1, c-fos and V-ATPase-d2, as well as the mRNA expression of TRAcP and Ctsk. Furthermore, Asperpyrone A strongly attenuated the RANKL-induced intracellular Ca\(^{2+}\) oscillations and ROS (reactive oxygen species) production in the process of osteoclastogenesis and suppressed the activation of MAPK and NF-κB signalling pathways. Collectively, Asperpyrone A attenuates RANKL-induced osteoclast formation via suppressing NFATc1, Ca\(^{2+}\) signalling and oxidative stress, as well as MAPK and NF-κB signalling pathways, indicating that this compound may become a potential candidate drug for the prevention or treatment of osteoporosis.

KEYWORDS
Asperpyrone A, Ca\(^{2+}\) signalling, osteoclast, osteoporosis, reactive oxygen species

1 | INTRODUCTION

In the adults, bone undergoes constant remodelling through the balance of osteoblast-mediated bone formation and osteoclast-induced bone resorption.1,2 Disruption of the balance may result in various bone-related diseases, in particular osteoporosis, which is characterized by reduction of bone mass or bone mineral density (BMD), as well as deterioration of the bone structure, leading to fragility fracture.3,4 Osteoporosis has become a major social health problem as ageing population increased rapidly all over the world despite currently available treatments.5 As derived from...
the monocyte/macrophage haematopoietic lineage, osteoclast plays an essential role in bone resorption. Thus, suppressing or inhibiting the osteoclast formation or activity was concerned to be the therapeutic potential for preventing the development of osteoporosis. Although bisphosphonates (eg alendronate) are widely prescribed for senile osteoporosis through inhibiting osteoclast formation and activities, the adverse effects limited the use of this drug. Therefore, screening and finding new natural compounds which had the effects on inhibiting osteoclastogenesis may serve as potential therapies to treat senile osteoporosis and have become an attractive research topics.

Two key cytokines involved in the process of osteoclastogenesis: (a) RANKL (receptor activator of NF-κB ligand) is an essential cytokine in the process of the osteoclast differentiation, which interacts with RANK to regulate signalling pathways such as NF-κB, MAPKs and calcium signalling in osteoclasts and then consequently stimulates the downstream factor-related osteoclastogenesis such as NFATc1 (nuclear factor of activated T cells, cytoplasmic 1) and c-Fos (a protein of Aspergillus niger. As one of BNPs (Bis-naphtho-γ-pyrones), it has biological activities including antitumour, antimicrobial and antioxidant. Based on our initial compound screening, we found that Asperpyrone A was a candidate drug for attenuating osteoclast formation. Therefore, we investigated whether Asperpyrone A could inhibit osteoclast formation or activation and explored its effects on RANKL-induced signalling pathways such as MAPK, NF-κB, Ca2+ signalling and ROS products in the present study. This study also provides evidence that Asperpyrone A may become a potential candidate drug for the prevention or treatment of osteoporosis.

2 | MATERIALS AND METHODS

2.1 | Materials

Asperpyrone A, with a purity >99%, was obtained from Professor RenXiang Tan in Nanjing University’s (China). DMSO (dimethylsulphoxide), Trizol reagent, FBS (foetal bovine serum), α-MEM (alpha-modified minimal essential medium) and Ca2+ oscillations assay were purchased from Thermo Fisher Scientific. Recombinant M-CSF was produced from R&D Systems, and RANKL protein (GST-rRANKL) was expressed and purified as previously described. The MTS cytotoxicity assay and luciferase report assay were purchased from Promega. The antibodies of phosphorylated ERK (p-ERK), NFATc1, V-ATPase-d2, c-fos, IκB-α and JNK were obtained from Santa Cruz and the antibodies of P38, phosphorylated P-38 (P-P38), phosphorylated JNK(p-JNK), c-fos and ERK from Cell Signaling Technology.

2.2 | Cell culture

Six-week-old C57BL/6J mice were used for bone marrow macrophage cells (BMMs) isolation. After flushing the femur and tibia by α-MEM medium, the cells were cultured in the complete culture medium (10% FBS and 100 U/mL penicillin, 100 g/mL streptomycin and 2 mmol/L L-glutamine) and supplied with M-CSF (50 ng/mL). The BMMs were passed when they reached 70%-80% confluence, and the 3rd passage was used for further research. RAW264.7 cells were purchased from the American Type Culture Collection and cultured in complete culture medium without M-CSF. Then, the RAW264.7 cells were prepared for the luciferase reporter assay by stably constructed of the NF-κB- or the NFATc1-responsive luciferase reporter.

2.3 | Osteoclastogenesis assay and compounds screening

Bone marrow macrophage cells were seeded into 96-well plates at a density of 6000 cells/well and cultured with complete medium supplemented with M-CSF (50 ng/mL) and RANKL (50 ng/mL) for 5 days. TRAcP (tartrate-resistant acid phosphatase) staining was used for osteoclastogenesis assay. Natural compound screening was performed to detect whether they can inhibit RANKL-induced osteoclastogenesis using BMM culture (the compounds varying concentrations from 0.5 μmol/L to 10 μmol/L).

2.4 | Cytotoxicity assays

MTS was used to detect the cell cytotoxicity of candidate compound Asperpyrone A. BMMs were seeded into a 96-well plate at

| Genes       | Forward (5'-3') | Reverse (3'-5') |
|-------------|----------------|----------------|
| NFATc1      | CAACGCCCTGACCACCGATAG | GCCGTGCCTTCGCTCATAGT |
| c-Fos       | GCGAGCAACTGAGAAGAC   | GCGAGCAACTGAGAAGAC   |
| V-ATPase-d2 | GTGAGACCTTGGAGACCTGAA | GAGAATGTGCTCAGGGGCTT |
| TRAcP       | TGGGCCATCTTTAGCT     | GTCTTCTTCTTGGGCCTT   |
| Ctsk        | GGGAGAAAACCTGGAAGC   | ATTCTGGAGACTCAGAGC   |
| β-actin     | CACTGTGCCCACTTACGGA  | TGATGCACGCCCAGATT    |
| B2m         | TTCTTGCTCTGCTCTCAG   | CAGTATGTTCCGCTTCCCCATT |

TABLE 1 The primer sequences for real-time PCR
a density of 6000 cells/well. After overnight, the cells were treated with Asperpyrone A varying concentrations from 0.5 to 10 μmol/L and cultured for 48 hours. Then, MTS solution (20 mL/well) was added and incubated for 2 hours. The 490 nm absorbance was read for every well (Thermo Labsystem Multiscan Spectrum, Thermo Lab System).
2.5 | Bone resorption assay

Bone resorption assay was used to evaluate the effect of Asperpyrone A on inhibiting osteoclast activity. BMMs were seeded into a 6-well plate with collagen-coated at a density of $1 \times 10^5$ cells/well and induced osteoclastogenesis by M-CSF and RANKL. When the mature osteoclasts were generated, cells were detached and seeded into 96-well plates with hydroxypatite-coated (Corning, Inc) with the same number of cell per well; then, cells were cultured in complete medium with M-CSF and RANKL for 48 hours. Half of the wells were used to assess the resorbed areas by bleaching the cells; the remaining half of the wells were used to perform TRAcP staining to assess the number of mature osteoclasts. ImageJ software for Windows (version 1.49) was used to evaluate the resorbed area of hydroxypatite surface according to the photographs captured by microscopy.

2.6 | Osteoblast cell culture and differentiation assays

The osteoblast cells were obtained from the calvaria of neonatal mice as previously described. In briefly, collagenase (0.1%) and dispase (0.2%) were used to digest the calvaria tissue, and then, the isolated cells were cultured in DMEM with 10% FBS, 100 U/mL penicillin and 100 g/mL streptomycin. The 3rd passage was used for osteoblast differentiation assay. Osteoblast cells were seeded in 48-well plate and cultured with 50 µg/mL ascorbic acid, 10 mmol/L ß-glycerophosphate and 10−7 mmol/L dexamethasone (DXM). The cells were treated with BMP, 2.5 and 5 µmol/L Asperpyrone A, respectively. ALP staining was performed for bone differentiation assay on day 7.

2.7 | Luciferase reporter assays

The RAW264.7 cells were used for luciferase reporter assays as the previous study. In brief, the RAW264.7 cells with either the NF-kB- or the NFATc1-responsive luciferase reporter were prepared and seeded into 48-well plates, respectively. RANKL stimulation (50 ng/ml) followed 1 hour after pre-treated with various concentrations of Asperpyrone A. Luciferase activity was detected according to the protocol of luciferase reporter assays (Promega).

2.8 | Reverse transcription (RT)-PCR analysis

The BMMs were stimulated with M-CSF (50 ng/mL) and RANKL (50 ng/mL) in the presence or not of Asperpyrone A for 1, 3 and 5 days, respectively; then, Trizol was used to isolate the total RNA of cells. The cDNA was synthesized from the RNA template, and the quantitative real-time PCR reactions were performed. The Ct value (cycle threshold) was used to assess the gene expression according to the $2^{-\Delta\Delta C_T}$ method. The expression of ß-actin and B2M mRNA was used for normalization. The detailed information of the primers used in RT-PCR analysis was shown in Table 1.

2.9 | Western blotting

The BMMs were cultured in 6-well plates with M-CSF (50 ng/mL) until the cells reached confluence. The cells were pre-treated with Asperpyrone A or not and then stimulated with RANKL (50 ng/mL) for 10, 20, 30 and 60 minutes, respectively. The protein was harvested by RIPA lysis buffer, and the concentration of each sample was equalled (BCA Protein Assay Kit). Each sample’s protein was separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred to PVDF membranes. The membranes were blocked with 5% fat-free milk for 1 hour and incubated with primary antibodies at 4°C overnight. After removal of the primary antibodies, the membranes were subsequently incubated with the secondary antibody with HRP (horseradish peroxidase)-conjugated for 2 hours at room temperature. The immunoreactive signals were detected by ECL reagent kit on an Image-quant LAS 4000 (GE Healthcare). The grey value was calculated by the software of Image J for Windows (version 1.49).

2.10 | Intracellular Ca$^{2+}$ oscillation assay

Ca$^{2+}$ oscillations were investigated by Fluo4-AM Kit (Thermo Fisher Scientific) as previously reported. In brief, the BMMs were seeded into a 48-well plate, and 2.5 or 5 µmol/L of Asperpyrone A was added when the cells were stimulated with M-CSF and RANKL for 24 hours. After washed by an assay buffer, the cells were incubated in Fluo4 staining solution at 37°C for 45 minutes. Then, changed assay buffer incubated for 20 minutes at room temperature. Visualization of fluorescence was detected by the fluorescent microscope (Nikon) for 3 minutes (every 2 seconds captured one image) at an excitation wavelength of 488 nm. Ca$^{2+}$ oscillation was calculated by the difference between the peak and baseline fluorescence intensity in the cells which had more than two peaks of fluorescence intensity.

2.11 | Intracellular ROS production detection

As described previously, the BMMs were pre-treated with Asperpyrone A (2.5 or 5 µmol/L) or not, followed by M-CSF and RANKL (50 ng/mL), and then incubated in H2DCFDA for 1 hour. As DCF (2',7'-dichlorofluorescein) converted from H2DCFDA when oxidation showed highly fluorescent, confocal microscope (NIKON A1Si) was used to detect the fluorescence of DCF in the cells and the mean fluorescence intensity (ROI) of ROS-positive cells was analysed using Image J software.

2.12 | Statistical analysis

All the results are presented as the mean ± standard deviation (SD). One-way ANOVA test was used for the comparing the means of all the groups, followed by Bonferroni’s post hoc test for every two groups analysis. Statistical software IBM SPSS for Windows (version 21) was used to perform all the statistical analyses, and GraphPad
Prism 5 (version 5.01) was used to prepare the bar figures. \( P < .05 \) was considered as significant differences.

3 | RESULTS

3.1 | Asperpyrone A inhibited RANKL-induced osteoclast formation

The results of compounds screening showed that Asperpyrone A (Figure 1A) had a potent inhibitory effect on osteoclast formation. At concentration of 1 \( \mu \text{mol/L} \), Asperpyrone A could suppress RANKL-induced osteoclast formation, and increasing concentration of Asperpyrone A had more efficacy (1 to 10 \( \mu \text{mol/L} \)) in suppressing osteoclastogenesis (Figure 1B,C). Then, the toxicity assay was performed, and Asperpyrone A had shown no cytotoxicity on BMM cells until the concentration reached 10 \( \mu \text{mol/L} \), only with slight cytotoxicity when the concentration reached 10 \( \mu \text{mol/L} \) (Figure 1D).

Interestingly, the Asperpyrone A exhibited no significant inhibitory effect on osteoclast activity as the bone resorption assay showed that no more resorption area was found when the concentration of Asperpyrone A reached 5 \( \mu \text{mol/L} \) (Figure 1E). Thus, Asperpyrone A mainly affects osteoclast formation.

3.2 | Asperpyrone A attenuated the expression of the osteoclast-related genes

We then explored the expression of osteoclast-specific marker genes in BMMs during osteoclast formation with or without the presence of Asperpyrone A. We found that treatment with 5 \( \mu \text{mol/L} \) of Asperpyrone A down-regulated the mRNAs expression of NFATc1, c-fos, V-ATPase-d2 (Vacuolar-type H+ATPase d2), TRAcP and Ctsk in BMMs during osteoclast formation induced by RANKL, and even 2.5 \( \mu \text{mol/L} \) of Asperpyrone A inhibited c-fos, V-ATPase-d2 and TRAcP mRNA expression (Figure 2).

3.3 | Asperpyrone A down-regulated osteoclast-related proteins and NFATc1 activity

The osteoclast-related proteins including NFATc1, c-fos and V-ATPase-d2 were investigated in BMMs when induced by RANKL for 5 days. The findings demonstrated that the protein level of NFATc1, c-fos and V-ATPase-d2 significantly increased during osteoclastogenesis, whereas Asperpyrone A down-regulated the protein level of NFATc1, c-fos and V-ATPase-d2 either with the concentration of 2.5 or 5 \( \mu \text{mol/L} \), and 5 \( \mu \text{mol/L} \) exhibited more efficacy (Figure 3A).

**FIGURE 2** Asperpyrone A inhibits the mRNA expression of osteoclast marker genes. Gene expression was normalized to \( \beta \)-actin and B2m. A, The relative mRNA expression of NFATc1. B, The relative mRNA expression of c-fos. C, The relative mRNA expression of V-ATPase-d2. D, The relative mRNA expression of TRAcP. E, The relative mRNA expression of Ctsk. All the data are presented as mean ± SD. (n = 3) *\( P < .05 \), **\( P < .01 \)
NFATc1 activity also was evaluated in RAW264.7 cells by luciferase reporter assay. Consistent with the mRNA and protein level in BMMs, the results showed that NFATc1 activity in RAW264.7 cells was also suppressed by Asperpyrone A either with 2.5 or 5 μmol/L concentration (Figure 3B).

3.4 | Asperpyrone A down-regulated RANKL-induced MAPK and NF-κB signalling pathways

To further understand the mechanism of Asperpyrone A on suppressing RANKL-induced osteoclast differentiation in BMMs, MAPK...
and NF-κB signalling pathways were explored by Western blot and Luciferase reporter assay. As shown in Figure 4, the ratio of p-ERK to ERK (p-ERK/ERK) and p-JNK to JNK (p-JNK/JNK) BMMs decreased when treated with 5 μmol/L of Asperpyrone A during RANKL-induced osteoclast differentiation, which indicated MAPK signalling pathways was suppressed by Asperpyrone A. Moreover, Asperpyrone A also had an inhibitory effect on NF-κB pathway during osteoclastogenesis as IκBα degradation was attenuated when treated with Asperpyrone A in BMMs (Figure 5A). Consistent with the results of Western blot, luciferase reporter assay also exhibited the suppressing effect of Asperpyrone A on NF-κB activity in RAW264.7 cells.

3.5 | Asperpyrone A inhibited RANKL-induced Ca^{2+} oscillations

RANKL stimulates Ca^{2+} oscillations in BMM cells through activation of the Ca^{2+} signal pathways. In the present study, we discovered that the intensity of Ca^{2+} oscillations in BMMs increased and Asperpyrone A had the inhibitory effect during RANKL-induced osteoclast formation (Figure 6). It appears that both 2.5 and 5 μmol/L concentration of Asperpyrone A exhibited a similar efficacy.

3.6 | Asperpyrone A suppressed RANKL-induced intracellular ROS products

As RANKL stimulation increases ROS production in BMMs during osteoclast differentiation, we then investigated whether Asperpyrone A could reduce ROS products in BMMs during RANKL-induced osteoclast formation. In this study, the results of fluorescent signal which reflects the ROS products showed increased during RANKL-induced osteoclast formation and reduced when treated with either 2.5 or 5 μmol/L concentration of Asperpyrone A (Figure 7), which indicated that Asperpyrone A increased the ability to clear intracellular ROS products during RANKL-induced osteoclastogenesis.

3.7 | Asperpyrone A had no effect on the osteoblast differentiation

The result of ALP staining showed that there was no significant difference between Asperpyrone A (2.5 and 5 μmol/L) and control group (Figure S1), indicating that Asperpyrone A did not affect the osteoblast differentiation.

4 | DISCUSSION

As RANKL has been identified as one of the critical cytokines that regulates osteoclast formation and activity, inhibiting RANKL-induced osteoclastogenesis was considered to be a potential therapeutic strategy for osteoporosis. For example, Zhou et al demonstrated that dihydroartemisinin suppressed both osteoclast formation and resorption in vitro, as well as reversed the bone loss in ovariectomized mice. Song et al showed that eriodictyol was potentially useful for the prevention of osteoporosis through inhibiting osteoclast formation and function. Achyranthes bidentata polysaccharide, berberine sulphate, nitidine chloride, artesunate and so on were also found to be potential therapeutic candidates for the prevention or treatment of osteoporosis. Other studies also investigated the osteoporotic effects of compounds such as magnolol and polysaccharides.

As one of BNPs, the compound of Asperpyrone A was isolated from Aspergillus niger with various biological activities including antitumour, antimicrobial and antioxidant. Its effects on attenuating RANKL-induced osteoclast formation were identified by our
compound screening assay using TRAcP staining. Therefore, we further investigated the effects of Asperpyrone A on suppressing RANKL-induced osteoclast formation and its cellular mechanisms in the present study. The findings demonstrated that Asperpyrone A significantly decreased both the number and the size of osteoclast, but had no inhibitory effect on the osteoclast function, which indicated Asperpyrone A mainly affects osteoclast formation but not osteoclast activity. In addition, the results of MTS assay exhibited no cytotoxicity on BMM cells until the concentration reached 10 μmol/L. Furthermore, the result of ALP staining showed that the Asperpyrone A had no effect on osteoblast differentiation. These findings suggest that Asperpyrone A has a major effect on osteoclasts and could be a potential candidate anti-resorptive drug for osteoporosis.

Then, we explored the mechanisms by which Asperpyrone A inhibited osteoclast formation. NFATc1 and c-fos were identified as the critical regulator in the process of osteoclast formation. In addition, NFATc1 can amplify its effectiveness via improving other osteoclastogenesis-related transcription factors in an auto-amplification loop, including c-fos, NF-κB and NFATc2. Thus, we then investigated the effects of Asperpyrone A on NFATc1 and c-fos, and found that Asperpyrone A attenuated the expression of the mRNA and protein of NFATc1 and c-fos in BMMs induced by RANKL. The analysis of luciferase report gene assay also indicates

**FIGURE 6** Asperpyrone A attenuates the intensity of RANKL-induced Ca^{2+} oscillation. (A-D) The representative image of the intensity of Ca^{2+} fluctuations within 3 minutes after 24 hours pre-treated with RANKL and Asperpyrone A. E, The quantification of the intensity of Ca^{2+} oscillation per cell (the data showed as mean ± SD, n = 3). **P < .01 compared with the positive group.

**FIGURE 7** Asperpyrone A suppresses RANKL-induced intracellular ROS production. A, The representative images of ROS production induced by RANKL in BMMs when pre-treated with or without Asperpyrone A. B, The quantification of ROS production was calculated. (n = 3). The data are presented as mean ± SD, (n = 3). *P < .05, **P < .01 compared with the positive group (with RANKL and M-CSF but without Asperpyrone A treated). Scale bar = 200 μm.
that Asperpyrone A inhibited NFAT activation induced by RANKL in RAW264.7 cells. Moreover, previous studies also showed that NFATc1 can stimulate osteoclasts to express specific proteins which play critical roles in bone matrix resorption, including V-ATPase-d2, Ctsk (Cathepsin K) and TRAcP. Therefore, we also investigated the mRNA expression of TRAcP, V-ATPase-d2 and Ctsk, and the results showed that the expression of the above genes was also suppressed when treated with Asperpyrone A during RANKL-induced osteoclast formation. These results exhibited that Asperpyrone A was a potential therapeutic candidate for the prevention of osteoporosis through inhibiting the expression of NFATc1 and other related transcription factors during osteoclastogenesis.

MAPK signalling pathways including ERK, JNK, P38 and NF-xB signalling pathway were known as the important signal transduction pathways in the process of osteoclast formation. It was also shown that the signalling pathways of ERK, JNK and P38 activated the expression of NFATc1 and c-fos in osteoclasts, and then triggered the downstream osteoclastogenesis-related gene such as Ctsk, V-ATPase-d2 and TRAcP. In this study, the results demonstrated that Asperpyrone A attenuated the activation of ERK and JNK signalling pathways during RANKL-induced osteoclast formation. In addition, the findings also exhibited that Asperpyrone A inhibited the NF-xB signalling cascade during the process of osteoclastogenesis as the level of ixBa protein degradation decreased and NF-xB activity measured by the luciferase report gene assay in RAW264.7 cells decreased when pre-treated with Asperpyrone A. Taken together, Asperpyrone A suppressed the expression of NFATc1, c-fos, Ctsk, V-ATPase-d2 and TRAcP in osteoclasts via the signalling pathways of MAPK/NF-xB/NFATc1/c-fos. Furthermore, Ca2+ signalling can induce the activation of NFATc1, and long-lasting Ca2+ oscillation was observed during the process of osteoclastogenesis. Therefore, intracellular Ca2+ oscillation is crucial for osteoclast formation. In this study, we observed that Asperpyrone A significantly reduced the intensity of Ca2+ oscillation both in the concentration of 2.5 and 5 μmol/L, suggesting that down-regulation of Ca2+ signalling by Asperpyrone might also contribute to the inhibitory effects of NFATc1 and subsequently to RANKL-induced osteoclast formation.

Recent studies have elucidated that intracellular ROS (reactive oxygen species) is involved in the processing of osteoclast formation, and high level of ROS was found in osteoclast precursors when stimulated by RANKL. Other studies demonstrated that osteoporotic postmenopausal women had high oxidative stress index when compared to the healthy group, suggesting a high level of oxidative stress might contribute to the development of osteoporosis and suppressing oxidative stress would be a potential treatment for osteoporosis. Therefore, we investigated the ROS level in the process of RANKL-induced osteoclast formation and discovered that Asperpyrone A suppressed the ROS production in a dose-dependent manner. Previous studies had shown that Asperpyrone A hugely inhibited COX-2 activities and exhibited an antioxidant effect, which might contribute to the inhibition of the ROS level. Moreover, ROS productions can also stimulate the MAPK and NF-xB activation, indicating Asperpyrone A suppressed MAPK and NF-xB activation in the present study might through attenuating the intracellular ROS production during RANKL-induced osteoclast formation.

Previous studies showed that natural compounds not only suppressed the osteoclast formation in vitro, but also reversed the bone loss in ovariectomized (OVX) model mice. One of the limitations in this study was that the animal experiments had not been performed. However, based on the evidence of Asperpyrone A inhibited osteoclast formation and did not affect osteoblast differentiation, it is highly possible that Asperpyrone A should be effective for the treatment of osteoclast -related conditions such as OVX-induced osteoporosis.

Taken together, our study had shown that natural compound Asperpyrone A inhibited RANKL-induced osteoclast formation via suppressing the intracellular Ca2+ signalling and ROS level, subsequently leading to attenuating MAPK and NF-xB signalling pathways as well as the expression of NFATc1 and other osteoclast-related factors. In addition, Asperpyrone A had little effect on osteoblast differentiation. These findings might provide evidence for the use of Asperpyrone A as a novel candidate anti-resorptive drug for the therapeutic treatment or prevention of osteoporosis.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Chen X and Wang C performed experiments and wrote the manuscript; Qiu H and Yuan Y performed experiments; Chen K and Cao Z analysed the data and prepared the Figures; Tickner J, Xu J and Zou J designed and guided all the experiments. All authors reviewed the manuscript. Tickner J, Xu J and Zou J revised the manuscript.

Data Availability Statement

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

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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