Thioacetamide promotes osteoclast transformation of bone marrow macrophages by influencing PI3K/AKT pathways

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Abstract

Background

Osteoclast cell increase is a major risk factor for osteoporosis and degenerative bone and joint diseases. Thioacetamide (TAA) can lead to many types of liver and kidney damage, but less attention has been paid to the association of TAA with bone damage. In this work, we investigated the effects of TAA on the osteoclastogenesis and differentiation of bone marrow macrophages (BMMs).

Methods

Bone marrow mononuclear macrophages of SD rat suckling mice were taken for primary culture. CCK-8 was used to detect the toxic effects of TAA on BMMs, and flow cytometry was used to detect the effects of TAA on the cell cycle, cell viability, apoptosis and intracytoplasmic Ca^{2+} concentration of BMMs. TRAP immunohistochemistry and fluorescence staining were used to detect the effect of TAA on osteoclast differentiation of BMMs. Western Blot was used to detect the expression level of PI3K/AKT pathway and osteoclast-specific proteins (TRAP and Cts-K).

Results

The results suggested that TAA inhibited the proliferation of BMMs, while enhancing osteoclastogenesis at 0.5 mg/mL and 1 mg/mL as assayed by TRAP staining, and BMMs could differentiate into osteoclast-like cells with overexpression of cathepsin K and TRAP proteins after TAA treatment. Western blot results showed that TAA can activate the expression levels of P-PI3K, P-AKT, P-P38, and P-JNK, accompanied by apoptosis of BMM cells and increase of intracellular Ca^{2+} .

Conclusion

TAA may induce osteoclast formation in BMMs by activating the expression of PI3K/AKT pathway proteins, which is comparable to the classic osteoclast differentiation inducer RANKL. This suggests that we may find a cheap osteoclast inducer.

Introduction

TAA is a widely used commercial chemical, and it is used to establish an animal model for liver cirrhosis due to its hepatotoxic effects ^[1]. TAA-induced cirrhosis has similar pathologic changes to human liver cirrhosis and can be used to reproduce the human disease ^[2,3]. Additionally, the effects of TAA are not limited to the liver, as profound structural and functional changes have been described in the kidney,

spleen, lung, intestine, stomach, brain and bone marrow upon TAA treatment ^[4–11]. However, less attention has been paid to the association of TAA with bone damage.

In a previous experiment, operation staff found an interesting phenomenon in the classic liver cirrhosis animal model administered with TAA. Approximately 40–50% of the animals, even animals that accidentally expired at night, presented open fractures, arthroncus and arthrorrhagia. No reports have expressly described bone damage, such as fractures, arthroncus and arthrorrhagia, to be caused by TAA, possibly because we used Zealand rabbits for our experiment animal, whereas others used rats or mice for liver cirrhosis animal models, and the weight of the model animal could possibly be related to the cause of the fracture. Lassila V ^[12,13] found that osteoblastic activity and osteoid were distinctly decreased, and found robust osteoclastic resorption, when the alveolar bone was under occlusal stress and TAA traumatization. Nakano A ^[14] also demonstrated that the bone volume in TAA-treated cirrhotic rats was significantly lower, with a combination of low bone formation rates and high resorption rates. TAA may induce osteoclast proliferation or increase the activity of these cells to enhance osteolysis in vivo, causing bone damage. Our experimental group wanted to know whether TAA could induce osteoclastic transformation of BMMs in vitro, so as to find evidence for the possible osteotoxicity of TAA.

Bone homeostasis in vivo depends on osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption ^[15]. These cell populations are tightly coordinated with each other under normal conditions. Osteoclasts are the only multinucleated giant cell with the ability to resorb bone, and they play a very important role in maintaining the steady state of bone mass in vivo ^[16]. Increased formation and abnormal activities of osteoclasts, with respect to bone resorption, can lead to osteoporosis ^[17–19]. Considering the important role of osteoclasts in bone homeostasis, it is pivotal to determine whether TAA can upregulate osteoclastogenesis and bone resorption to induce osteoporosis. Therefore, we sought to demonstrate that bone damage could be caused by TAA using rapid experiments to examine its effect on osteoclastogenesis in BMMs in vitro.

Bone marrow macrophages possess self-renewal capacity and play a crucial role in modulating normal bone homeostasis, with the potential to differentiate into osteoclasts in vitro ^[20]. It is widely believed that osteoclasts differentiate from the monocyte/macrophage lineage in response to two essential cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL). The classic method of inducing osteoclast differentiation from osteoclast precursors in vitro uses RANKL in combination with M-CSF ^[21–23]. However, RANKL is expensive and the preparation process is complicated, which complicates the study of osteoclasts in vitro. Therefore, if we successfully verify in vitro that TAA can induce BMMs to differentiate into osteoclasts, this suggests that TAA may cause bone damage and even osteoporosis. We hypothesized that TAA can enter the body and induce the transformation of BMMs into osteoclasts, thereby breaking the dynamic balance of osteoblasts and osteoclasts generated in the bone marrow, resulting in bone damage. To test this hypothesis, we investigated the effects of TAA on osteoclast differentiation of BMMs in vitro. We found that the osteoclastogenesis of BMMs were enhanced upon treatment by TAA. The number of osteoclast cells

were increased, the expression of osteoclast-specific genes was upregulated. Our study shows that TAA have the significant effect on the induction of osteoclast differentiation from BMMs, and TAA has a stronger bone-damaging effect on BMMs. We will discover a new method to obtain osteoclasts in vitro, which will provide experimental evidence for the bone damage caused by TAA. In addition, this can help us identify potential hazards and help prevent human health hazards.

Methods

Cells and cell culture

Sprague-Dawley rats were purchased from Shanghai SLAC Laboratory Animal Co, Ltd. The BMMs were isolated from 2-day-old SD rats. The cells were cultured in growth media containing Dulbecco's modified Eagle's medium/Nutrient Mixture F-12(Ham) (DMEM/F-12(1:1) basic(1X); Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma). The cultures were maintained at 37°C in a humidified 5% CO₂ incubator. When the cells reached 80–90% confluency, cultures were harvested with Trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA; Sigma). The media was changed every 2–3 days.

Preparation of TAA

Thioacetamide (TAA, CAS No.62-55-5, > 98.0% purity; Sang on Biotech, Shanghai, China) was prepared by dissolving 500 mg pure TAA, which is in crystal form, in 50 mL of culture media until all the crystals were dissolved. Filter with a 0.22µm microporous membrane, and then dilute with culture medium to the corresponding concentration.

Cell proliferation assay (CCK-8)

To assess the cytotoxic effect of TAA on BMMs, CCK-8 cell viability assay was performed. BMMs (2×10³ cells/well) were seeded into 96-well plates (Corning, USA) in a volume of 100 µl and cultured overnight. Then, the culture media was replaced by media containing different concentrations of TAA (0, 0.5, 1, 1.5, and 2mg/mL), and each concentration was used in 5 parallel wells after adherence. The media was replaced with 100 µL of fresh media and 10 µL of CCK-8 solution (CCK-8; Beyotime Institute of Biotechnology, Shanghai, China) after culture for 12, 24, 36, 48, 60, 72, 80 and 96 h, the cells were then incubated for an additional 2 h, and the absorbance was measured at 450 nm with a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA). The cell viability and IC₅₀ values were then calculated. The survival rate of BMMs (%) = experimental group A value/control group A value× 100%. At least 3 independent experiments were performed.

CFSE (5, 6 - carboxy fluorescein diacetate succinimidyl oxy ester) assay

BMMs were labeled for 15–20 min at 37°C with 3µM carboxy fluorescein diacetate succinimidyl ester (CFSE; Cell Trace CFSE Cell Proliferation Kit, Invitrogen) in PBS supplemented with 0.1% BSA. CFSE-labeled cells were washed three times with PBS for 5 min at 400g at room temperature and then cultured

overnight. Then, the culture media was replaced by media containing different concentrations of TAA (0, 0.5 and 1mg/mL). The cells were then incubated for 24 h. Cell fluorescence was evaluated by flow cytometry (Novo Cytex, ACEA, California, USA), and the data were analyzed using Flow Jo 7.6 software. At least 3 independent experiments were performed.

Cell cycle analyses

BMMs were treated with TAA (0, 0.5 and 1mg/mL) for 24 h. The cells were subsequently collected, washed with PBS and fixed with 75% ethanol overnight. The cells were then centrifuged for 5min at 400g, incubated with 10 mg/mL RNase and 1 mg/mL PI (Multi Sciences, CCS012) at 37°C for 30 min away from light. Finally, the cell cycle distribution was analyzed by flow cytometry (Novo Cytex, ACEA, California, USA). At least 3 independent experiments were performed.

Wright-Giemsa stain and tartrate-resistant acid phosphatase (TRAP) stain

BMMs (1×10^6 cells/well) were cultured with RANKL (50ng/mL) or varying concentrations of TAA (0, 0.5, and 1 mg/mL) in a 6-well plate for 7days. Cells were fixed and stained with the wright-giemsa stain (C190805) and TRAP/ALP stain kit (code No.294-67001), according to the instructions of the manufacturer. Osteoclasts were defined as TRAP-positive cells being stained purple under a light microscope.

Immunofluorescence

BMMs were plated onto 35mm glass-based dishes (801002; NEST Biotechnology, New Orleans, LA, USA) one day before treatment with RANKL or TAA. Cells were then fixed in 4% polyformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 15 min. They were then blocked with 5% BSA for 1 h. Primary antibody incubations were with the following antibodies: anti-tartrate resistant acid phosphatase (TRAP) (Abcam, ab191406, 1:1,000) in PBS containing 2% FBS overnight at 4°C, followed by incubation with goat anti-mouse IgG H&L (DyLight488) (Abcam, ab96871, 1:400) in PBS containing 2% FBS as a secondary antibody for 2 h at room temperature. Finally, the cell nuclei were stained with DAPI (0.5 µg/ml) for 15 min, and images were obtained using a confocal fluorescence microscope. PBS was used for all washing steps.

Western blot

Cells were rinsed twice with PBS and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology), along with PMSF and Phosphatase inhibitor. The lysates were centrifuged, and the supernatants were collected. The protein was separated by 10% SDS PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with skim milk (5%) in Tris-buffered saline and Tween-20 for 2 h at room temperature, the membrane was incubated overnight at 4°C with the following primary antibodies: anti-PI3K (Abcam, ab151549, 1:1,000), anti-P-PI3K (Abcam, ab182651, 1:1,000), Anti-AKT1 (Abcam, ab81283, 1:10,000), Anti-P-AKT1 (Abcam, ab179463, 1:10,000), Anti-JNK1 + JNK2 + JNK3 (Abcam, ab18841-95, 1:1,000), Anti-P-JNK1 + JNK2 + JNK3 (Abcam, ab124956, 1:10,000), anti-P38 (Abcam, ab170099, 1:5,000),

anti-P-P38 (Abcam, ab4822, 1:1,000), anti-Tartrate Resistant Acid Phosphatase (Abcam, Ab191406, 1:1,000), anti-cathepsin K (Abcam, ab190277 1:1000), or anti- β -actin monoclonal antibody (Multi Sciences Biotech, Mab1445, 1:1,000). The membranes were then incubated with secondary antibodies conjugated to horseradish peroxidase (HRP), goat anti-mouse IgG, (GAR0072, Multi Sciences Biotech) or goat anti-rabbit IgG (GAR0072, Multi Sciences Biotech) for 2h at room temperature. The membranes were then visualized using an ECL substrate kit (P1425; Multi Sciences Biotech) on the Omega Lum G Imaging System. β -actin levels were used to standardize protein loading. ImageJ software was used to quantify band intensities. At least 3 independent experiments were performed.

Flow cytometric analysis of apoptosis using Annexin V

The apoptosis of BMMs was analyzed by flow cytometry (Novo Cytex, ACEA, California, USA) using an Annexin V-FITC/PI apoptosis kit (Multi Sciences Biotech, AP101-100), according to the manufacturer's protocol. Briefly, cells were washed twice with cold PBS and resuspended in 1 $\times$ binding buffer at a density of 1 $\times$ 10⁶ cells/mL; 100 μ L of the cell suspension was mixed with 5 μ L FITC Annexin V and 10 μ L propidium iodide (PI) and, then gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Then, 400 μ L of 1 $\times$ binding buffer was added to each tube, and apoptosis was analyzed within 1h. At least 3 independent experiments were performed.

Detection of intracellular Ca²⁺

The cells were treated with different concentrations of TAA for 24h. Then, the cells were incubated with 400 μ L 3 μ M Fluo-3 AM at 37°C for 30 min, with vertical shaking every 5 min. The fluorescent intensity of Fluo-3 AM was measured using flow cytometry (Novo Cytex, ACEA, California, USA) with excitation and emission wavelengths at 488 and 540–570 nm, respectively. The fluorescence intensity was proportional to the concentration of Ca²⁺. At least 3 independent experiments were performed.

Statistical analysis

Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) software 19.0. Statistical analysis of all data was performed using GraphPad Prism 7(GraphPad Software). $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered statistically significant. Each experiment was repeated at least three times in vitro. The results from at least three independent experiments were presented as the mean $\pm$ SD.

Results

Morphology and Characterization of BMMs Culture in Vitro

We observed that freshly harvested BMMs were a uniform population; as cells continued to proliferate, the majority of cells became larger, and binuclear or even trinuclear can be seen. The cell morphology changed gradually, the cell volume increased, gradually became irregular, radially adherent disk growth (Fig. 1A).

BMMs proliferation and viability were inhibited by TAA

We then determined the inhibitory effect of TAA on BMMs using a CCK-8 assay upon treatment of BMMs with TAA at concentrations of 0, 0.5, 1, 1.5 and 2 mg/mL. Cell survival was found to be decreased in a dose and time-dependent manner, and cell viability reached a stable period after 72 h (Fig. 2B). The results showed that TAA reduced cell viability. We observed that cell viability was significantly decreased (to approximately 50%) after 24 h treatment with TAA at a dose of 1.5 and 2 mg/mL. In all the following experiments, 0.5 mg/mL and 1 mg/mL of TAA were selected for further investigation of the effects of TAA on BMMs and exploration of the related mechanisms.

The CFSE distribution assay is an effective method for detecting cell division and proliferation. CFSE can pass through the cell membrane and is irreversibly coupled to intracellular proteins by covalently binding to intracellular amino groups. Fluorescence-labeled CFSE can be evenly distributed to two progeny cells, resulting in a continuous fluorescence intensity reduction that can be detected by flow cytometry. We observed that the division index of 0.5 mg/mL and 1 mg/mL TAA-treated BMMs decreased to $54.1\% \pm 1.5\%$ and $51.8\% \pm 1.9\%$, respectively, compared to the control ($63.2\% \pm 3.3\%$) after 24 h in TAA conditions (Fig. 2C, 2D, $*P < 0.05$). The results indicated that TAA could inhibit cell division.

To gain insight into the mechanism of TAA-induced growth inhibition in cells, we analyzed the TAA-dependent changes in the cell cycle distribution (Fig. 2E). Flow cytometric analysis showed that stimulation with TAA for 24 h caused a substantial increase in the ratio of S-phase cells relative to the whole cell population. The percentage of cells in S-phase was significantly increased to $19.4\% \pm 1.04\%$ and $25.74\% \pm 1.77\%$ in cells treated with TAA at 0.5 mg/mL and 1 mg/mL, respectively, compared to $15.98\% \pm 1.16\%$ in control cells ($*P < 0.05$). The percentage of cells in G0/G1-phase decreased compared to the control ($67.6\% \pm 2.54\%$, $64.04\% \pm 3.05\%$ vs. $72.78\% \pm 1.88\%$, $*P < 0.05$, $**P < 0.01$, shown in Fig. 2F).

BMMs Differentiated by Osteoclastogenesis upon TAA Treatment

To investigate the effects of TAA on the osteoclastogenesis differentiation process of BMMs, Immunofluorescence and TRAP staining were used to examine the osteoclast differentiation status of BMMs.

We observed cell fusion leading to larger cells, the TRAP staining results of cells treated with TAA for 7 days showed that the cells were irregular, with two or more nuclei (Fig. 3C). Also, BMMs induced with TAA began to show osteoclast-like morphology, with 1mg/mL TAA TRAP staining was deeper. After induced differentiation, TRAP staining results showed that there were few positive purple cells in the control group, while there were a large number of positive purple particles in the cytoplasm of the experimental group. The immunofluorescence results also confirmed this point (Fig. 3A). There was almost no TRAP green fluorescence in the control group, while the TRAP fluorescence intensity in RANKL and TAA treatment groups was significantly higher than that in the control group, and the higher the TAA concentration, the stronger the fluorescence intensity. Quantitative analysis showed that the numbers of

TRAP positive cells in the 0.5 mg/mL, and 1 mg/mL TAA groups were significantly higher than that in the control group ($***P < 0.001$ vs control Fig. 3B for immunofluorescence; Fig. 3D for immunohistochemistry).

Effects of TAA on PI3K/AKT pathway and osteoclast-specific protein expression

To research the effect of TAA on PI3K/AKT pathway and osteoclast-specific protein expression in BMMs, we used western blot analysis (Fig. 4). Western blotting showed that TAA exposure activated the protein expression of P-PI3K, P-AKT, P-P38, P-JNK, and promoted the expression of osteoclast specific protein TRAP and cathepsin K. These results demonstrate that TAA exposure may promote osteoclast transformation of bone marrow cells by activating PI3K/AKT pathway related proteins.

TAA exposure increases intracellular Ca^{2+} and promotes apoptosis

Next, we explored whether TAA induced BMMs apoptosis. Figure 5A and 5B showed that, as the concentration of TAA increased, the percentage of apoptotic BMMs increased. To further explore whether TAA was a calcium channel blocker, intracellular Ca^{2+} was detected by evaluating the fluorescent intensity of Fluo-3 AM. As shown in Fig. 5C and 5D, as the TAA concentration increased from 0 mg/mL to 0.5 mg/mL and 1 mg/mL, the fluorescent intensity of Fluo-3 AM increased from 4.16–12.2% and 27.81%, respectively. The results indicated that BMMs intracellular Ca^{2+} were increased upon treatment with TAA.

Discussion

Osteoporosis has become a major public problem with a significant population burden, especially in the aging population [24,25]. Overactivation of bone resorption plays a critical role in the pathological mechanisms of osteoporosis [26]. Until now, osteoclasts were known large multinucleated cells with exclusive bone-resorbing ability [27]. TAA is a potent experimental hepatotoxin and hepato-carcinogenic compound that is often used to induce fulminant hepatic failure in experimental animal models [28]. In this study, we show for the first time that TAA is directly associated with bone damage and can induce BMMs to differentiate into osteoclast-like cells. As we hypothesized, our results show that osteoclastogenesis differentiation from BMMs into osteoclast-like cells was enhanced by TAA treatment in vitro.

First, we investigated the cytotoxic effects of TAA on BMMs. We found that TAA inhibited the proliferation of BMMs in vitro in a dose- and duration-dependent manner (Fig. 2B) [29]. We found that cell viability reached a stable phase by 72 h when BMMs were treated with TAA. The CFSE results showed that the cell division index was significantly reduced when cells were treated with TAA at concentrations of 0.5 mg/mL or 1 mg/mL, indicating that the proportion of cells in the 0 and 1 generation was increased after

TAA stimulation and that TAA could inhibit cell proliferation and division (Fig. 2C, 2D). A substantial increase in the ratio of S-phase cells relative to the whole BMMs cell population was seen with TAA stimulation, which indicated that the number of cell nuclei was increasing during the cell cycle (Fig. 2E, 2F). Osteoclasts are terminally differentiated cells responsible for bone resorption under physiological and pathological conditions^[30]. TRAP staining was performed to confirm the formation of mature osteoclasts^[31]. High expression of TRAP has been used as a cytochemical marker for osteoclast detection^[32]. Our results showed that the number of TRAP-positive cells increased after TAA treatment (Fig. 3). Osteoclasts, derived from macrophages, are characterized by their unique, multinucleated morphology, as well as the expression of TRAP and cathepsin K. Western blot results showed that TAA upregulated the expression of osteoclastogenesis-related marker genes, such as TRAP and cathepsin K, by 7 days of TAA treatment (Fig. 4A). Taken together, these experiments allowed us to conclude that TAA could enhance osteoclast-like cell formation. Although TAA significantly increased the level of osteoclastogenesis in our study, the functions of these osteoclast-like cells, such as bone resorption, remain to be investigated in our future studies.

P2X7/PI3K/AKT pathway has been shown to regulate osteoclast survival and differentiation. Yonggang Ma^[33] found that the suppression of PI3K/AKT signaling was further inhibited osteoclast and osteoblast differentiation after long-term Cd exposure. PI3K, AKT, P38 and JNK are activated in osteoclast precursor cells and modulate osteoclastogenesis and osteoclast activity^[34–37]. Similar to previous reports, our results showed that TAA promoted the expression of these genes, indicating that the mechanism of osteoclast like cell formation induced by TAA may be the same as that induced by RANKL (Fig. 4). In addition, flow cytometry assays showed that TAA can induce BMMs apoptosis (Fig. 5A). Additionally, we have shown that TAA significantly increased the intracellular Ca^{2+} levels in these cells after 2 h of TAA treatment. Intracellular Ca^{2+} rose as the concentration of TAA increased, indicating that TAA induced apoptosis may because of the overloading of cellular Ca^{2+} ^[38] (Fig. 5B).

TAA is widely used in industrial applications and has become a commercial chemical^[39–42]. As a chemical, the preparation methods and distribution channels of TAA are well established, allowing mass-production in a relatively inexpensive manner. Humans are exposed intentionally and unintentionally to a variety of diverse chemicals that harm the bone. Drugs, natural products, industrial chemicals, and environmental pollutants that can cause bone damage have increased. Toxic, chemical-induced bone damage tends to be more common among certain patients and in specific clinical situations. In daily life, people can come into contact with electroplating additives, photographic drugs, pesticides and dyeing aids, all of which contain TAA. The elderly is the main group of people who dye their hair and also is the primary population who are overweight. Hair dye could be a new cause of osteoporosis, and, as a constituent, TAA maybe the main cause. It will be of great social significance in the future to study the existence and transformation of TAA in nature and the ways that it enters the human body. It is also crucial to begin a discussion of the major social and environmental problems of TAA, such as its specific accumulation in the human body and subsequent harm to human organs.

Our findings strongly indicate that TAA is worthy of new evaluation as the cause of various bone diseases associated with osteoclast overdifferentiation and bone destruction. TAA may promote the osteoclast transformation of BMMs by activating the PI3K/AKT pathway, which is consistent with the effect of the osteoclast inducer RANKL, so we boldly guess that TAA may have a similar effect to RANKL, which can induce the formation of osteoclasts. RANKL is expensive and not easy to obtain, while TAA is cheap as a chemical. The potential of TAA to induce osteoclasts suggests that we may have found a cheap osteoclast inducer. At the same time, in order to prove the bone injury toxicity of TAA, our laboratory plans to continue research in animals.

Abbreviations

TAA: Thioacetamide; BMMs: Bone marrow macrophages; M-CSF: macrophage colony-stimulating factor; RANKL: receptor activator of nuclear factor kappa B ligand

Declarations

Data availability

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

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The contributions of each author

XL.J, Y.L. and J.C and Y.Y.Y participated in the planning and design of the experiments. XL.J and Y.L performed the experiments and wrote the manuscript. XL.J, Y.Y.Y performed the experiments. J.X and B.X designed and discussed the study data. B.X and J.X supervised the entire project, and edited the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no competing interests.

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Figures

Figure 1

Characterization of Sprague-Dawley BMMs. (A): The cells were observed in the bright field, which grew into confluence after 3day, 7day and 10day.

Figure 2

Effects of TAA on BMMs proliferation and viability. (A) Chemical structure of TAA. (B) Effects of TAA on the cell viability of BMMs. Cells were treated with the indicated concentrations of TAA for the indicated times. Cell viability was determined by the CCK-8 assay. Dates are expressed as mean $\pm$ SD. (C) BMMs were treated with the indicated concentrations of TAA for 24h, then, cells were labeled for 15-20 min at 37°C with 3 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE). Cell fluorescence was evaluated by flow cytometry. (D) The bar chart represented cells divided index. Dates are expressed as mean $\pm$ SD, *P<0.05 vs control. (E) Cell cycle distribution was measured in BMMs by flow cytometry analysis after TAA treated with indicated concentrations for 24 h. (F) The bar chart represented the percentage of cells in G0/G1, S, or G2/M phase, as indicated. Values are expressed as mean $\pm$ SD of three individual experiments; * P<0.05 and ** P<0.01 indicates significant differences between TAA-treated groups and control groups.

Figure 3

TAA promoted osteoclastogenesis in BMMs. (A) Representative images of immunofluorescent microscopy showing the TARP (green) and nuclear (blue) in RANKL-treated or TAA-treated osteoclasts. (C) Effects of TAA at different concentrations (0, 0.5, 1mg/mL) on the formation of osteoclast-like cells derived from BMMs. (B–D) The number of osteoclasts showed TRAP-positive multinucleated cells (B for Immunofluorescence–D for TRAP staining). Values are expressed as mean $\pm$ SD of three individual

experiments; *P<0.05 and **P<0.01 indicates significant differences between RANKL-treated or TAA-treated groups and control groups.

Figure 4

TAA promote the expression of osteoclast-specific protein and PI3K/AKT pathway related proteins. (A, B) Cells were cultured with TAA at the indicated concentrations for 7 days and lysed for western blot analysis with antibodies against PI3K, P-PI3K, AKT, P-AKT, P38, P-P38, JNK, P-JNK, TRAP, cathepsin k and actin. Values are expressed as mean $\pm$ SD of three individual experiments; **P<0.01 and ***P<0.001 indicate significant differences between TAA-treated groups and control groups.

Figure 5

TAA exposure increased intracellular Ca^{2+} and promotes apoptosis. (A) Quantification of cell apoptosis by flow cytometry. BMMs were treated with TAA at the indicated concentrations for 24h, then, cells were incubated with FITC Annexin V in a buffer containing propidium iodide (PI) and analyzed by FCM. (B) Statistical bar graph showing the apoptosis ratio. (C) Analysis of intracellular Ca^{2+} in BMMs. Cells were treated with TAA at the indicated concentrations, then the cells were incubated with 400 μL 5 μM Fluo-3 AM at 37°C for 30 min. The fluorescent intensity of Fluo-3 AM was measured by the flow cytometry analysis at 488nm (excitation) and 540-570nm (emission). (D) The bar chart represented the intensity of fluo-3 AM in cells. Values are expressed as mean $\pm$ SD of three individual experiments; * $P < 0.05$ and ** $P < 0.01$ indicate significant differences between TAA-treated groups and control groups.