Beneficial effects of sulfonamide-based gallates on osteoblasts *in vitro*

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Received November 25, 2015; Accepted December 8, 2016

DOI: 10.3892/mmr.2017.6142

Abstract. Effective treatments for osteoporosis remain fairly elusive; however, studies have reported that antioxidants may aid in the maintenance of reactive oxygen species at a favorable level, in order to prevent osteoporosis. Gallic acid (GA) and its derivatives are potent antioxidative and anti-inflammatory agents that affect several biochemical and pharmacological pathways; however, GA is slightly cytotoxic and suppresses cell proliferation. The present study modified GA by the introduction of sulfonamide, in order to obtain a novel compound known as JEZ-C, and investigated its effects on osteoblasts by measuring cell proliferation, viability, morphology, alkaline phosphatase (ALP) activity, and the expression of relevant osteoblast markers. Results indicated that JEZ-C may effectively promote osteoblast growth. JEZ-C increased ALP activity, upregulated the expression of osteogenic-related genes, including runt-related transcription factor 2, bone sialoprotein, osteocalcin and alpha-1 type I collagen, thus indicating that JEZ-C enhances bone matrix production and mineralization. The recommended range of JEZ-C concentration is between $6.25x10^{-3}$ and $6.25x10^{-1}$ μg/ml, within which cell growth was promoted compared with the control. Specifically, treatment with $6.25x10^{-2}$ μg/ml JEZ-C is ideal. These findings may represent a novel approach to cell-based therapy for the treatment of osteoporosis.

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Key words: JEZ-C, gallic acid, osteoblast, proliferation, osteoporosis

Introduction

Osteoporosis is a common disease resulting from bone resorption by osteoclasts dominating over bone formation by osteoblasts, which is characterized by decreased bone mineral density and degraded bone fiber tissue. The disease can result in electrolyte imbalance and bone fracture, severely affecting quality of life in elderly patients. Current therapies for osteoporosis, including bisphosphonates, denosumab and teriparatide (parathyroid hormone), are associated with the following side effects: Osteonecrosis (1,2), prolonged union time for fractures (3), hypocalcaemia (4-6), headaches, nausea, dizziness, limb pain and transient severe hypotension (7). Furthermore, the cathepsin K inhibitor odanacatib, the anti-sclerostin antibody romosozumab and anti-Dickkopf-related protein 1 antibody exert their anti-osteoporotic effects via biological pathways, which may induce undesirable effects with long-term use. Traditional treatments, such as dietary control and supplementation of calcium and vitamin D, are generally unsatisfactory.

Gallic acid (GA) and its derivatives are a group of polyphenol compounds that are well known for their potent antioxidative (8) and anti-inflammatory abilities, which affect several biochemical and pharmacological pathways (9). However, GA has also been shown to suppress cell proliferation (10), which may influence its cell protective effects. Santamaria *et al* (11) suggested that modifying GA by introducing a sulfonamide group may enhance its bioactivity and hydrophobicity, thus allowing it to support cell growth. The introduction of sulfamethoxazole (SMZ), specifically, may then promote antibiotic ability and hydrophobicity of GA by displacing hydrogen atoms on amino para-positions to create different heterocyclic structures (Fig. 1).

Based on the hypothesis that synthetic gallate compounds and sulfonamides may improve GA multiplication and exert protective effects toward osteoblasts, the present study synthesized sulfonamide-based gallates. Subsequently, their biological effects were evaluated by examining the proliferation, morphology, viability and extracellular matrix (ECM) synthesis of osteoblasts, as well as osteoblast-specific gene expression. The findings of the present study may provide

a valuable reference point for enhancing the proliferative capacity of osteoblasts and the development of future therapies for the treatment of osteoporosis.

Materials and methods

Synthesis of JEZ-C. JEZ-C [3,4,5-trihydroxy-N-{4-[(5-methylisoxazol-3-yl)sulfamoyl]phenyl}benzamide; Fig. 1A] was prepared from GA and SMZ, as outlined in Fig. 1B. Subsequently, an appropriate amount of distilled water was added to the mixture, and the raw precipitated product was separated by vacuum filtration. The raw product was then recrystallized in a tetrahydrofuran-methanol solvent system. Electrospray ionization mass spectrum (ESI-MS) was detected on a Shimadzu LC-MS 2010A. ^{1}H and ^{13}C NMR spectra were assessed by using a Bruker Advance III 300 at 400 and 125 MHz, respectively.

JEZ-C has the following properties: White powder, mp: 217-218˚C, yield 63%, MS-ESI: 404.0[M-H]$^{-}$, ^{1}H-NMR (400 MHz, DMSO-d6) δ 11.33 (s, 1H, -SO_2-NH), 10.29 (s, 1H, -CO-NH), 7.95-7.77 (m, 4H, 4xAr-H), 6.94 (s, 2H, 2xAr-H), 6.13 (s, 1H, isoxazol-H), 2.28 (s, 3H, -CH_3). ^{13}C-NMR (125 MHz, DMSO-d6) δ170.30, 166.09, 157.64, 145.60, 144.06, 137.37, 132.96, 127.83, 124.31, 119.79, 107.47, 95.43, 12.10.

Primary osteoblast separation and culture. The study was approved by the ethics committee of Guangxi Medical University (Nanning, China; Protocol Number: 20141008A). A total of 6 newborn Sprague-Dawley rats (3-7 days old; 3 male, 3 female) were used in the present study. Specific Pathogen Free Sprague-Dawley rats were purchased from the Animal Resources Center of Guangxi Medical University (Nanning, China). Animals were housed in temperature a temperature controlled environment at 24˚C, with a 12 h light/dark cycle, and were provided with food and water *ad libitum*. After delivery, neonatal rats were carefully placed as close to the dams as possible. Following sacrifice by cervical dislocation, osteoblasts were acquired from neonatal rat parietal bones by enzymatic digestion with 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min at 37˚C, followed by 2 mg/ml collagenase type I (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in serum-free alpha-modified Eagle's medium (α-MEM; Gibco; Thermo Fisher Scientific, Inc.) for 4 h at 37˚C. The cells were resuspended following centrifugation at 800 x *g* for 5 min in α-MEM basal culture medium containing 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic mixture (100 U/ml penicillin, 100 U/ml streptomycin). The culture medium was changed every 2 days, and culture conditions in a humidified incubator (Thermo Fisher Scientific, Inc.) were maintained at 5% CO_2 and 37˚C. Cells were used for analysis upon reaching 80-90% confluence.

JEZ-C treatment. JEZ-C was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and diluted in PBS to obtain a final concentration of 6.25 mg/ml. The final concentration of DMSO was less than 0.1% (v/v) in all experiments. The stock solution was stored at 4˚C for one week and diluted with culture medium immediately before the experiment. Cells were treated with the obtained JEZ-C at various concentrations (0 μg/ml as control, $6.25x10^{-3}$, $6.25x10^{-2}$ and $6.25x10^{-1}$ μg/ml).

Cell viability assay. Osteoblast cell viability was determined by staining samples with fluorescein diacetate (FDA; Genway Biotech, Inc., San Diego, CA, USA) and propidium iodide (PI; Sigma-Aldrich; Merck Millipore) at 2, 4 and 6 days. Briefly, FDA and PI stock solutions were added to the cells at final concentrations of 2 μmol/l and 2 μg/l, respectively; the cells were then incubated in the dark for 5 min at 37˚C. Images were captured under a laser scanning confocal microscope (Nikon A1; Nikon Corporation, Tokyo, Japan).

Cell proliferation assay. To investigate the dose-dependent effects of JEZ-C on osteoblast proliferation, an MTT assay was used to determine the amount of cells in the samples. Cells were initially digested with 0.25% trypsin/EDTA, resuspended in culture medium, and seeded into 24-well plates at $5x10^{3}$ cells/well density. After a 24-h culture at 37˚C, the culture medium was replaced with various concentrations of JEZ-C (0, $6.25x10^{-3}$, $6.25x10^{-2}$ and $6.25x10^{-1}$ μg/ml). Assays were performed at 2, 4 and 6 days; 1 ml 0.5 mg/ml MTT (Sigma-Aldrich; Merck Millipore) was added to each well and the samples were incubated in an atmosphere containing 5% CO_2 (Forma™ Series II Water-Jacketed incubator; Thermo Fisher Scientific, Inc.) at 37˚C for 4 h. The formed formazan crystals were then dissolved in 1 ml dimethyl sulfoxide. After thoroughly and evenly mixing the samples, 200 μl was randomly extracted from three parallel wells at each JEZ-C concentration and transferred to a 96-well plate. Sample absorbance values were measured at 570 nm using a microplate reader (Multiskan™ GO Microplate Spectrophotometer; Thermo Fisher Scientific, Inc.). Results are presented as optical density absorbance values.

Cell morphological analysis. Samples in the three experimental groups were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich; Merck Millipore) after being cultured for 2, 4 and 6 days. Subsequently, cells were incubated with 1% bovine serum albumin (BSA; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as a blocking buffer for 30 min at 37˚C. Cells were then stained for 30 min at room temperature with rhodamine phalloidin (Invitrogen; Thermo Fisher Scientific, Inc.), followed by Hoechst 33258 (Beyotime Institute of Biotechnoloy, Haimen, China) for 5 min to visualize nuclei. All imaging was performed with a scanning confocal microscope.

Alkaline phosphatase (ALP) staining and activity assay. To determine the effects of JEZ-C on osteoblasts and to allow for subsequent staining, cells were seeded at a density of $1x10^{4}$ on to coverslips of 24-well plate and cultured in media with various JEZ-C concentrations. After 2, 4 and 6 days of culturing, the cells were washed with PBS and stained using an ALP staining kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's protocol. Staining was observed and images were captured with an inverted phase contrast microscope (Olympus Corporation, Tokyo, Japan) and imaging software for microscopy (cellSens Dimension 1.14; Olympus Corporation).

A second cell sample was lysed with 200 μl Radioimmunoprecipitation Assay Lysis Buffer (Beyotime Institute of Biotechnology) and phenylmethanesulfonyl fluoride to a final concentration of 1 mM for ALP activity

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer
β-actin	5'-CCCATCTATGAGGGTTACGC-3'	5'-TTTAATGTCACGCACGATTTC-3'
RUNX2	5'-TGTCATGGCGGGTAACGATG-3'	5'-CCCTAAATCACTGAGGCGGT-3'
BSP	5'-CAATCTGTGCCACTCACTGC-3'	5'-TGCCCTGAACTGGAAATCGTT-3'
OCN	5'-ACACTCCTCGCCCTATTGGC-3'	5'-CCATTGATACAGGTAGCGCCT-3'
COL1A1	5'-GTTCAGCTTTGTGGACCTCCG-3'	5'-GCAGTTCTTGGTCTCGTCAC-3'

β-actin was used as a control gene for normalization. RUNX2, runt-related transcription factor 2; BSP, bone sialoprotein; OCN, osteocalcin; COL1A1, alpha-1 type I collagen.

analysis. Total protein concentration (mg/ml) and ALP activity (units/ml) were measured with an improved bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) and ALP reagent kit (cat. no. A059-2; Nanjing Jiancheng Bioengineering Research Institute), respectively, according to the manufacturers' protocols. ALP levels were standardized to total protein content. All samples were examined in triplicate.

Alizarin red staining. The mineralization of osteoblast ECMs was determined using Alizarin red staining. After 2, 4 and 6 days of culturing, the cells were washed with distilled water and fixed in ice-cold 70% (v/v) ethanol for 1 h at 4˚C. The cells were then placed on coverslips and rinsed twice with deionized water [Tiangen Biotech (Beijing) Co., Ltd. Beijing, China], prior to staining with Alizarin red S (Sigma-Aldrich; Merck Millipore) solution (40 mM, pH 4.2) for 10 min at room temperature. Dyestuff was prepared in Tris-HCl (Sigma-Aldrich; Merck Millipore) buffer solution and adjusted to the target pH, then excess dye was gently removed with running water. Calcification deposits, typically stained red, were examined under an optical microscope (Nikon Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR assays were performed to measure osteogenic gene expression in cells cultured in 24-well plates with various concentrations of JEZ-C (0, $6.25x10^{-3}$, $6.25x10^{-2}$ and $6.25x10^{-1}$ μg/ml). Total RNA was extracted with an RNA isolation kit (Tiangen Biotechnology, Beijing, China) according to the manufacturer's instructions on days 2, 4 and 6. Subsequently, 300 ng total RNA was reverse transcribed to cDNA with an RT system (Promega Corporation, Madison, WI, USA). The total qPCR was performed using SYBR Green master mix (BioTeke Corporation, Beijing, China) and thermocycling conditions as follows: 95˚C for 10 min, then 40 cycles of 95˚C for 15s and 60˚C for 1 min. Details regarding the primers are listed in Table I. Marker gene expressions were analyzed by the $2^{-\Delta\Delta CT}$ method (12), using β-actin as the normalizing control. Each sample was repeated three times for each gene.

Immunohistochemical assay. After 2, 4 and 6 days of culturing, COL1 immunohistochemical staining was performed according to the manufacturer's protocol. Briefly, cells at density of $1x10^4$ on coverslips of 24-well plate were washed with PBS, rinsed with 0.01% Triton X-100, washed again thoroughly in PBS and treated with 3% hydrogen peroxide (Wuhan Boster Biological Technology, Ltd., Wuhan, China). The cells were then washed once more in PBS, and blocked with 3% BSA. After incubation with a 1:200 dilution of the COL1 primary antibody (cat. no. BA0325; Wuhan Boster Biological Technology, Ltd.) at 4˚C for 12 h, and the secondary antibody (cat. no. SP-0023; Zymed Technologines, Thermo Fisher Scientific, Inc.) at 37˚C for 20 min, biotin-labeled horseradish peroxidase was added to the cells at 37˚C for 20 min. Eventually, After color was developed using a 3'-3'-diaminobenzidine tetrahydrochloride (DAB) kit (Wuhan Boster Biological Technology, Ltd.) for 3 min and nucleus were stained with hematoxylin for 1 min, the coverslips were finally air-dried and sealed with neutral resin. Cells were observed and photographed with an inverted phase contrast microscope (Olympus Corporation) and imaging software for microscopy (cellSens Dimension 1.14; Olympus Corporation).

Statistical analysis. Data are presented as the mean ± standard deviation. All data were evaluated by one-way analysis of variance followed by least significant difference *post-hoc* tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability. FDA/PI staining was used to detect cell viability, as presented in Fig. 2A-L. JEZ-C had a marked effect on osteoblast cell viability. FDA/PI staining demonstrated that live cells in JEZ-C groups were more abundant compared with in the control group.

Cell proliferation. Cells were subjected to MTT assay after being cultured with three different concentrations of JEZ-C. The three sample groups produced quite diverse results, as presented in Fig. 2M. Osteoblast numbers increased over time, and cells cultured with $6.25x10^{-3}$ and $6.25x10^{-2}$ μg/ml grew significantly faster than other groups ($P<0.05$; Fig. 2M). These results were in accordance with the cell viability assay findings, thus suggesting that JEZ-C exerted a positive effect on osteoblast growth. Among all groups, JEZ-C at a concentration of $6.25x10^{-2}$ μg/ml had the most marked effect.

Cell morphology. The actin filaments in osteoblasts were stained with rhodamine phalloidin/Hoechst 33258, as presented in Fig. 3A-L. Cells treated with JEZ-C grew in

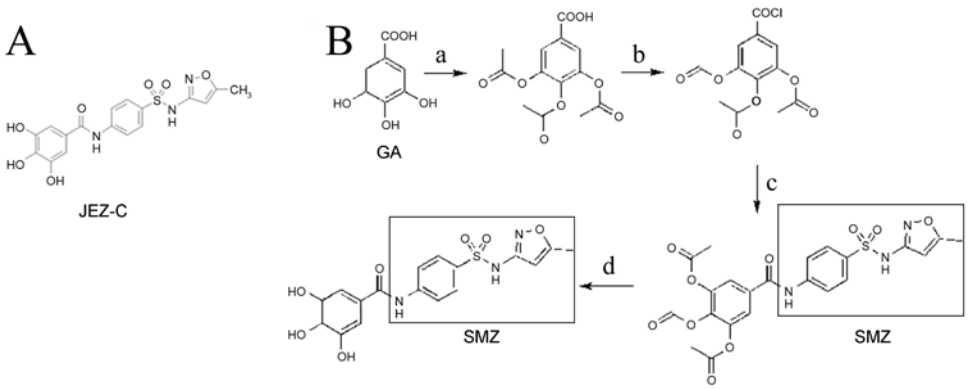

Figure 1. (A) Chemical structure of JEZ-C. (B) JEZ-C synthesis route. Reagents and conditions: (a) Acetyl oxide, oil bath, 120˚C; (b) $SOCl_2$, oil bath, 80˚C; (c) SMZ, THF, Pyridine, ice bath; (d) HCl, THF, 60˚C. JEZ-C, 3,4,5-trihydroxy-N-{4-[(5-methylisoxazol-3-yl)sulfamoyl]phenyl}benzamide; SMZ, sulfamethoxazole; THF, tetrahydrofuran.

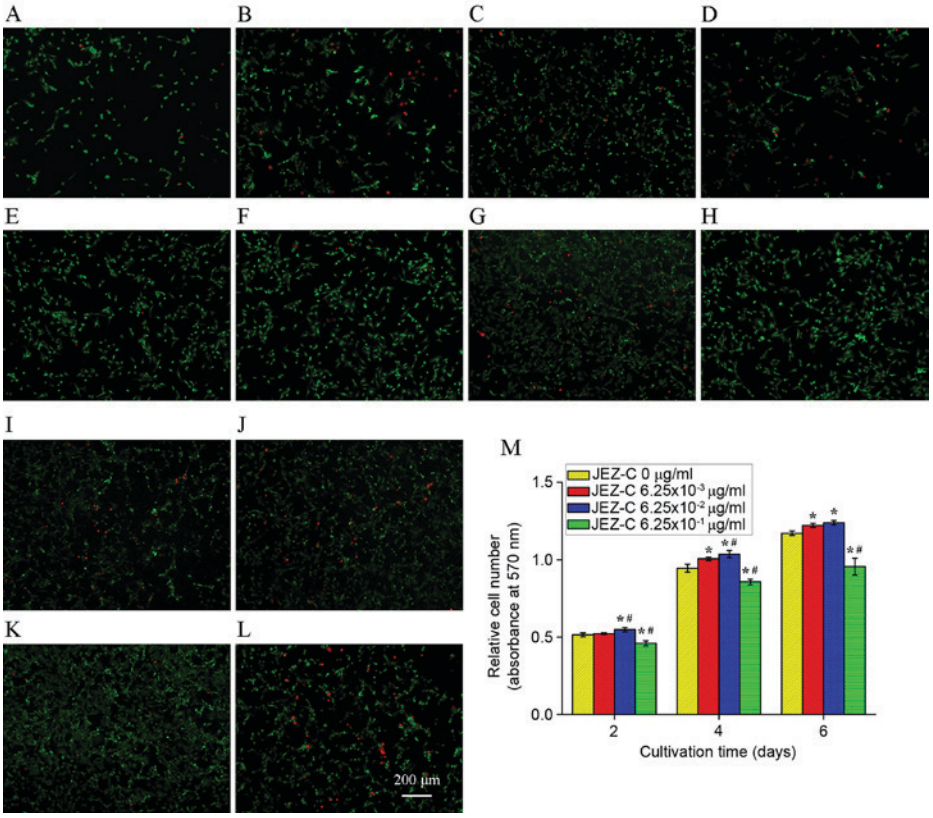

Figure 2. Fluorescein diacetate/propidium iodide staining of osteoblasts cultured with various concentrations of JEZ-C over time. Viable cells were stained green and dead cells were stained red. Staining of osteoblasts treated with (A) 0 μg/ml, (B) $6.25x10^{-3}$ μg/ml, (C) $6.25x10^{-2}$ μg/ml and (D) $6.25x10^{-1}$ μg/ml JEZ-C at day 2. Staining of osteoblasts treated with (E) 0 μg/ml, (F) $6.25x10^{-3}$ μg/ml, (G) $6.25x10^{-2}$ μg/ml and (H) $6.25x10^{-1}$ μg/ml JEZ-C at day 4. Staining of osteoblasts treated with (I) 0 μg/ml, (J) $6.25x10^{-3}$ μg/ml, (K) $6.25x10^{-2}$ μg/ml and (L) $6.25x10^{-1}$ μg/ml JEZ-C at day 6. Staining was markedly strengthened over time in all groups. Cells treated with JEZ-C exhibited stronger staining compared with the control group, particularly at $6.25x10^{-2}$ μg/ml. Scale bar=200 μm. (M) Relative cell number of osteoblasts treated with various concentrations (0, $6.25x10^{-3}$, $6.25x10^{-2}$ and $6.25x10^{-1}$ μg/ml) of JEZ-C. Cell proliferation was higher in the $6.25x10^{-2}$ μg/ml group compared with in the other groups. Data are presented as the mean ± standard deviation (n=9). *P<0.05 vs. JEZ-C 0 μg/ml; $^{\#}$P<0.05 vs. JEZ-C $6.25x10^{-3}$ μg/ml.

clumps distributed with dense ECMs, whereas in the control group there were fewer cells and actin filaments. This effect was particularly evident on day 6 with a concentration of $6.25x10^{-2}$ μg/ml JEZ-C (Fig. 3K) compared with 0 μg/ml (Fig. 3I).

ALP staining and activity. Osteoblasts secrete ALP, which likely causes enough inorganic pyrophosphate downregulation to provide sufficient local phosphate (13) for mineralization (14). ALP activity is commonly considered a marker of osteogenesis, and is assumed to represent the degree of osteogenic differentiation. The ALP activity assay and staining results are presented in Fig. 4. After being cultured for 4 days, JEZ-C groups exhibited much higher ALP activity compared with the control group. In particular, the $6.25x10^{-2}$ μg/ml JEZ-C group exhibited the highest ALP levels. ALP activity

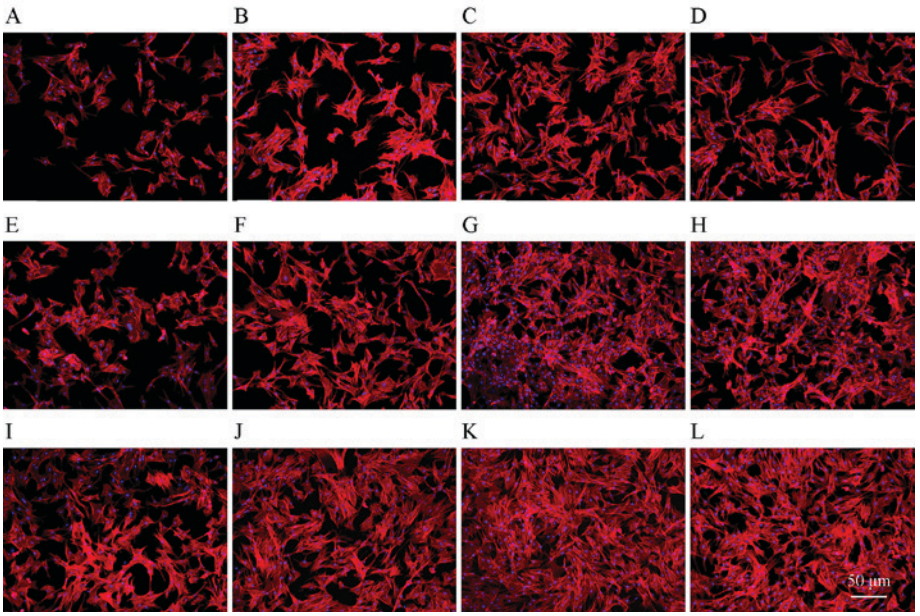

Figure 3. Rhodamine phalloidin/Hoechst 33258 staining of osteoblasts cultured with various concentrations of JEZ-C over time. Staining of osteoblasts treated with (A) 0 μg/ml, (B) 6.25x10^{-3} μg/ml, (C) 6.25x10^{-2} μg/ml and (D) 6.25x10^{-1} μg/ml JEZ-C on day 2. Staining of osteoblasts treated with (E) 0 μg/ml, (F) 6.25x10^{-3} μg/ml, (G) 6.25x10^{-2} μg/ml and (H) 6.25x10^{-1} μg/ml JEZ-C on day 4. Staining of osteoblasts treated with (I) 0 μg/ml, (J) 6.25x10^{-3} μg/ml, (K) 6.25x10^{-2} μg/ml and (L) 6.25x10^{-1} μg/ml JEZ-C on day 6. Scale bar=50 μm. Staining was markedly strengthened over time in all groups. Cells treated with JEZ-C exhibited stronger staining compared with the control, particularly when treated with 6.25x10^{-2} μg/ml JEZ-C.

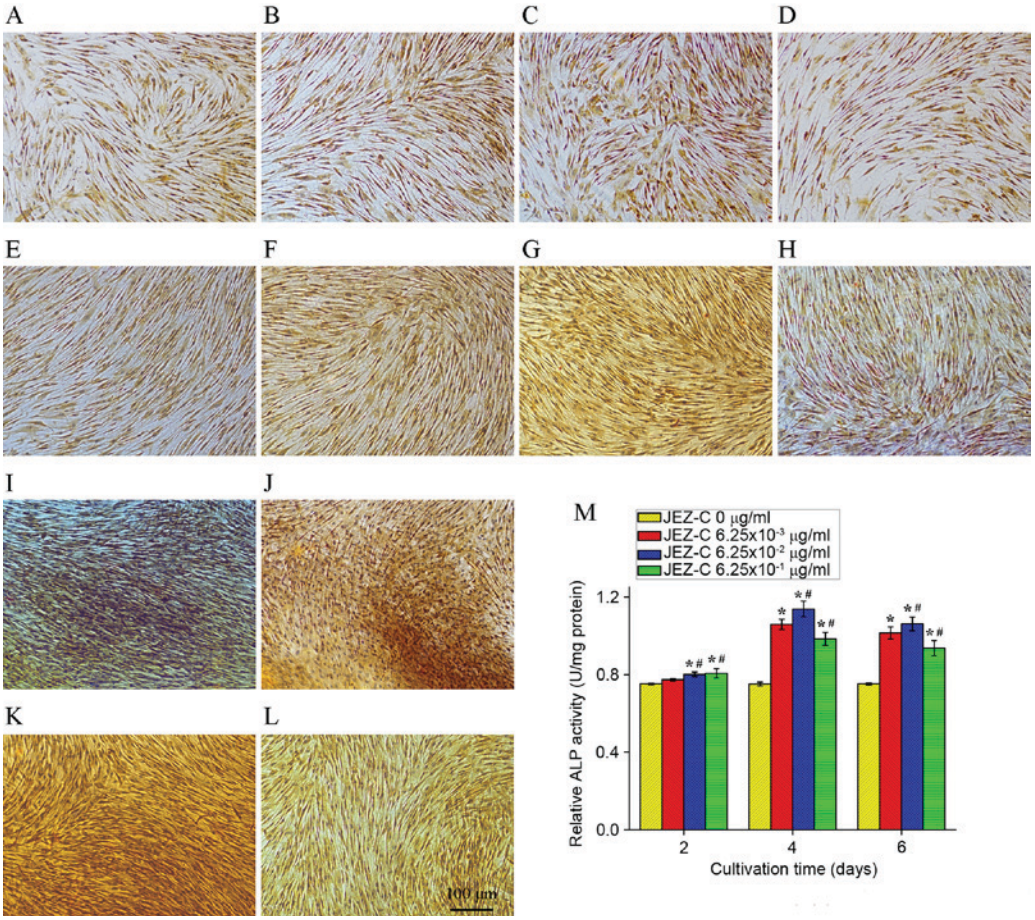

Figure 4. ALP staining of osteoblasts treated with various concentrations of JEZ-C. ALP staining of osteoblasts treated with (A) 0 μg/ml, (B) 6.25x10^{-3} μg/ml, (C) 6.25x10^{-2} μg/ml and (D) 6.25x10^{-1} μg/ml JEZ-C on day 2. ALP staining of osteoblasts treated with (E) 0 μg/ml, (F) 6.25x10^{-3} μg/ml, (G) 6.25x10^{-2} μg/ml and (H) 6.25x10^{-1} μg/ml JEZ-C on day 4. ALP staining of osteoblasts treated with (I) 0 μg/ml, (J) 6.25x10^{-3} μg/ml, (K) 6.25x10^{-2} μg/ml and (L) 6.25x10^{-1} μg/ml JEZ-C on day 6. Scale bar=100 μm. (M) Time-course of ALP activity of osteoblasts treated with various concentrations (0, 6.25x10^{-3}, 6.25x10^{-2} and 6.25x10^{-1} μg/ml) of JEZ-C. Relative ALP activity (units/mg protein) was expressed as mean ± standard deviation (n=3). ALP activity in the 6.25x10^{-2} μg/ml JEZ-C group was significantly higher than in the other groups. *P<0.05 vs. JEZ-C 0 μg/ml; $^{\#}$P<0.05 vs. JEZ-C 6.25x10^{-3} μg/ml. ALP, alkaline phosphatase.

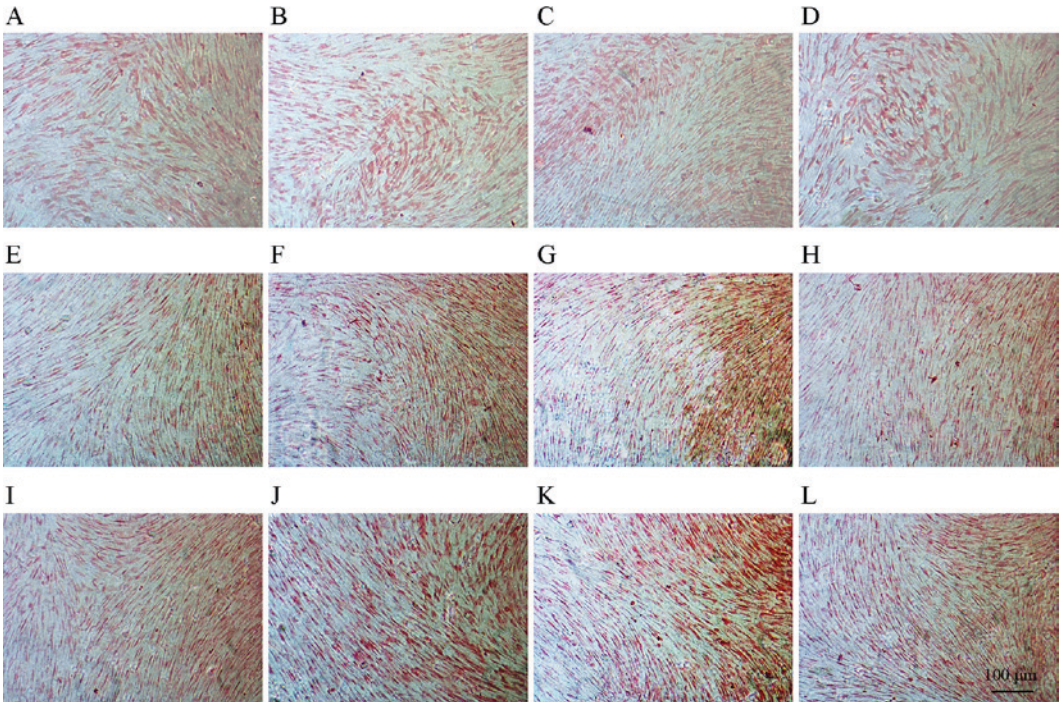

Figure 5. Alizarin red staining of osteoblasts cultured with various concentrations of JEZ-C over time. Staining of osteoblasts treated with (A) 0 μg/ml, (B) $6.25x10^{-3}$ μg/ml, (C) $6.25x10^{-2}$ μg/ml and (D) $6.25x10^{-1}$ μg/ml JEZ-C on day 2. Staining of osteoblasts treated with (E) 0 μg/ml, (F) $6.25x10^{-3}$ μg/ml, (G) $6.25x10^{-2}$ μg/ml and (H) $6.25x10^{-1}$ μg/ml JEZ-C on day 4. Staining of osteoblasts treated with (I) 0 μg/ml, (J) $6.25x10^{-3}$ μg/ml, (K) $6.25x10^{-2}$ μg/ml and (L) $6.25x10^{-1}$ μg/ml JEZ-C on day 6. Scale bar=100 μm. Staining was markedly strengthened over time in all groups. Cells treated with JEZ-C exhibited stronger staining compared with the control, particularly when treated with $6.25x10^{-2}$ μg/ml JEZ-C.

continuously increased over the course of the experiment from day 2 to 4 and slightly decreased from day 4 to 6 (Fig. 4M).

Alizarin red staining. The calcium content of each sample was determined by Alizarin red staining. The staining indicated that bone-like nodules formed in all groups in a time-dependent manner (Fig. 5), and that JEZ-C enhanced mineralization. In particular, treatment with JEZ-C at a concentration of $6.25x10^{-2}$ μg/ml resulted in the most enhanced levels of mineralization. Mineralization was not entirely complete in any sample by day 6.

RT-qPCR. The expression levels of the following genes: RUNX2, BSP, OCN and COL1A1, were used to validate the pro-osteogenic effects of JEZ-C on osteoblasts, as presented in Fig. 6. The expression levels of these genes were significantly upregulated in samples treated with JEZ-C ($P<0.05$), particularly at a dose of $6.25x10^{-2}$ μg/ml. The results further indicated that the effects of JEZ-C on osteogenic differentiation were dose-dependent between 0 and $6.25x10^{-2}$ μg/ml; however, for all genes detected, compared with the $6.25x10^{-2}$ μg/ml JEZ-C group, treatment with $6.25x10^{-1}$ μg/ml JEZ-C resulted in a slight downregulation. RUNX2 and COL1A1 gene expression peaked at day 4, whereas the other genes increased continuously throughout the culture period.

Immunohistochemical analysis. Immunohistochemical staining indicated that COL1 is expressed strongly in accordance with bone formation. As presented in Fig. 7A-L, secretion of COL1 increased over time and JEZ-C up-regulated the level. High staining confirmed that JEZ-C enhances osteoblast mineralization, most effectively at a concentration of $6.25x10^{-2}$ μg/ml (Fig. 7C, G and K).

Discussion

The present study synthesized JEZ-C by coupling sulfonamide groups with GA; subsequently, its effects on osteoblast growth at various concentrations were investigated. The results indicated that JEZ-C is able to increase osteoblast growth, as evidenced by rapid cell proliferation compared with in the control group (Fig. 2M). Furthermore, JEZ-C markedly promoted ALP secretion in cultured osteoblasts, as indicated by a biochemical assay (Fig. 4M). As an early marker of osteoblast growth, ALP is involved in matrix mineralization and organization (15,16). Throughout the culture period, the present study observed a progressive upregulation in ALP activity from day 2 to 4 in all groups, which is indicative of ECM development preceding full mineralization. ALP activity decreases once osteoblasts are fully mature (17); therefore, it may be hypothesized that ALP activity decreases with prolonged culture time, whereas the continuously increasing ALP detected in the present study marks the growth stage.

The present study demonstrated that JEZ-C upregulates the gene expression of RUNX2, BSP, OCN and COL1A1 (Fig. 6). As opposed to BSP and OCN, which exhibited continual upregulation, RUNX2 and COL1A1 expression increased gradually until it peaked at day 4 and subsequently declined. RUNX2, which is a critical regulator for osteoblast development and maturation, is essential in the early stages of bone calcification (18); furthermore, RUNX2 affects the expression of COL1A1, BSP

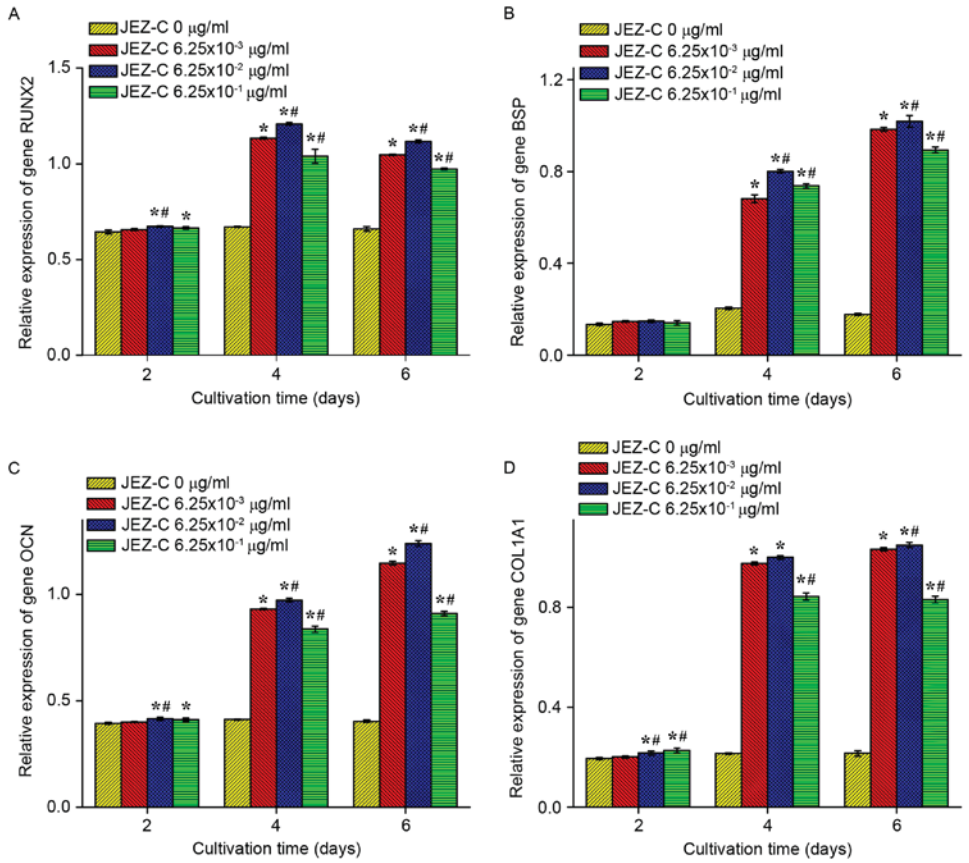

Figure 6. Reverse transcription-quantitative polymerase chain reaction was used to analyze the expression levels of osteogenic genes: (A) RUNX2, (B) BSP, (C) OCN and (D) COL1A1, in osteoblasts cultured with various concentrations of JEZ-C for 2, 4 and 6 days. These genes are all associated with the osteoblast phenotype and were all significantly upregulated following JEZ-C treatment ($P<0.05$), particularly when cells were treated with $6.25x10^{-2}$ μg/ml JEZ-C. Data are presented as the mean ± standard deviation (n=3). *$P<0.05$ vs. JEZ-C 0 μg/ml; #$P<0.05$ vs. JEZ-C $6.25x10^{-3}$ μg/ml. RUNX2, runt-related transcription factor 2; BSP, bone sialoprotein; OCN, osteocalcin; COL1A1, alpha-1 type I collagen.

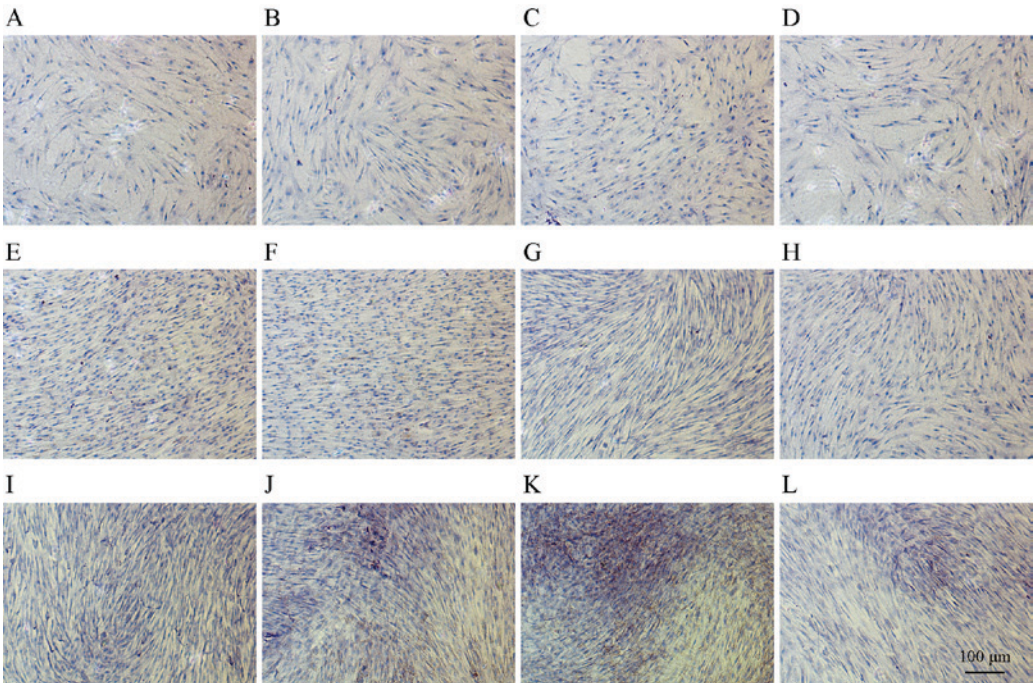

Figure 7. Immunohistochemical examination of COL1 in osteoblasts treated with various concentrations of JEZ-C over time. Staining of osteoblasts treated with (A) 0 μg/ml, (B) $6.25x10^{-3}$ μg/ml, (C) $6.25c10^{-2}$ μg/ml and (D) $6.25x10^{-1}$ μg/ml JEZ-C on day 2. Staining of osteoblasts treated with (E) 0 μg/ml, (F) $6.25x10^{-3}$ μg/ml, (G) $6.25x10^{-2}$ μg/ml and (H) $6.25x10^{-1}$ μg/ml JEZ-C on day 4. Staining of osteoblasts treated with (I) 0 μg/ml, (J) $6.25x10^{-3}$ μg/ml, (K) $6.25x10^{-2}$ μg/ml and (L) $6.25x10^{-1}$ μg/ml JEZ-C on day 6. Scale bar=100 μm. The expression of COL1 increased with time. The groups treated with JEZ-C exhibited stronger positive staining compared with the control, particularly when cells were treated with $6.25x10^{-2}$ μg/ml JEZ-C. COL1, collagen type 1.

and OCN to a certain degree (19). COL1A1, which is another marker of mature osteoblasts, influences cellular matrix production in osteoblasts (20). The results of the present study suggested that JEZ-C accelerates osteoblast growth and stimulates ECM secretion by regulating COL1, the key activator of the osteoblast-specific enhancer, which is further evidenced by the continuous upregulation of other genes, including BSP and OCN. In the control group, the genes were expressed at lower levels, thus indicating that JEZ-C exerts potent regulatory effects on gene expression. The results of an MTT assay also indicated that JEZ-C promotes cell proliferation in a dose-dependent manner. In particular, treatment with JEZ-C at a concentration of $6.25x10^{-2}$ μg/ml markedly promoted osteoblast proliferation compared with the other concentrations (Fig. 2).

GA and its derivatives have been reported to suppress cell growth. Epigallocatechin-3-gallate, one such GA derivative, has been reported to inhibit the degradation of human cartilage proteoglycan and type II collagen, and to selectively inhibit a disintegrin and metalloproteinase with thrombospondin motifs ADAMTS-1, ADAMTS-4 and ADAMTS-5 (21). Sulfonamides also exhibit slight cytotoxicity in human keratinocytes and rat hepatocytes (22), and inhibitory effects on cell wall synthesis (23). In the present study, JEZ-C, a novel derivative of GA, was able to effectively support osteoblast growth and phenotypic maintenance, indicating that appropriately modifying GA with sulfonamides may improve its pharmacological effects. The present study demonstrated that the JEZ-C concentrations most effective for enhancing osteoblast proliferation ranged between $6.25x10^{-3}$ and $6.25x10^{-1}$ μg/ml, among which $6.25x10^{-2}$ μg/ml was considered the optimal concentration.

In conclusion, the present study demonstrated that JEZ-C effectively promotes osteoblast proliferation, increases the secretion and ECM synthesis of osteoblasts, and maintains cell phenotype. Osteogenic-related genes, including ALP, RUNX2, COL1A1, BSP and OCN, were upregulated after JEZ-C treatment. Treatment with JEZ-C at $6.25x10^{-2}$ μg/ml proved the most favorable of all doses tested. These results suggested that JEZ-C is a useful pro-osteogenic agent, and may be considered an attractive potential cell-based therapy for the treatment of osteoporosis.

Acknowledgements

The present study was financially supported by the National Key Research and Development Program of China (grant no. 2016YFB0700804), the Guangxi Scientific Research and Technological Development Foundation (grant no. Guikegong 1598013-15) and the Guangxi Science Fund for Distinguished Young Scholars (grant no. 2014GXNSFGA118006).

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