Original Article

Effects and mechanism of aromatic aminoketone SY0916 on osteoclastic bone destruction

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Aim: To study the effects and mechanism of aromatic aminoketone (SY0916) on bone destruction *in vitro*.
Methods: MC3T3-E1 cells and bone marrow cells were co-cultured to obtain purified osteoclasts. The proliferation of osteoclast-like cells (OCLs) was determined by MTT assay. The number of osteoclasts was measured by tartrate-resistant acid phosphatase (TRAP) staining. The functioning of osteoclasts was determined by measuring the area of bone resorption pits on bone slices. MMP-9 secretion by osteoclasts was measured by an ELISA kit. Osteoclast apoptosis was detected by flow cytometry using an AnnexinV-FITC kit. Gene expression of *RANK* and *MMP-9* in osteoclasts as well as *RANKL* and *OPG* in MC3T3-E1 cells was determined by real-time PCR.
Results: SY0916 significantly inhibited the proliferation of OCLs, decreased both the total and average area of bone resorption pits, and dramatically inhibited the number of osteoclasts between concentrations of 0.01 and 10 µmol/L. Furthermore, SY0916 reversed IL-1β-mediated inhibition of osteoclast apoptosis and shortened osteoclast lifespan. In addition, SY0916 significantly inhibited the mRNA expression of *RANK*, *RANKL*, *OPG*, and *MMP-9*. However, the inhibition of *OPG* was weaker than that of *RANKL*. Accordingly, the ratio of *RANKL* to *OPG* mRNA expression in MC3T3-E1 cells was significantly decreased by SY0916. Meanwhile, the expression of MMP-9 protein in osteoclasts was inhibited by SY0916 between 0.01 and 10 µmol/L.
Conclusion: SY0916 prevents osteoclastic bone destruction by inhibiting the proliferation and function of osteoclasts. The underlying mechanism for this effect involves the regulation of the RANKL-OPG-RANK axis, which determines the direction of bone metabolism.

Keywords: SY0916; osteoclast; bone destruction; RANKL; RANK; OPG

Introduction

The destruction of bone and cartilage is the major cause of progressive disabilities culminating in the crippling of patients suffering from rheumatoid arthritis (RA). Extensive bone erosion is often seen as marginal joint erosions radiographically, and it is predictive of a poor prognosis. Prevention of the development of bone destruction is an important objective in the treatment of RA.

Osteoclasts are the cell type primarily responsible for resorption during both physiologic bone turnover and inflammatory bone disease[1]. They are formed by the fusion of mononuclear cells derived from osteoclast precursors. Bone-resorbing osteoclasts exhibit highly polarized morphological features such as actin rings, clear zones, and ruffled borders, which are markers of functional osteoclasts. The resorption lacuna is formed by targeted secretion of protons and proteases from the ruffled border after tight attachment of osteoclasts to mineralized bone surfaces, which is mediated by integrin receptors expressed on the cell surface following polarization[2]. Multiple kinds of inflammatory cytokines are involved in the regulation of osteoclasts, including IL-1β.

Aromatic aminoketone (SY0916) had been demonstrated to be a new type of PAF receptor antagonist and is considered a novel anti-inflammation drug. The anti-inflammatory effects of SY0916 have been shown by our previous studies both *in vivo* and *in vitro*[3]. Notably, we found that SY0916 prevented bone erosion from occurring in CIA rats[4]. However, we did not study the mechanism underlying this effect of SY0916. The aim of the present study was to investigate the effects and mechanism of SY0916 on bone destruction *in vitro*.

Materials and methods
Animals
Male C57BL/6 mice (H-2^d, 18±2 g, 6−8 weeks old) were provided by the Experimental Animal Center, Chinese Academy of Medical Sciences & Peking Union Medical College (SPF,

certificate N<u>o</u> SCXK2004-0001). All animals were housed in groups under a 12-h light/dark regime (lights on from 7:00 to 19:00) at 23±2 °C prior to the experiments, and were given standard laboratory chow and tap water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee before any actual experimentation began.

Reagents

Rh IL-1β, M-CSF, and sRANKL were purchased from Peprotech. FITC-phalloidin, MTT, and 1,25-dihydroxyvitamin D3 were purchased from Sigma. ELISA kits for murine MMP-9 were from R&D systems. αMEM and FCS were from Hyclon. TRIzol reagent was purchased from Promega. SYBR® Prime-Script[TM] RT-PCR kit was from Takara. Annexin V-FITC kit for apoptosis detection was from BAOSAI Reagent.

Test compound

SY0916 was synthesized as described previously[3], its structure is shown in Figure 1. The purity was more than 99% upon HPLC analysis. SY0916 was prepared in a 0.01 mol/L stock solution with DMSO and stored at -20 °C. Before using, the stock solution was diluted to the appropriate concentration in αMEM.
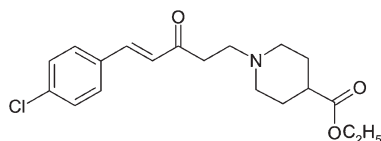

Figure 1. The structure of SY0916, M_W=386.31.

Cell culture

The MC3T3-E1 cell line (an osteoblastic cell line derived from mice) was purchased from the cell center at the Chinese Academy of Medical Sciences & Peking Union Medical College. MC3T3-E1 cells were cultured in αMEM containing 10% FCS at 37 °C in 5% CO_2. When the cells were confluent (after approximately 3 d in culture), a mixture of 0.05% trypsin and 0.02% EDTA was used to passage the cells. The collected cells were then seeded on culture dishes.

The preparation of bone marrow cells

The femur and tibia were collected from 6–8 week-old C57BL/6 mice sacrificed under sterile conditions. A single cell suspension was prepared by gently pressing bone marrow segments through a glass injector. Bone marrow cells were separated from red blood cells by centrifugation at 790×g for 20 min in a Histpopaque 1077 centrifuge. The collected cells were washed twice and seeded on culture plates in αMEM containing 10% FCS at 37 °C in 5% CO_2 for about 6 h and then collected.

Induction and purification of osteoclasts

MC3T3-E1 cells (1×10⁶ cells/mL) and bone marrow cells (2×10⁷ cells/mL) were co-cultured in αMEM containing 10% FCS in 100 mm tissue culture dishes (15 mL/dish) precoated with 2.5 mL of 0.2% collagen gel matrix. After 2 d, half of the medium was changed with fresh medium. Subsequently, 1,25-dihydroxyvitamin D3 (10^{-8} mol/L) and M-CSF (50 ng/mL) were added to the media as stimulants. After culturing for 5 d, the non-adherent cells were removed and the mononuclear cells attached to the osteoblastic cell layer were recovered as pOCs by gentle pipetting with fresh αMEM. pOCs were collected by centrifugation (110×g for 5 min) and 2×10⁵ cells were placed in 48 well culture plates, which were then cultured with M-CSF (50 ng/mL) and sRANKL (50 ng/mL)[5]. OCLs were formed within 48 h of culturing, which then differentiated to osteoclasts after stimulation with sRANKL (100 ng/mL) for 24 h.

MTT assay for the proliferation of OCLs

OCLs were placed in 96-well culture plates and pre-cultured for 4 h. Then, the incubation continued for another 48 h with SY0916 at concentrations ranging from 0.01 to 10 µmol/L. Proliferation of OCLs was determined by MTT assay. MTT was added to each well for 4 h before the addition of DMSO, and the absorbance value of each well was measured at 570 nm.

Determination of the activity and function of osteoclasts

Purified osteoclasts were obtained according to the method presented above. Sterile bone slices (2 cm²) and coverslips were pre-positioned in the plates. Except for the blank control group (without the addition of IL-1β), IL-1β (280 ng/mL) was added into each group. SY0916 was added to experimental groups at concentrations ranging from 0.01 to 10 µmol/L. After culturing for 3 d, the coverslips were dislodged and the number of osteoclasts with more than three nuclei were measured by TRAP staining following the protocol provided by the TRAP staining kit (Sigma)[6]. Each group was divided in three parallel wells and five random fields were counted for each well. The bone slices were stained by toluidine blue as reported previously and the functioning of osteoclasts was determined by measuring the area of bone resorption pits. Images of osteoclasts and bone slices were captured by a digital camera. The osteoclast count and the bone lacuna area were analyzed by Image 5.0 software.

ELISA method for MMP-9 expression assay

Osteoclasts were placed in 48-well culture plates and pre-cultured for 4 h. Except for the blank control group (without the addition of IL-1β), IL-1β (280 ng/mL) was added into each group and SY0916 (from 0.01 to 10 µmol/L) was added to the experimental groups. Subsequently, all groups were incubated for 48 h. Then, the cell supernatants were collected and the level of MMP-9 was measured by an ELISA kit according to the manufacturer's protocol.

Determination of osteoclasts apoptosis by AnnexinV-FITC kit

Osteoclasts were placed in 48 well culture plates and pre-cultured for 4 h. Except for the blank control group (without

the addition of IL-1β), IL-1β (280 ng/mL) was added into each group and SY0916 (from 0.01 to 10 µmol/L) was added to the experimental groups. All groups were incubated for 72 h. The cells were washed twice with cold PBS and resuspended in 1× binding buffer at a concentration of $1×10^6$ cells/mL. Subsequently, 100 µL of cell suspension was transferred to a 5-mL culture tube. Ten µL of Annexin V-FITC and 5 µL of PI were added to the cell suspension[7]. The tube was vortexed gently and incubated for 30 min at room temperature (20−25 °C) in the dark. Then, 400 µL of 1×binding buffer was added into each tube. The cells were immediately analyzed by flow cytometry.

Real-time PCR for *RANK*, *RANKL*, *OPG*, and *MMP-9* gene expression

Except for the blank control group (without the addition of IL-1β), IL-1β (280 ng/mL) was added into each group and SY0916 (from 0.01 to 10 µmol/L) was added to the experimental groups. All groups were incubated for 15 h. To detect the mRNA expression of *RANK* and *MMP-9*, total cellular RNA was extracted from $1×10^5$ osteoclasts. To detect the mRNA expression of *RANKL* and *OPG*, total cellular RNA was extracted from $5×10^6$ MC3T3-E1 cells stimulated with IL-1β. The RNA was extracted with TRIzol according to a previously reported method[8]. DNA synthesis was performed following the protocol of the SYBR® PrimeScript™ RT-PCR kit. Real-time PCR for β-actin, *RANKL*, *OPG*, *RANK,* and *MMP-9* was performed in triplicate following the manufacturer's protocol. The samples were placed into 8 stripe tubes (Axygen). PCR amplifications were performed using the ABI PRISM 7000HT Real-Time PCR system. Thermal cycling conditions were 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 31 s at 60 °C. The primers used for the reaction are shown in Table 1. Gene expression was measured by relative quantitation[9], which compared the threshold cycles (Ct) of the sample of interest to the Ct generated by a reference sample that was referred to as the calibrator (non-stimulated cells that were incubated for the same time period as cells in the experimental groups). Gene expression was normalized to β-actin expression by subtraction of Ct to provide ΔCt values. The ΔΔCt was calculated as the difference

between ΔCt values for IL-1β-stimulated and non-stimulated cells. The relative difference in the expression of the gene of interest between stimulated and unstimulated cells was determined using the equation of $2^{-ΔΔCt}$. This equation was considered valid only if the amplification efficiencies of the gene of interest and β-actin were approximately equal. To confirm the validity of the method, we performed the reaction over a range of DNA template concentrations and the resulting slope of the log of the template DNA concentration plotted against the ΔCt calculated for β-actin and each gene of interest was less than 0.1 (data not shown).

Statistical method for analysis

All values were expressed as the mean±SD of at least three independent experiments. Statistical analysis was carried out using SPSS-13.0 software. One-way ANOVA with *post hoc* analysis by an LSD test or by the Dunnett's C test was applied, which compared treatment groups to a specific control group. A value of *P*<0.05 was considered statistically significant.

Results

The effect of SY0916 on the proliferation of OCLs

The proliferation of OCLs was significantly decreased by SY0916 between 0.01 and 10 µmol/L. The value of OD: 0.40±0.07 (0.01 µmol/L), 0.30±0.08[b] (0.1 µmol/L), 0.39±0.06[b] (1 µmol/L), 0.31±0.09[b] (10 µmol/L), 0.48±0.05 (control), *n*=5, [b]*P*<0.05 *vs* control group.

The effect of SY0916 on the activity and function of osteoclasts

IL-1β significantly increased the number of osteoclasts and bone resorption pits. Typical photos of osteoclasts stained by TRAP and bone slices stained by toluidine are shown in Figure 2. IL-1β also increased the total area and average area of bone resorption pits, which was in accordance with previous reports[10, 11]. SY0916 significantly decreased both the total area and average area of bone resorption pits at concentrations ranging from 0.01 to 10 µmol/L. In addition, SY0916 also inhibited the number of osteoclasts at 0.01–10 µmol/L (Table 2).

The effect of SY0916 on the MMP-9 secretion of osteoclasts

The level of MMP-9 in osteoclasts increased approximately 2-fold after stimulation with IL-1β for 48 h. The increase in MMP-9 was also observed in osteoclasts after stimulation with IL-1β for 24 h, but it was not a significant difference. SY0916 notably inhibited the expression of MMP-9 in osteoclasts stimulated by IL-1β at 0.1–10 µmol/L (Figure 3).

The effect of SY0916 on the apoptosis of osteoclasts

Under normal conditions, osteoclasts extensively undergo apoptosis after five days in culture. However, IL-1β inhibited the apoptosis of osteoclasts and extended their lifespan. SY0916 significantly blocked the effect of IL-1β at 0.01−10 µmol/L (Figure 4).

Table 1. Primer sequences used in real-time RT-PCR.

| Gene		Primers	Product (bp)
RANK	sense	5′-ATG GTG GGC TAC CCA GGT GA-3′	150
	anti-sense	5′-ACT TGC GGC TGC ACA GTG A-3′	
MMP-9	sense	5′-GGT GTA GCA CAA CAG CTG ACT ACG A-3′	130
	anti-sense	5′-GAG CGG CCC TCA AAG ATG AA-3′	
β-actin	sense	5′-CAT CCG TAA AGA CCT CTA TGC CAA C-3′	171
	anti-sense	5′-ATG GAG CCA CCG ATC CAC A-3′	
RANKL	sense	5′-CAT GTG CCA CTG AGA ACC TTG AA-3′	107
	anti-sense	5′-CAG GTC CCA GCG CAA TGT AAC-3′	
OPG	sense	5′-CAA TGG CTG GCT TGG TTT CAT AG-3′	118
	anti-sense	5′-CTG AAC CAG ACA TGA CAG CTG GA-3′	
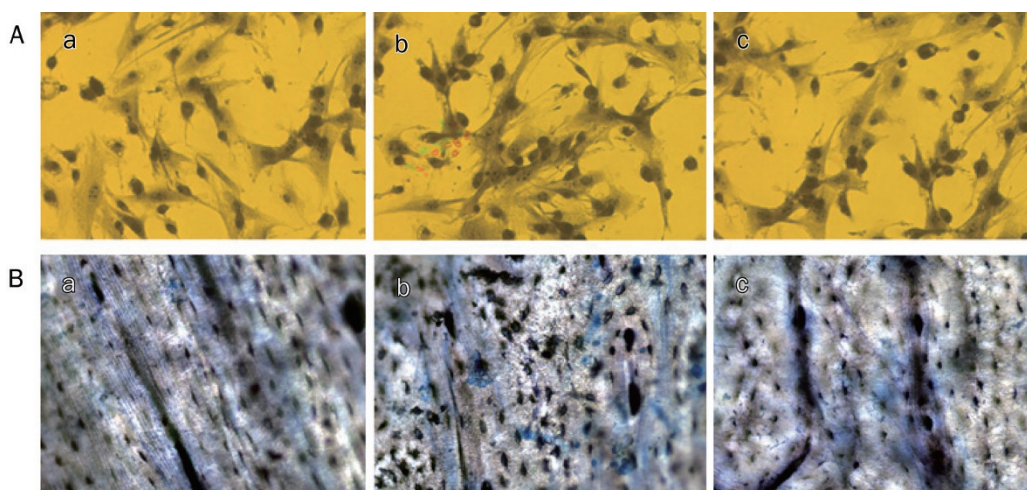
Figure 2. The photographs of osteoclasts stained by TRAP and bone resorption pits stained by toluidine blue. Sterile bone slices (2-cm^2) and coverslips were pre-positioned in the plates. Except for the blank control group (without the stimulator of IL-1β), IL-1β (280 ng/mL) was added into each group and SY0916 at 0.01−10 μmol/L were added into the experimental groups. After being cultured for 3 d, osteoclasts were measured by TRAP staining. The bone slices were stained by toluidine blue. Images of osteoclasts and bone slices were captured by the digital camera of the microscope. (A) The typical photographs of the osteoclasts (TRAP stain, ×400). (B) The typical photographs of bone slices (toluidine blue stain, ×400). a: blank control group; b: IL-1β stimulated group; c: SY0916 10 μmol/L group.

Table 2. Effect of SY0916 on the activity and function of osteoclasts. Except for the blank control group (without the stimulator of IL-1β), IL-1β (280 ng/mL) was added into each group and SY0916 at 0.01–10 μmol/L were added into the experimental groups. After culturing for 3 d, the coverslips were dislodged and the number of osteoclasts with more than three nuclei was measured. The function of osteoclasts was determined by measuring the number and area of bone resorption pits. Images of osteoclasts and bone slices were captured on the digital camera and analyzed by Image 5.0 software. Values were expressed as mean±SD. *n*=5. [b]*P*<0.05 *vs* blank control group. [e]*P*<0.05 *vs* IL-1β group.

| Group	The number of osteoclasts (counts/field)	Bone reporption pits (counts/field)	Average area of pits (area/pit)	Total area of pits (area/field)
Control	13±2	11±2	1798±773	19906±2496
IL-1β	22±3[b]	56±1[b]	3679±928[b]	95427±12640[b]
SY0916 0.01 μmol/L	11±2[e]	34±10[e]	1950±1071[e]	57409±10301[e]
SY0916 0.1 μmol/L	8±0[e]	32±13[e]	1675±127[e]	48318±3020[e]
SY0916 1 μmol/L	8±1[e]	22±6[e]	1596±145[e]	44295±17995[e]
SY0916 10 μmol/L	8±1[e]	19±8[e]	1464±149[e]	33644±7221[e]
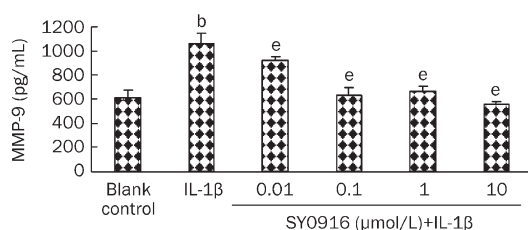
Figure 3. Effect of SY0916 on the secretion of MMP-9 in osteoclasts. Osteoclasts were placed on the 48-well culture plates and pre-cultured for 4 h. Except for the blank control group (without the stimulator of IL-1β), IL-1β (280 ng/mL) was added into each group and SY0916 at 0.01−10 μmol/L were added int o the experimental groups. Then the incubation continued for another 48 h. The cell supernatants were collected and the level of MMP-9 was measured by ELISA kit according to the manufacturer's protocol. Values were expressed as mean±SD. *n*=3. [b]*P*<0.05 *vs* blank control group. [e]*P*<0.05 *vs* IL-1β group.

The effect of SY0916 on the gene expression of *RANK*, *RANKL*, *OPG*, and *MMP-9*

IL-1β significantly increased the mRNA expression of *RANK*, *RANKL*, *OPG*, and *MMP-9*. IL-1β stimulation increased the mRNA expression of *RANKL* and *OPG* by approximately 6-fold and 2-fold, respectively, which caused the ratio of *RANKL* to *OPG* to significantly increase in MC3T3-E1 cells. SY0916 between 0.01 and 10 μmol/L significantly inhibited the mRNA expression of *MMP-9* and *RANK* in osteoclasts. In addition, SY0916 inhibited the expression of *RANKL* and *OPG* in MC3T3-E1 cells, whereas the inhibition of the *OPG* gene was weaker. As a result, the ratio of *RANKL* to *OPG* in MC3T3-E1 cells was significantly decreased (Figure 5A−5B).

Discussion

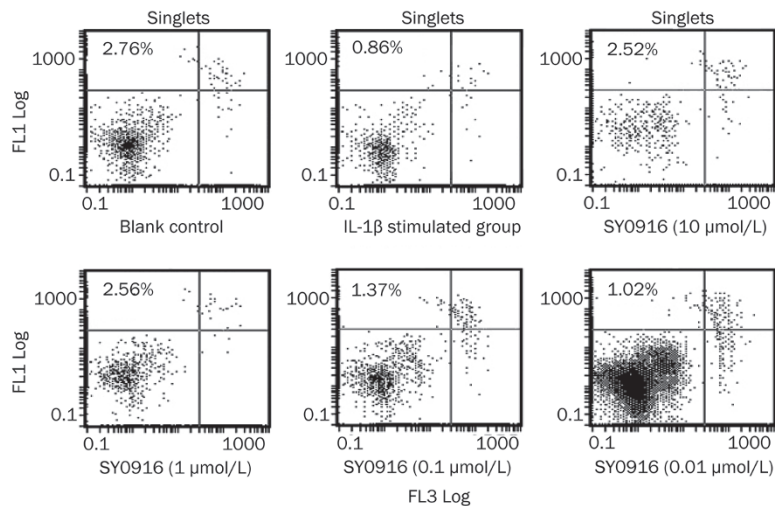SY0916 was developed as a novel type of anti-inflammatory

Figure 4. Effect of SY0916 on the apoptosis of osteoclasts. Except for the blank control group (without the stimulator of IL-1β), IL-1β (280 ng/mL) was added into each group and SY0916 at 0.01–10 μmol/L were added into the experimental groups. After being cultured, the cells were resuspend in 1×Binding Buffer at a concentration of $1×10^6$ cells/mL. 100 μL ($1×10^5$ cells) was transfered into a 5 mL culture tube, then added 10 μL of Annexin V-FITC and 5 μL of PI. The tubes were gently vortexed and incubated for 30 min at room temperature in dark. Four hundred μL of 1×Binding Buffer was added into each tube. All samples were analyzed on a FACS flow cytometry (EPICS XLHP). Apoptotic cells, viable cells and necrotic cells were detected as An^+/PI^- cells, An^-/PI^- cells and PI^+ cells, respectively. The An^+/PI^- cells represent the annexin-V positive and propidium iodide negative cells, that is, the apoptosis cells. The experiment was repeated three times and the typical result was presented.
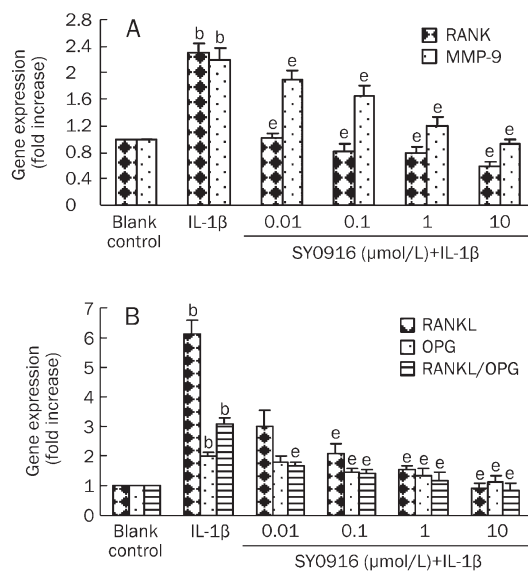

Figure 5. Effect of SY0916 on gene expression of *RANKL* and *OPG* in MC3T3-E1 cells and gene expression of *RANK* and *MMP-9* in osteoclasts. SYBR® PrimeScript™ RT-PCR kit was applied for cDNA synthesis and real-time PCR for β-actin, *RANKL*, *OPG*, *RANK*, *MMP-9*. The detection was performed in triplicate following the protocol of the kit. Gene expression was measured by relative quantitation. The relative difference in the genes of interest expression between stimulated and unstimulated cells was determined using the equation of $2^{-\Delta\Delta Ct}$. Results were expressed as fold increase in gene expression by IL-1β stimulated cells compared to unstimulated (media alone) cells. The y-axis showed the means of $2^{-\Delta\Delta Ct}$ of each group, which reflected the fold increase in the genes of interest expression. The x-axis showed the treatment given to each group. (A) Effect of SY0916 on the gene expression of *RANK* and *MMP-9* in osteoclasts; (B) Effect of SY0916 on the gene expression of *RANKL* and *OPG* in MC3T3-E1 cells. $^bP<0.05$ *vs* blank control, $^eP<0.05$ *vs* IL-1β.

drug. In a previous pharmacodynamic study, Wang *et al* found that SY0916 treatment had a beneficial effect in CIA rats, especially in preventing bone erosion in this model[4]. However, little was known about the mechanism underlying the compound's effect on bone protection.

Osteoclasts, the only somatic cells with bone-resorbing capacity, play a critical role in the pathogenesis of bone destructive disorders such as rheumatoid arthritis and osteoporosis[12]. Therefore, osteoclasts were chosen as the study targets for this research. The present study showed that SY0916 concentrations from 0.1 to 10 μmol/L could significantly inhibit the proliferative and functional responses of osteoclasts stimulated by IL-1β. Furthermore, Wang *et al* also observed the effect of SY0916 on the apoptosis of osteoclasts. The result showed that SY0916 between 0.01 and 10 μmol/L significantly decreased the lifespan of osteoclasts after treatment with IL-1β. Therefore, the inhibitory effect of SY0916 on osteoclasts may be a critical pathway associated with its prevention of bone destruction.

The system constituted by RANKL, OPG, and RANK plays an important role in bone metabolism. RANKL and OPG are expressed by osteoblasts, whereas RANK is expressed by osteoclasts. RANKL and RANK affect bone metabolism by stimulating osteoclast differentiation, function and survival. In contrast, OPG is the decoy receptor for RANKL. OPG inhibits RANKL function by competing with RANK for RANKL[13, 14]. In the case of increased levels of RANKL, patients present with symptoms characteristic of abnormal bone resorption, which appear similar to osteoporosis[15]. In the case of increased OPG levels, patients present with symptoms of hyperosteogeny or osteosclerosis. SY0916 directly inhibit the function of osteoclasts. Meanwhile, we further investigated the influence of SY0916 on gene expression of *OPG*, *RANK,* and *RANKL.* These results showed that SY0916 significantly inhibited IL-1β-mediated increases in mRNA expression of *RANKL* and *RANK* in osteoblasts and osteoclasts, respectively. Noticeably, we found that the ratio of RANKL to OPG was significantly decreased, although *OPG* mRNA expression in osteoblasts was also decreased by SY0916. Therefore, we concluded that the mechanism underlying the effects of SY0916 on osteoclastic bone destruction involved regulation of the balance between RANKL and OPG, and the inhibition of RANK.

It is well known that matrix metalloproteinase 9 (MMP-9) is responsible for bone matrix degradation and is highly expressed both at early stages of osteoclast development and in mature osteoclasts. Moreover, many researchers have reported that RANKL stimulated MMP-9 gene expression in osteoclasts through TRAF6[16]. In the present study, we found that SY0916 could inhibit the expression of MMP-9 induced by IL-1β at the level of both RNA and protein. The inhibitory effect of SY0916 on MMP-9 expression was in line with that of SY0916's effect on RANKL expression.

In conclusion, our study indicated that SY0916 could inhibit the inflammatory diseases associated with bone destruction by restoring the normal state of osteoclasts, which included the generation, activation, functioning and apoptosis of osteoclasts. In addition, the regulation of the RANKL-OPG-RANK system was also involved in this process. The regulation of a RANKL associated signal transduction network influenced protein expression that was associated with bone destruction, which included increases in MMP-9. In summary, this study is an important contribution to elucidating the mechanism underlying the effect of SY0916 on bone protection.

Acknowledgements

Author contribution

Wen-jie WANG and Lin WANG designed the research; Lin WANG, Shan-ying PENG, Ping LI, and Yang LIU performed the research; Lin WANG, Shan-ying PENG, and Ping LI analyzed the data; Lin WANG and Wen-jie WANG wrote the paper.

Abbreviations

RA, rheumatoid arthritis; CIA, collagen-induced arthritis; PAF, platelet-activating factor; pOC, pre-osteoclast; OCL, osteoclast-like cell; RANKL, receptor activator of NF-KB ligand; RANK, receptor activator of NF-KB; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase; MMP-9, matrix metalloproteinases 9.

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