LY900009 Regulates RANKL-Induced Osteoclast Formation and LPS-Induced Bone Resorption

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Abstract

To investigate the suppressive function of LY900009, a potent-secretase inhibitor, on RANKL-induced osteoclastogenesis. The cytotoxicity of LY900009 was evaluated. The suppressive effect and possible molecular mechanism of LY900009 on RANKL-induced osteoclastogenesis was evaluated both in vitro and in vivo. The IC₅₀ of LY900009 was 2.93 mM. LY900009 treatment at different doses (100 nM, 200 nM, and 400 nM) effectively reduced osteoclast formation (number and area) in a dose-dependent manner. The qPCR result shows that LY900009 attenuates RANKL-induced osteoclast formation and NFATc1 protein expression. The in vivo experiments demonstrated the inhibitory effect of LY900009 on LPS-induced bone resorption. LY900009 could potentially inhibit osteoclastogenesis and bone resorption by down-regulating Notch/MAPK/Akt - mediated NFATc1 reduction in vitro. In accordance with the in vitro observations, we confirmed that LY900009 attenuated LPS-induced osteolysis in mice. In conclusion, our findings indicate that Notch was a potential therapeutic target which could be used for osteolytic diseases treatment.

1. Introduction

Bone metabolism is a dynamic process that involves the simultaneous processes of bone resorption and formation^{1; 2}. Thus, osteoblast and osteoclast disequilibrium can undermine bone integrity and normal functionality, promoting a series of pathological lytic bone disorders³⁻⁵. Thus, recent research has centered on the investigation and identification of novel agents (synthetic or natural) which may inhibit the pathological formation of osteoclasts and bone resorption^{6; 7}.

Osteoclasts (OCs) are a type of multinucleated giant cell derived from macrophage precursors. OC differentiation is the first stage of the bone resorptive process, which requires stimulation of receptor activator of NF- κ B ligand (RANKL) and macrophage colony stimulating factor (M-CSF). Previous studies have shown that RANKL plays an essential role in OC multinucleated formation^{8; 9}. After the interaction between RANKL and receptor activator of NF- κ B (RANK), various downstream signaling pathways (e.g., MAPKs and PI3K/Akt) are activated. These specific pathways are able to upregulate the key regulator of the osteoclast process: nuclear factor of activated T cells c1 (NFATc1)¹⁰⁻¹³.

In previous studies, Notch signaling has been shown to play an essential role in multiple developmental pathways¹⁴. The interaction between Notch receptors and Delta-like1/3/4 (Dlk 1/3/4) protein causes the release of tumor necrosis factor α -converting enzyme (TACE), and γ -secretase¹⁵. Former research confirmed the inhibition effect of γ -secretase down-regulation on Notch receptor cleavage¹⁶. Both enhance and prohibitive effect of Notch on RANKL-induced bone resorption was reported in relative research¹⁷, suggesting that it may represent a promising targetable pathway for the development of anti-resorptive agents^{18; 19}. Moreover, LY900009, a γ -secretase inhibitor suppressing Notch cleavage, has not yet been confirmed its ability and possible signaling in regulation of osteoclast formation.

In this research, we assessed effect of LY900009 in suppressing RANKL-induced osteoclast formation and elucidated the potential molecular mechanisms associated with this process. We confirmed that LY900009 suppressed osteoclastogenesis and bone resorption by blocking the Notch/Hes1/MAPK(Erk and p38)/Akt pathway. This conclusion was also confirmed by the in vivo results.

2. Materials And Methods

2.1. *BMMs and Culture System*

BMMs were obtained and cultured according to former relative research²⁰⁻²². Primary BMMs were isolated from mice (five-week-old C57/BL6 male) femurs and tibiae. All process were under the supervision of Shanghai General Hospital Animal Center Committee of Animal Care and Use (2018371A218). All procedures were performed under the guidelines of the Ethical Conduct in the Care and Use of Nonhuman Animals in Research. To select primary BMMs, Cell were then cultured in complete α -MEM (10% Gibco FBS) with 33.3 ng/mL M-CSF (R&D Systems, Inc.).

2.2. *Cell Cytotoxicity Assay*

Cytotoxicity of LY900009 (Selleck, Inc.) was determined by MTT method. The BMMs were seeded into 96-well plates(1×10^4 cells per well) in complete medium with different concentrations of LY900009 (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 μ M). Following LY900009 treatment, the cells were incubated for different timepoint(24,48,96h). At the end of the process, CCK-8 solution (Dojindo Molecular Technologies, Inc) was used according to specification.

2.3. *Osteoclast Formation and TRAP Staining Assay*

The function of LY900009 on osteoclast formation was assessed. The BMMs were seeded into 96-well plates (1×10^4 cells per well) and stimulated by RANKL (100 ng/mL, R&D Systems, Inc.), M-CSF (33.3 ng/mL) and different concentrations of LY900009 (0, 100, 200, and 400 nM). The culture medium was replaced every 48 h for 6 days. After the osteoclast was observed in control group, 4% paraformaldehyde was used for OC fixation. TRAP (Sigma-aldrich; Merck, Inc.) staining solution was added into each of the wells at 37°C for 1 h. TRAP⁺ cells with at least three nuclei were considered to be osteoclasts. Image J software (National Institutes of Health) was used for cell enumeration.

2.4. *Bone Resorption Assay*

M-CSF-dependent BMMs were incubated in six-well plates (20×10^5 cells/well). After 24 h, the cells were supplied with stimulated by RANKL (100 ng/mL), M-CSF (33.3 ng/mL) until OC formation was observed. The OCs (1×10^4 cells/well) were then incubated on Corning Osteo Assay Surface plates (Corning, Inc.) and cultured with M-CSF (33.3 ng/mL), RANKL (100 ng/mL)and LY900009 (0, 100, 200, and 400 nM) for four days. A BioTek Cytation 3 Cell Imaging Reader (BioTek, Winooski, VT) and Image J software were used to photograph and analyze the total resorption pits.

2.5 Immunofluorescence analysis of the podosomal actin belt

OCs derived from BMMs were generated and processed. On days 5 - 7, pancake-like osteoclasts were observed in the RANKL-treated control group, fixed with 0.1% Triton X-100 (Sigma-Aldrich; Merck, Inc.), and permeabilized for 5 min. After blocking with 1% BSA-PBS for 1 h, the cytoskeletal actin structure was stained with rhodamine-conjugated phalloidin. The immunofluorescence images were obtained by using BioTek Cytation 3 Cell Imaging Reader. Image J software was used to analyze the size (diffusion area) and number of the dental actin bands.

2.6. Real-time PCR Analysis

Cell total RNA (2×10⁶ cells) was extracted using the TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) was used to assess concentration of the total RNA. The following process was performed according to former research.¹⁶ The following mouse primer sets were used: mouse NFATc1: forward, 5'-TGCTCCTCCTCCTGCTGCTC-3' and reverse, 5'-GCAGAAGGTGGAGGTGCAGC-3'; mouse C-fos: forward, 5'-CCAGTCAAGAGCATCAGCAA-3' and reverse, 5'-AAGTAGTGCAGCCCGGAGTA-3'; mouse Cath-K: forward, 5'-CTTCCAATACGTGCAGCAGA-3' and reverse, 5'-TCTTCAGGGCTTTCTCGTTC-3'; mouse Trap: forward, 5'-CAAAGAGATCGCCAGAACCG-3' and reverse, 5'-GAGACGTTGCCAAGGTGATC-3'; mouse GAPDH: forward, 5'-CACCATGGGAGAAGGCCGGGG-3' and reverse, 3'-GACGGACACATTGGGGGTAG-5'.

2.7. Western Blotting Analysis

The total cellular proteins (TCPs) were extracted at different time points. Following treatment with 400 nM LY900009 for 2 h, RANKL (100 ng/mL) was used to stimulate the cells for different periods of time (short time course). The membrane was blocked in 1% TBST (tri-buffered saline, Tween 20) in 5% skim milk at room temperature for 1 h, and subsequently reacted with primary antibodies NFATc1(cat no. 5861S; 1:1,000; Cell Signaling Technology, Inc.) ; Cleaved-Notch1(cat no. 4147S; 1:1,000; Cell Signaling Technology, Inc.) ; HES1(cat no.11988; 1:1,000; Cell Signaling Technology, Inc.) GAPDH(cat no.174S; 1:1,000; Cell Signaling Technology, Inc.); p-Akt (cat no.4060S; 1:1,000; Cell Signaling Technology, Inc.); Akt (cat no.4685S; 1:1,000; Cell Signaling Technology, Inc.); p-ERK (cat no.4370S; 1:1,000; Cell Signaling Technology, Inc.); Erk (cat no.9194S; 1:1,000; Cell Signaling Technology, Inc.) ; p65 (cat no.8242S; 1:1,000; Cell Signaling Technology, Inc.); p-p38 (cat no.4511; 1:1,000; Cell Signaling Technology, Inc.); p38 (cat no.8690; 1:1,000; Cell Signaling Technology, Inc.); p-p65 (cat no.3033; 1:1,000; Cell Signaling Technology, Inc.). After incubating the samples overnight at 4°C, the secondary antibody was incubated at room temperature for 1 h using Odyssey V3 .0 image scan (Li-COR. Inc., Lincoln, NE, USA) to observe antibody reactivity.

2.8. Mouse Model of LPS-Induced Calvarial Bone resorption

All process were was carried out in compliance with the ARRIVE guidelines and under the supervision of Shanghai General Hospital Animal Center Committee of Animal Care and Use. All procedures were performed under the guidelines of the Ethical Conduct in the Care and Use of Nonhuman Animals in Research. C57BL/6 male mice, aged 7 to 8 weeks, were randomly assigned to four groups: LPS (control), and LPS with low(400nM) or high(800nM) concentrations of LY900009. 1% pentobarbital sodium (10 mg/kg) was intraperitoneally injected to anaesthetize the mice. Mice were injected with 100 µl LPS(10 mg/kg), and with or without LY900009, respectively, for two weeks. During the experiment period, no significant adverse effects and toxic effects were observed. The mice showed no significant weight loss and lack of spirit, and no mice died during the experiment period. After the operation, euthanasia was performed using pentobarbital sodium(100 mg/kg)²³. The knee joints were resected and fixed in 4% paraformaldehyde for 48h. After that, the specimens were prepared for micro-CT (Skyscan 1072; Skyscan, Inc.) and histological analyses.

2.9. Histological Staining and Histomorphometric Analysis

The 10% EDTA was used to decalcified PFA-fixed mice knees for two weeks and embedded in paraffin. Next, the samples were sliced into specific sections(4µm thickness) and subjected to H&E and TRAP staining. Digital images were obtained using Axio ScopeA1 light microscope (ZEISS, Inc.). The osteoclasts amount were calculated by Image J software.

2.11. Statistical Analysis

All result were shown as the mean ± standard deviation (SD).The Student's *t*-test was used to assess the differences between control and therapeutic group. The results for the multiple group comparisons were analyzed using a Scheffe's test and one-way analysis of variance (ANOVA) with SPSS 22.0 software (SPSS, Inc.). Values were determined to be significant at **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

3. Results

3.1. RANKL-Induced Osteoclast formation in vitro was down-regulated by LY900009

Cytotoxicity of LY900009 was firstly evaluated. The results showed that the IC₅₀ of LY900009 in BMMs at 24 h, 48 h, and 96 h was 4.44 mM, 3.66 mM, and 2.93 mM respectively (Figure 1A and B). Figure 1C was the chemical structure formula of LY900009 (provided by Selleck). Compared with control group, treatment of LY900009 effectively reduced the number and area of OCs in a dose-dependent manner (Figure 1D, E and F). Accordingly, LY900009 could inhibit osteoclast formation in a specific concentration under IC₅₀.

3.2. Podosome actin belt formation and OC-mediated bone resorption activity was inhibited by LY900009

In order to assess the effect of LY900009 on OC-mediated bone resorption activity, resorption area on the hydroxyapatite-coated Osteo Assay plates was calculated (Figure 2A). In accrodance with the osteoclast

reduction, the bone resorption area also reduced in a dose-dependent manner (Figure 2B). The immunofluorescence results showed that the podosome actin belt formation was suppressed LY900009 in a dose-dependent manner (Figure 2C). In summary, these part of data shows that LY900009 could effectively induce podosome actin belt formation(Figure 2D).

3.3. Osteoclastogenesis Relative Gene Expression was Depressed by LY900009

Quantitative PCR(qPCR) was used to evaluate the relative expression of osteoclastogenesis genes at the mRNA level. It was observed that relative Gene, including NFATc1, C-fos, Cath-K, and TRAP, was markedly downregulated by LY900009 in a dose-dependent and time-dependent manner. (Figure 3 A and B)

3.4. LY900009 mediate Osteoclastogenesis by inhibiting Notch, MAPK and Akt signalings in BMMs

To investigate the potential molecular mechanisms of LY900009 in osteoclastogenesis inhibition, the RANKL-induced NF- κ B, MAPK, and PI3K/Akt pathways were examined using a Western blot analysis. BMM treatment with LY900009 during RANKL-induced osteoclast formation induced cleaved - Notch1, Hes1 and NFATc1 protein expression (Figure 4 A and B). In addition, short-term stimulation of LY900009 significantly reduced the phosphorylation of p38, ERK1/2, p65, and Akt (Figure 4 C). Accordingly, our experiment suggests that LY900009 blocked osteoclastogenesis inhibited osteoclastogenesis by preventing Notch receptor cleavage and inhibiting Notch signaling, and perturbing the activation of ERK1/2, p38 and AKT signaling cascades.

3.5. LY900009 Administration Prevents LPS-Induced Bone Resorption in vivo

The in vitro experiments were used to investigate the effect of LY900009 on RANKL-induced osteoclastogenesis and bone resorption function by studying the phenotype and associated mechanism. We investigated the function of LY900009 in mice with LPS-induced bone resorption. According to the micro-CT three-dimensional reconstruction shown in Figure 5A, compared with control group, LY900009-treated groups display fewer and smaller resorption pits. A significant reduction in BV/TV ,Tb.n and BMD was also detected in LPS group (Figure 5B).

The histological analysis also reveal the inhibition of LY900009 on LPS-induced bone resorption in vivo. In accordance with former result, extensive osteolysis was observed in the HE-stained images of the control group, whereas a lower osteolytic level was observed in the LY900009-treated groups (Figure 6A). The TRAP staining results confirmed a decreased amount of OCs under LY900009 treatment (Figure 6B and 6C). Accordingly, the in vivo results confirmed the potential therapeutic application of LY900009 in inflammation-relative osteolytic disease.

4. Discussion

A pathological bone homeostasis promotes the over-formation and activation of OCs, leading to massive bone disruption, which is a feature of several osteolytic diseases³⁻⁵. Medical therapies that suppress

osteolysis are considered to be a potential treatment strategy for these diseases^{24; 25}. However, a great number of these treatment have various side-effects^{26; 27}. Accordingly, the development of medications that can inhibit osteoclastogenesis without side-effects are urgently required.

Our research reveals some potential signaling which may regulate RANKL-induced osteoclast formation. Notch signaling is important in many function, such as morphogenesis and stem cell niche maintenance²⁸⁻³⁰. However, the function of Notch signalling on osteoclastogenesis is no clear, with evidence supporting both a stimulatory and inhibitory role. According to former research, Notch has been shown to work in conjunction with MAPK, NF- κ B, and PI3K/Akt to possibly regulate NFATc1 expression during osteoclast differentiation^{18; 31-34}. In addition, suppression of Notch1 in BMMs has been confirmed to promote osteoclastogenesis in vitro³⁵, whereas suppression of Notch2 attenuates osteoclastogenesis^{17, 36}.

Former research has confirmed the synergistic function between Notch and NF- κ B signaling³⁷. According to our research, NFATc1 expression was significantly decreased under the LY900009 treatment. LY900009, a potent γ -secretase inhibitor, doesn't change expression but blocked cleavage of Notch. Accordingly, expression of cleaved notch1 (Notch intracellular domain-1; NICD1) and hairy enhancer of split 1 (HES1), a targeted gene regulated by cleaved-Notch, were evaluated in long-term stimulated BMMs. In accordance with previous studies³⁸, we also observed a reduction of ERK, p38, Akt, and p65 phosphorylation under the treatment of LY900009. Herein, there is a significant crosstalk between Notch, ERK, and Akt signaling. Our data do suggest that the LY900009 can suppress bone loss and osteoclast formation by means of Notch mediated NFATc1 expression.

In this study, a widely-accepted LPS-induced osteolytic experimental model was established to assess the effect of LY900009. During the design of this research, Ovx, RANKL-induced bone resorption and LPS-induced bone resorption model were taken into consideration. Ovx model is inappropriate to be used in this research, because Ovx regulates not only osteoclast but also osteoblast formation. The reason of using RANKL in vitro is that RANKL is mainly produced by osteoblast and necessary for the formation of osteoclast. However, there is already has osteoblast in vivo. LPS-induced bone resorption, as a general approach, has been widely used for osteoclast relative in vivo study.³⁹⁻⁴³ Accordingly, LPS-induced bone resorption was chosen as the in vivo model. Not only the micro-CT analysis but also the histological staining of TRAP confirmed a downregulated osteopenia in the LY900009-treated groups. However, there are still a series of limitations in present research. Our research didn't discuss the possibility of LY900009 regulating osteoclast via other signaling pathways. Secondly, the influence of LY900009 on osteoblast and bone formation should be further assessed.

In conclusion, our data demonstrate that LY900009 can effectively attenuate RANKL-induced osteoclastogenesis in vitro via downregulating Notch/MAPK (ERK and p38)/Akt signaling. In accordance with the in vitro observations, prevention of LPS-induced osteolysis in vivo also confirmed the possibility

of using LY900009 as a potential treatment strategy for osteolytic diseases in relation to excessive osteoclast-mediated bone resorption.

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are available in an open access repository

Authors' contributions

Donghua Hang responsible for the analysis and interpretation of the data. Lei Wang and Yuan Zhou recorded the data . Yi Zhao built the 3-D model together. Congyunn Zhao collected samples. Tao Huang is one of the first authors responsible for conception and Critical revision of the article for important intellectual content. All the authors read and approved the manuscript.

Ethics approval and consent to participate

The study design was carried out in compliance with the ARRIVE guidelines and approved by the Ethical Committee of Shanghai General Hospital .

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Figures

Figure 1

RANKL-Induced Osteoclast formation in vitro was down-regulated by LY900009 (A) The cytotoxicity of LY900009. (B) The results showed that the IC₅₀ of LY900009 at different timepoint in the BMMs was 4.44 mM, 3.66 mM, and 2.93 mM, respectively. (C) The chemical structural formula of LY900009 (provided by Selleck). (D) BMMs were simulated with different concentration LY900009, M-CSF(33.3 ng/mL) and RANKL(100 ng/mL) for five days. Cells were fixed in 4% paraformaldehyde and stained for TRAP. (E and F) The area and number of TRAP-positive cells. The data were presented as the mean $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 2

Podosome actin belt formation and OC-mediated bone resorption activity was inhibited by LY900009. (A) OCL were seeded onto Osteo Assay Stripwell Plates with RANKL (100 ng/mL), M-CSF (33.3 ng/mL), and LY900009 (0,100, 200, and 400 nM) for four days (B) The resorption area in Osteo Assay Stripwell Plates. (C) BMMs were planted onto 48-well plates with 0, 100, 200, and 400 nM LY900009 for five days. The cells were fixed and stained for immunofluorescence. (D) Area of OCs with an F-actin belt. The data were presented as the mean $\pm$ SD.

Figure 3

Osteoclastogenesis Relative Gene Expression was Depressed by LY900009. (A) BMMs were treated with M-CSF (33.3 ng/mL), RANKL (100 ng/mL) in the presence of 0, 100, 200, and 400 nM LY900009 for five days. The expression of the osteoclast-specific genes, including NFATc1, Cath-K, TRAP, and C-fos were analyzed using quantitative real-time PCR. (B) The BMMs were treated with M-CSF (33.3 ng/mL) and RANKL (100 ng/mL) in the presence 400 nM LY900009 for 1, 3, and 5 days. Osteoclast-specific gene expression was analyzed using quantitative real-time PCR. RNA expression levels were normalized to the expression of GAPDH. The data were presented as the mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 4

LY900009 suppresses osteoclastogenesis via Notch inhibition and AKT phosphorylation regulation. (A) LY900009 treatment suppressed the expression of cleaved - Notch1. (B) LY900009 treatment suppressed the expression of Hes1 and NFATc1. (C) LY900009 treatment suppressed the expression of AKT phosphorylation. The data were presented as the mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 5

LY900009 attenuated lipopolysaccharide-induced knees bone resorption in vivo. (A) Images of three dimension reconstruction based on micro-CT scanning. (B) Result of BMD, bone BV/TV and Tb.n.

Figure 6

(A,B) HE and TRAP results (C) Amount of TRAP-positive OCs. The data are presented as the mean $\pm$ SD (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).