Mevalonates restore zoledronic acid-induced osteoclastogenesis inhibition

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Abstract

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is likely to be caused by continuous imperfection of bone healing after surgical treatments in patients with long-term administration of nitrogen-containing bisphosphonates (NBPs). NBPs inhibit osteoclastic bone resorption by impairing the mevalonic acid sterol pathway in osteoclasts. Thus, we hypothesized that exogenous mevalonic acid metabolites restore the inhibitory effects of NBPs on osteoclastogenesis and bone remodeling. To clarify the effects of mevalonic acid metabolites, especially geranylgeranyl pyrophosphate (GGPP) and geranylgeranyl transferase substrate geranylgeranyl acid (GGOH), we examined that the effects of zoledronic acid with or without GGOH or GGPP on osteoclast differentiation, multinucleation, and bone mineral deposition in tooth-extracted sockets. Zoledronic acid decreased the number of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells derived from mouse osteoclast precursors treated with receptor activator of nuclear factor of kappa B ligand and macrophage colony-stimulating factor. Zoledronic acid simultaneously suppressed not only the expressions of osteoclastic differentiation-related molecules such as TRAP, cathepsin K, calcitonin receptor and vacuolar H-ATPase, but also those of multinucleation-related molecules such as dendrocyte expressed seven transmembrane and osteoclast stimulatory transmembrane protein. Treatment with GGOH or GGPP, but not farnesyl acid, restored the zoledronic acid-inhibited number of TRAP-positive multinuclear cells together with the expressions of these molecules. Although intraperitoneal administration of zoledronic acid and lipopolysaccharide into mice appeared to induce BRONJ-like lesions with empty bone lacunae and decreased mineral deposition in tooth-extracted socket, both GGOH and GGPP partially restored the inhibitory effects on zoledronic acid-related mineral deposition. These results suggest the potential of mevalonic acid metabolites as therapeutic agents for BRONJ.

Introduction

Osteoclasts are derived from hematopoietic precursors of a monocyte/macrophage lineage and differentiate into multinucleated cells through interactions with the surface of osteoblastic cells (Suda *et al*., 1999; Teitelbaum *et al*., 2003). Receptor activator of nuclear factor of kappa B (NF-κB) -ligand (RANKL), identified as a member of the TNF superfamily, acts as a crucial regulator of osteoclast differentiation, function, and survival (Anderson *et al*., 1997; Li *et al*., 2000). When RANK binds to RANKL, osteoclast differentiation is induced via activation of transcription factors such as nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), NF-κB, and c-fos, resulting in the expressions of osteoclast differentiation- and function-related molecules, such as tartrate-resistant acid phosphatase (TRAP), cathepsinK, calcitonin receptor, and vacuolar H-ATPase (V-ATPase) (Takayanagi *et al*., 2002).

Nitrogen-containing bisphosphonates (NBPs) are frequently used as effective pharmacological agents for the treatment of bone diseases, including osteoporosis, hypercalcemia, metastatic bone disease, and Paget's disease (Coxon *et al*., 2000; Benford *et al*., 2001). NBPs have been shown to directly inhibit bone resorption and promote the apoptosis of mature osteoclasts (Hughes *et al*., 1995; Parfitt *et al*., 1996; Jilka *et al*., 1998). The main pharmacological mechanism of NBPs is the inhibition of an enzyme in mevalonic acid metabolism, via inactivation of farnesyl diphosphate synthase (FDPS) (Zhang and Casey, 1996). This leads to decreased formation of mevalonic acid metabolites such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate, and reduced protein prenylation (Zhang and Casey, 1996). Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is one of the main side effects in patients treated with NBPs. Although several possible pathological mechanisms for BRONJ have been discussed (Allegra *et al*., 2010; Landesberg *et al*., 2011; Ziebart *et al*., 2011), the mechanism of BRONJ remains unclear. Furthermore, little is known about how to inhibit the onset of BRONJ in patients with long-term administration of NBPs. NBPs were reported to inhibit the proliferation and survival of oral

epithelial cells, osteoblasts, and fibroblasts (Reszka *et al*., 2001; Kim *et al*., 2011; Ziebart *et al*., 2011). Denosumab is a novel and clinically effective agent that inhibits osteoclast differentiation, survival, and function by binding to RANKL and inhibiting its interaction with RANK on osteoclasts (Baron, 2011). It was recently reported that denosumab may also be associated with osteonecrosis of the jaw (ONJ) (Stopeck *et al*., 2010; Fusoco *et al*., 2011; Henry *et al*., 2011). These results suggest that ONJ induced by NBPs or denosumab might be caused by decreases in osteoclast differentiation, survival, and bone turnover ability.

Recently, animal tooth extraction models have been able to replicate BRONJ-like lesions, in rodents (Bi *et al*., 2010; Kikuiri *et al*., 2010; Aguirre *et al*., 2012). The BRONJ were observed with empty bone lacunae, increase in periosteal thickness, decrease angiogenesis and infiltration of inflammatory cells. Among them, the symptom in BRONJ frequently observed some empty of bone lacunae with osteocytic apoptosis in rodnts (Kikuiri *et al*., 2010; Aguirre *et al*., 2012). BRONJ mouse model used long-term high-dose NBP treatment in combination with experimentally-induced periapical disease, such as inflammation induced by lipopolysaccharide (LPS) in the tissues surrounding the tooth, thus emphasizing the importance of dental disease in BRONJ pathology (Kim *et al*., 2013).

Geranylgeranyl acid (GGOH) is a natural molecule that can be isolated from various plants or biochemically synthesized, and is also found in humans as a metabolite of the mevalonic acid pathway. GGOH is converted into GGPP (Crick *et al*., 1997), an intermediate of the mevalonic acid pathway, downstream from FDPS. GGPP serves as a substrate for geranylgeranylation of small GTP proteins. GGOH can antagonize the effects of NBPs on bone resorption (Fisher *et al*., 1999).

Thus, we hypothesized that exogenous mevalonic acid metabolites restore the negative effects of NBPs on osteoclast differentiation and bone remodeling. To clarify this hypothesis, we investigated whether GGOH and GGPP can neutralize the negative effects of zoledronic acid, a potent NBP, *in vitro* as well as *in vivo*, in terms of the expressions of osteoclast

differentiation-related molecules, multinucleation-related molecules, and mineral deposition in tooth-extracted sockets of alveolar bone using BRONJ-like mice continuously treated with zoledronic acid and LPS.

Materials and methods

Cell Culture

Bone marrow cells (BMCs) isolated from the tibias of 3–5-week-old male *ddY* mice were suspended in alpha-minimal essential medium (Sigma, MO, USA) containing 10% fetal bovine serum. Bone marrow-derived macrophages (BMMs) were also prepared as osteoclast precursors from 3–5-week-old male *ddY* mice. Briefly, BMMs were obtained from tibial BMCs isolated from the mice by culture in the presence of macrophage colony-stimulating factor (M-CSF; 50 ng/ml, Pepro Tec Inc. NJ, USA) for 3 days. The obtained BMMs were then cultured for a further 3 days with RANKL (80 ng/ml, PeproTec Inc). The cultured cells were fixed with 3.7% formaldehyde and subjected to TRAP (marker enzyme for osteoclasts) and DAPI staining. TRAP-positive multinucleated cells ($TRAP^+$ MNCs) containing more than three nuclei were observed under a microscope and counted as osteoclasts. All procedures using animals were approved by the Council on Animal Care of Fukuoka Dental College.

Semiquantitative RT-PCR

Total RNA from cells was prepared with Trizol (Invitrogen, Carlsbad, CA, USA), reverse-transcribed by Superscript II (Invitrogen), and amplified by Taq polymerase (Invitrogen) using gene-specific primers (Supplemental Table1). The cDNA was amplified by PCR under the following conditions: 1 min of denaturation at 95°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C for a total of 30 cycles. The PCR products were subjected to electrophoresis in a 2% (w/v) agarose gel and visualized with ethidium bromide. The each PCR product was detected

with a Image Analyzer (FLA-2000F; Fuji Film, Tokyo, Japan). The signals of the targeted mRNAs were normalized to the corresponding GAPDH mRNA expression levels using Image J software (version 1.67; NIH, Bethesda, MD, USA).

Western blot analysis

Cells were lysed in TNT buffer comprising 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1% Triton X-100, 1 mM DTT, and protease inhibitors (Roche, Basel, Switzerland). The protein contents of the samples were measured using BCA reagents (Pierce, Rockford, IL, USA), following the manufacturer's protocol. Protein samples of 20 μg were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to PVDF membranes (100 V, 1 h, 4°C). The membranes were incubated with primary antibodies overnight at 4°C. The membranes were incubated with appropriate secondary antibodies for 30 min at room temperature. The immunoreactive proteins were visualized using enhanced ECL chemiluminescence reagents (GE Healthcare, Tokyo, Japan).

Micro-CT analysis

Male C57BL/6J mice at 6 weeks of age were intraperitoneally injected with zoledronic acid (250 μg/kg) and LPS (250 μg/kg; Salmonella; Re595; Sigma, MO, USA) twice a week. The right first molars in the maxilla were extracted at 2 weeks after onset of the intraperitoneal injections under deep anesthesia using isoflurane. In some experiments, GGOH (250 μg/kg; Sigma) or GGPP (250 μg/kg; Sigma) was simultaneously injected twice a week with zoledronic acid and LPS treatment after the tooth extraction. After 4 weeks, the maxillary bones were collected and fixed with 4% paraformaldehyde for 24 h. Images were obtained using a micro-CT device (SkyScan 1176; TOYO Corporation, Tokyo, Japan). The image parameters were set at 50-kV X-ray source with 0.5-mm aluminum filter. Corrected images set at 9 μm for one slice were reconstructed with NRecon

software (SkyScan). The bone mineral density (BMD) and bone volume/ total volume (BV/TV) in the samples were analyzed using CTAN software (SkyScan).

Statistical Analysis

Data were expressed as means ± SEM. The statistical significance of differences in values was analyzed by one-way analysis of variance (ANOVA) and Scheffe's multiple comparison test. Values of $P<0.05$ and $P<0.01$ were considered to indicate statistical significance.

Results

Zoledronic acid decreases the number of TRAP-positive cells with multinucleation during osteoclast differentiation

It is well-known that bisphosphonates directly inhibit bone resorption and promote apoptosis in mature osteoclasts. However, the effects of bisphosphonates on osteoclast precursors, especially BMMs, remain unclear. Previous studies have shown that zoledronic acid suppresses osteoclast formation in the macrophage cell lines RAW264.7 and C7 (Kimachi *et al*., 2011; Abe et al., 2012). To clarify the inhibitory effects of NBPs on osteoclast differentiation in more detail, we examined the effects of zoledronic acid on the number of $TRAP^+$ MNCs using mouse BMMs. Treatment with RANKL (80 ng/ml) increased the number of $TRAP^+$ MNCs differentiated from BMMs (Fig. 1A and 1B). In contrast, zoledronic acid (1–7 μM) significantly suppressed the number of RANKL-induced $TRAP^+$ MNCs in a dose-dependent manner. Zoledronic acid not only decreased the ratio of $TRAP^+$ MNCs containing more than 11 nuclei per total $TRAP^+$ MNCs, but also increased the ratios of $TRAP^+$ MNCs with less than 10 nuclei (3–5 and 6–10) in the total $TRAP^+$ MNCs, indicating that zoledronic acid suppressed osteoclast multinucleation during osteoclast differentiation in a dose-dependent manner (Fig. 1A and 1C). Zoledronic acid had no effects on the cell attachment and viability of RAW264.7 cells until 30 μM and also had no effects on the cell attachment of BMMs at less than 10 μM.

GGOH and GGPP restore the zoledronic acid-induced inhibition of osteoclast differentiation and multinucleation during osteoclastogenesis

Zoledronic acid is well-known to inhibit the mevalonic sterol pathway in osteoclasts, thus reducing protein prenylation and inhibiting the polymerization of the cytoskeleton structure during bone resorption (Zhang and Casey, 1996). In our previous paper, we demonstrated that the inhibitory effects of zoledronic acid on the activity of osteoclastic bone resorption and precursor migration were recovered by replenishing the cells with GGOH, a mevalonic sterol pathway metabolite. In the present study, we attempted to further clarify whether mevalonic pathway metabolites can restore the inhibition of osteoclast differentiation and multinucleation induced by zoledronic acid during osteoclastogenesis. Although addition of GGOH alone had no effects on the number of TRAP^{+} MNCs differentiated from BMMs, the inhibitory effect of zoledronic acid on TRAP^{+} MNC formation was dramatically neutralized by addition of GGOH (Fig. 2A and 2B), which was considered to arise through restoration of the geranylgeranylated proteins. Furthermore, treatment of BMMs with GGOH increased the number of TRAP^{+} MNCs with more than 11 nuclei compared with the number after treatment with zoledronic acid alone (Fig. 2B). In contrast, farnesyl acid (FOH) did not recover the inhibitory effects of zoledronic acid on the number of TRAP^{+} MNCs. Similar to the neutralizing effects of GGOH, treatment with GGPP restored the zoledronic acid-induced inhibition of the differentiation and multinucleation during osteoclastogenesis. Although GGPP alone slightly increased the number of TRAP^{+} MNCs compared with non-treated cells, the neutralizing effect of GGPP on the zoledronic acid-induced inhibition was less than that of GGOH. These results indicated that GGOH and GGPP, but not FOH, restored the zoledronic acid-induced inhibition of the differentiation and multinucleation during osteoclastogenesis.

Zoledronic acid suppresses the expressions of osteoclast differentiation molecules, and its

inhibitory effects are restored in the presence of GGOH in BMMs

Next, we examined the effects of zoledronic acid with or without GGOH on the expressions of osteoclast differentiation- and multinucleation-regulating molecules during osteoclast differentiation. RANKL increased the mRNA expression of TRAP, an osteoclast differentiation molecule, in a time-dependent manner (Fig. 3A). Zoledronic acid significantly inhibited the RANKL-induced expression of TRAP mRNA on day 2 (early stage of osteoclastogenesis) and day 4 (late stage of osteoclastogenesis) after RANKL treatment of BMMs (Fig. 3A and B). In contrast, GGOH prevented the zoledronic acid-induced inhibition of TRAP mRNA expression at the late stage of osteoclastogenesis. Multinucleation is a key cellular event in osteoclastogenesis, and is regulated by osteoclast fusion gene products. RANKL also upregulated the expressions of osteoclast fusion genes such as dendrocyte expressed seven transmembrane (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP) in a time-dependent manner (Fig. 3A). Zoledronic acid significantly inhibited the RANKL-induced upregulation of the STAMP mRNA expressions at the early stage and late stage of osteoclastogenesis after RANKL treatment of BMMs (Figs. 3A and 4A). Likewise, GGOH neutralized the inhibition of the DC-STAMP mRNA expressions by zoledronic acid at the late stage of osteoclastogenesis. GGPP also restored the zoledronic acid-induced inhibition of DC-STAMP mRNA expression. Similar to the RT-PCR data, RANKL increased the protein levels of osteoclast differentiation-related molecules such as TRAP, NFATc1, calcitonin receptor, and V-ATPase in a time-dependent manner, and treatment with zoledronic acid significantly suppressed the RANKL-induced expressions at the late stage of osteoclast differentiation (Fig. 4A). Furthermore, zoledronic acid inhibited the expression level of DC-STAMP protein, especially at the late stage of osteoclast differentiation. The inhibitory effects of zoledronic acid on the expressions of TRAP and DC-STAMP proteins were partially restored by addition of GGOH or GGPP (Fig. 4B). In contrast, FOH did not restore the inhibitory effects of zoledronic acid on these protein expressions during osteoclast differentiation (data not shown).

GGOH and GGPP partially restore the delayed bone deposition in tooth-extracted sockets induced by zoledronic acid administration in mice

Given that the overall purpose of this study was to develop a therapy for BRONJ by recovering osteoclast functions, we applied GGOH and GGPP to an animal model that was reported to reproduce a BRONJ-like disease in mice. The right first molars in the maxilla of the mice were extracted at 2 weeks after intraperitoneal injections of zoledronic acid together with LPS, which is a risk factor for BRONJ expected to induce inflammatory reactions resembling a bacterial infection (Kim *et al*., 2013), twice a week. We then examined the effects of the mevalonic pathway metabolites GGOH and GGPP on the alveolar bone deposition after tooth extraction using micro-CT. According to a previous report (Bi *et al*., 2010), we expected that BRONJ-like lesions, would occur in the alveolar bone of these mice. BRONJ-like lesions with empty osteocytic lacunae and infiltration of inflammatory cells were observed in alveolar bone at 4 weeks after both treatments (supplemental Fig. 1A). Bone minerals were gradually deposited in control mice at 4 weeks after tooth extraction compared with day 1 after extraction (Fig. 5A). Intraperitoneal injections of both agents partially decreased the BMD and BV/TV in the tooth-extracted sockets (Fig. 5A and 5B). Intraperitoneal injections with zoledronic acid and LPS decreased the BMD in the sockets compared with control mice. In contrast, simultaneous injection with GGOH or GGPP in zoledronic acid- and LPS-administered mice significantly increased the BMD and BV/TV with upregulation in TRAP^{+} cells (supplemental Fig. 1B), indicating that GGOH or GGPP restored the zoledronic acid-induced decrease in bone deposition in BRONJ-like model mice.

Discussion

The aim of this study was to elucidate the neutralizing effects of mevalonic acid metabolites, especially GGOH, a substrate for geranylgeranyl transferase, and GGPP, on the zoledronic

acid-induced inhibition of osteoclastogenesis and alveolar bone remodeling in tooth-extracted sockets in zoledronic acid-injected mice. We showed that zoledronic acid inhibited osteoclast differentiation and multinucleation and decreased the rate of mineral deposition in tooth-extracted sockets under inflammation, all of which were partially restored by GGOH or GGPP. Our results suggest that mevalonic acid metabolites have potential for treatment of BRONJ by restoring osteoclastogenesis.

NBPs are known to inhibit farnesyl pyrophosphate synthase in the mevalonic acid pathway in osteoclasts, resulting in disturbance of the polymerized cytoskeleton structure in bone resorption (Coxon *et al.*, 2000; Benford *et al.*, 2001). Recently, NBPs were reported to inhibit osteoclast differentiation as well as bone resorbing activity in the macrophage cell lines RAW264.7 and C7 *in vitro* (Kimachi *et al.*, 2011; Abe *et al.*, 2012). We previously showed that zoledronic acid inhibited RANKL-induced upregulation of RANK in RAW264.7 cells (Kimachi *et al.*, 2011). Furthermore, zoledronic acid was reported to increase the expression of osteoprotegerin, a RANKL decoy receptor, in osteosarcoma and osteoblasts, leading to the suppression of osteoclast activity (Benassi *et al.*, 2007; Mori *et al.*, 2007). We also found that zoledronic acid reduced the expressions of osteoclast differentiation-related molecules such as NFATc1, calcitonin receptor, cathepsinK, and TRAP in the present experiments, thereby suppressing the formation of $TRAP^{+}$ MNCs from mouse osteoclast precursor BMMs.

The RANK-RANKL signaling pathway in osteoclast precursors plays an essential role in osteoclast differentiation, fusion, multinucleation, and bone resorption (Takayanagi *et al.*, 2002). The fusion process in osteoclast mononuclear precursors may be initiated by adhesive interactions through DC-STAMP and OC-STAMP (Yang *et al.*, 2008; Miyamoto *et al.*, 2012). DC-STAMP expression is promoted by RANKL and plays an important role in osteoclast differentiation. DC-STAMP-negative cells were found to be unable to initiate cell-cell fusion with each other, but were able to fuse with DC-STAMP-positive cells, thus providing the first mechanistic insights into

the process of fusion (Vigney, 2005; Yagi *et al*., 2005). In the present experiments, we found that zoledronic acid decreased the expressions of DC-STAMP and OC-STAMP, suggesting that zoledronic acid-inhibited RANK-RANKL signaling not only suppresses osteoclast differentiation, but also prevents the multinucleation through DC-STAMP in BMMs. It seems to be a new finding that multinucleation of osteoclast precursors is also blocked by NBPs.

Continuous administration of NBPs was reported to involve BRONJ as the main side effect (Bi *et al*., 2010; Kikuiri *et al*., 2010; Aguirre *et al*., 2012). Mouse and rat models for BRONJ-like diseases were recently shown to develop ONJ-like lesions (Bi *et al*., 2010; Aguirre *et al*., 2012). A variety of pathologies for the occurrence of BRONJ have been proposed, including toxicity to the oral epithelium, altered would healing after tooth extraction, delayed bone turnover of the jaw, promoted oral biofilm formation, and suppressed angiogenesis (Landesberg *et al*., 2011). In the present experiments, we observed the bony changes in a mouse model continuously treated with zoledronic acid and LPS. Although we found slight ONJ-like lesions with empty osteocytic lacunae at 4 weeks after both treatments, the BMD was decreased in the extracted tooth sockets. This seems to be inconsistent with the notion that zoledronic acid is a drug for treatment of osteoporosis by increasing the BMD. In this mouse model, the zoledronic acid-impaired bone turnover may lead to the delayed mineral deposition observed in the extracted sockets. LPS seems to worsen the lowered BMD by inducing systemic inflammatory reactions and resulting in tissue damage. Importantly, we found that GGOH or GGPP partially suppressed the zoledronic acid-induced effects with upregulation of $TRAP^+$ cells in these mice. One of the etiologies for BRONJ is assumed to be bisphosphonate-induced lower bone turnover via inhibition of osteoclast differentiation (Reid *et al*., 2007). Our data suggest that these geranylgeranylated substrates can restore the osteoclast differentiation and bone turnover damped by bisphosphonate administration even *in vivo*.

GGPP is an essential compound for inducing the proliferation of many types of cells through activation of Rho small GTPase (Tatsuno *et al*., 1997; Nishimura *et al*., 1999). In contrast, GGPP

was reported to suppress the differentiation of human osteoblasts through activation of Rho and Rho-kinase (Ohnaka *et al*., 2001). These results indicate that GGPP may have a positive effect on the proliferation and a negative effect on the differentiation of osteoblasts, depending on the concentration of GGPP (Yoshida *et al*., 2006). Furthermore, GGOH was rescued the BP-induced inhibition on proliferation and angiogenesis in human gingival cells (HGFs; Zafer et al., 2014). Thus, the recovery of bone formation in the tooth-extracted sockets in the presence of GGPP or GGOH may arise through osteoclast recruitment as well as HGFs, rather than osteoblastic differentiation. Further experiments are required to elucidate the therapeutic potential of GGPP and GGOH on ONJ, but not bone mineral deposition, by reproducing the osteonecrosis mimicking BRONJ in animal models.

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Figure legends

Figure1. Inhibition of the formation and multinucleation of $TRAP^+$ MNCs induced by zoledronic acid A) BMM osteoclast precursors were cultured with RANKL (80 ng/ml) in the presence or absence of zoledronic acid (0–7 μM) for 4 days. After 4 days, the cells were subjected to TRAP and DAPI staining. The images in right column indicated the merged of TRAP and DAPI staining (white circles). Scale bars: 100 μm. (B) The numbers of $TRAP^+$ MNCs with 3–5, 6–10, and ≥11 nuclei were counted. Data are means ± SEM from six culture wells. *$P<0.05$. **$P<0.01$.

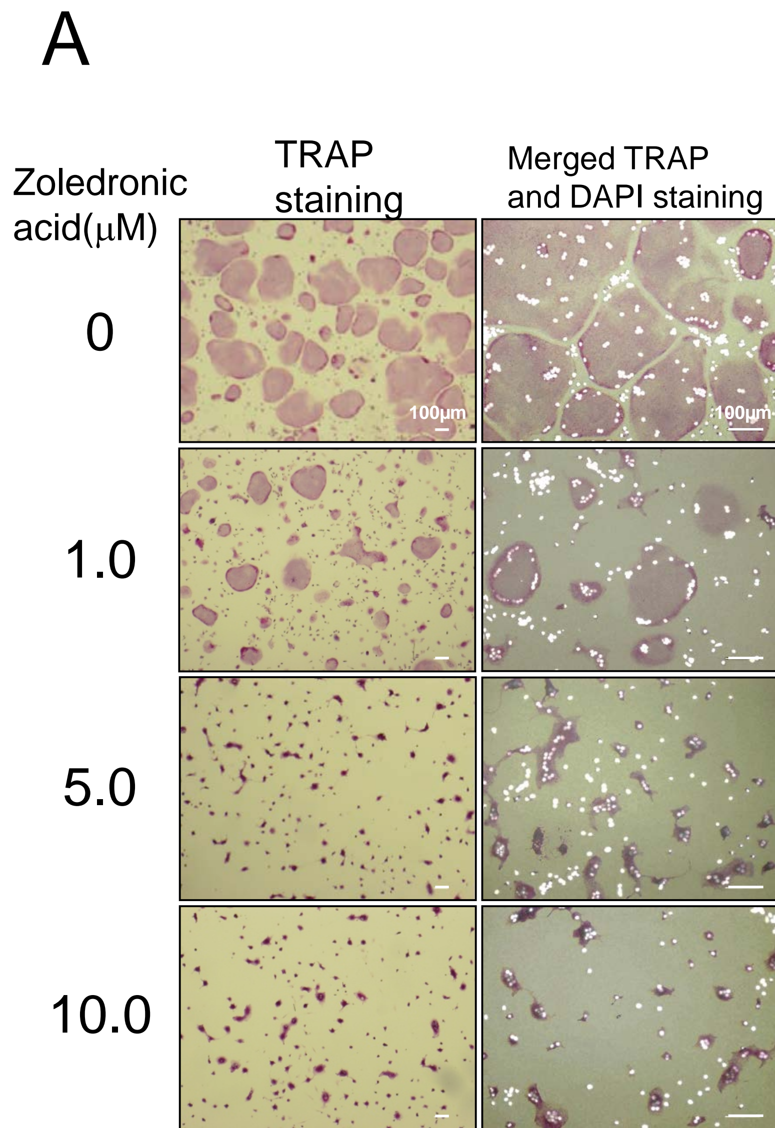
Figure2. Partial suppression of the zoledronic acid-induced inhibition of $TRAP^+$ MNC formation by GGOH or GGPP. (A) BMMs were cultured with RANKL (80 ng/ml) and zoledronic acid (0–10 μM) in the presence or absence of GGOH (3 μM) or GGPP (3 μM) for 4 days. After 4 days, the cells were subjected to TRAP and DAPI staining. Scale bars: 100 μm. (B) The numbers of $TRAP^+$ MNCs with 3–5, 6–10, and ≥11 nuclei were counted. Data are means ± SEM from six culture wells. *$P<0.05$. **$P<0.01$.

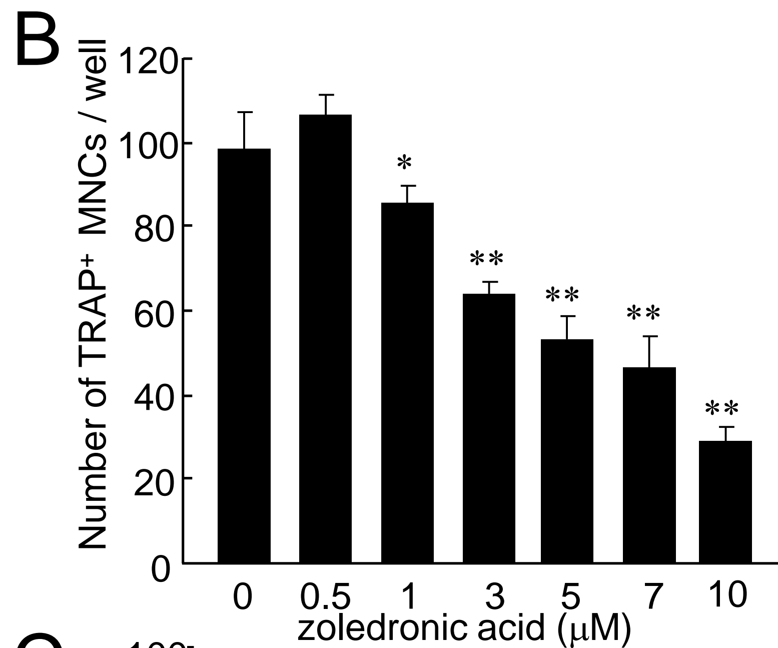
Figure3. Effects of zoledronic acid with or without GGOH (3 μM) on the mRNA expressions of TRAP, DC-STAMP, and OC-STAMP. (A) BMMs were cultured with RANKL (80 ng/ml) and zoledronic acid (5 μM) with or without GGOH (3 μM) or GGPP (3 μM) for 0–4 days. The mRNA expression levels of the targeted genes and β-actin were analyzed by semiquantitative RT-PCR. The numbers below the gels represent the intensities of the targeted gene mRNAs relative to β-actin mRNA. (B) The mRNA expression levels of the targeted genes were analyzed by semi-quantitative RT-PCR. Data are means ± SEM from 10 culture wells. *$P<0.05$.

Figure4. Zoledronic acid-induced inhibition of osteoclastogenesis-related molecules in BMMs and its suppression by GGOH and GGPP. Zoledronic acid inhibits the expressions of RANKL-induced

osteoclastogenesis-related molecules in BMMs and its inhibition is suppressed in the presence of GGOH or GGPP. (A) BMMs were cultured with RANKL (80 ng/ml) and zoledronic acid (5 μM) for 0–4 days. Total cell lysates were prepared and analyzed by western blotting using antibodies against osteoclastogenesis-related molecules. Similar results were obtained in three independent experiments. (B) BMMs were cultured with RANKL (80 ng/ml) and zoledronic acid (5 μM) in the presence or absence of GGOH (3 μM) or GGPP (3 μM) for 0–4 days. Total cell lysates were prepared and analyzed by western blotting using anti- TRAP, DC-STAMP, cathepsinK, and calcitonin receptor antibodies. Similar results were obtained in three independent experiments.

Figure5. Decrease of bone deposition induced by zoledronic acid in extracted teeth sockets and its suppression by GGOH and GGPP. (A) Male C57BL/6J mice at 6 weeks of age were administered intraperitoneal injections of zoledronic acid (250 μg/kg) and LPS (250 μg/kg; Salmonella; Re595) twice a week. The right first molars in the maxilla were extracted at 2 weeks after the onset of the intraperitoneal injections under deep anesthesia using isoflurane. In some experiments, GGOH (250 μg/kg) or GGPP (250 μg/kg) was simultaneously injected with the zoledronic acid and LPS treatment twice a week after the tooth extraction. (B) The BMD and BV/TV in the samples were analyzed using CTAN software. Data are means ± SEM from six culture wells. *P<0.05. **P<0.01.





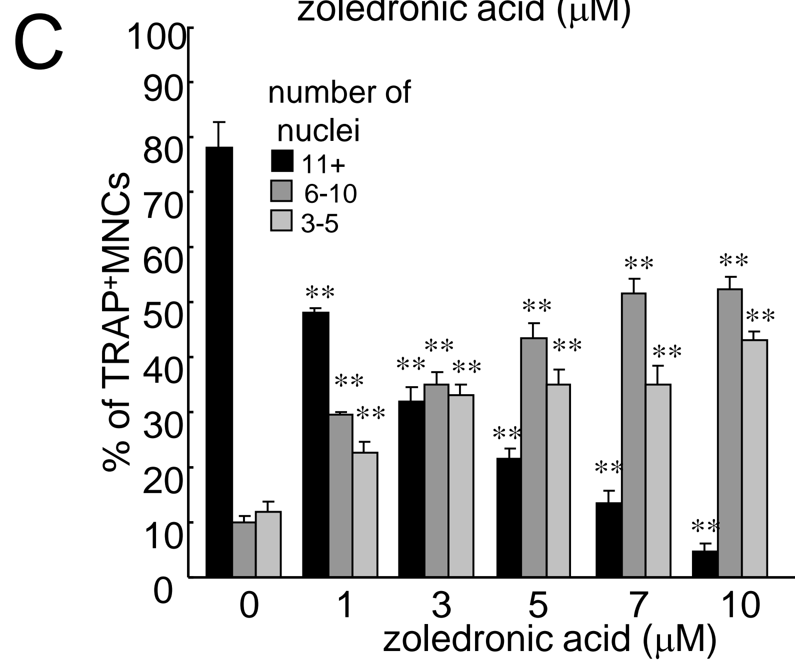
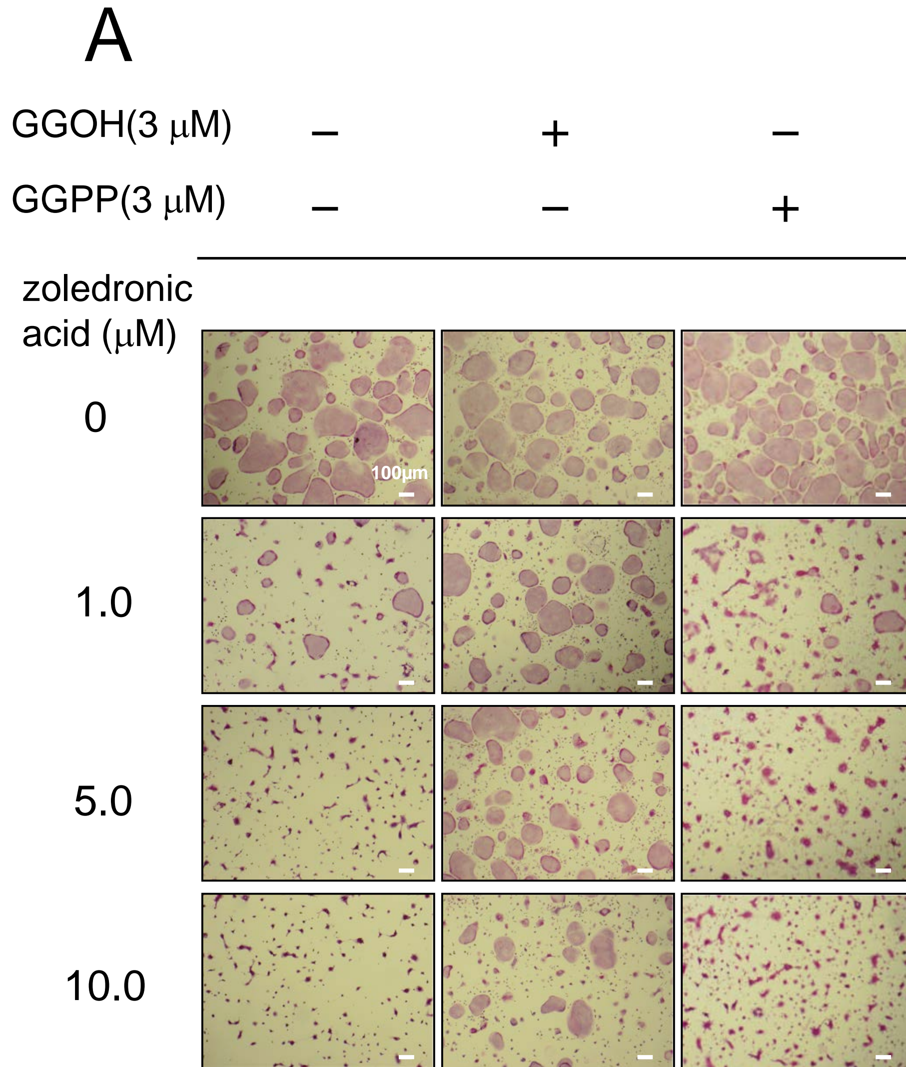


Fig. 1



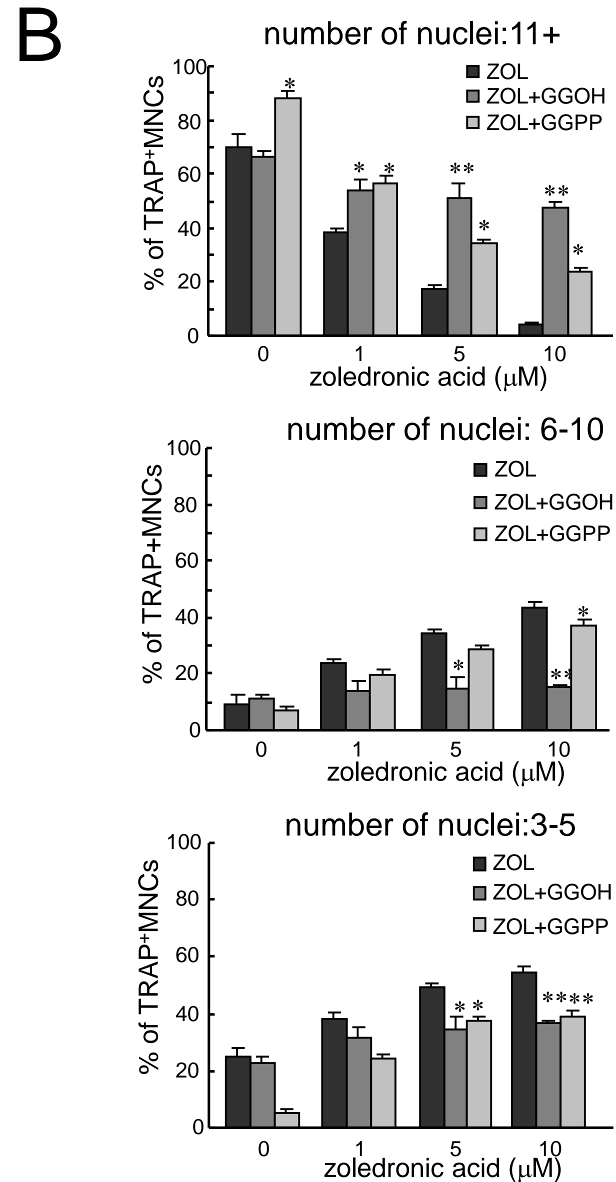


Fig. 2

A

incubation time (day)	control RANKL(80 ng/ml) 0	2	4	zoledronic acid (5 μM) RANKL(80 ng/ml) 0	2	4	zoledronic acid(5 μM) +GGOH(3 μM) RANKL(80 ng/ml) 0	2	4	zoledronic acid (5 μM) +GGPP(3 μM) RANKL(80 ng/ml) 0	2	4
TRAP	0.12	0.54	0.63	0.12	0.30	0.20	0.12	0.16	0.45	0.13	0.37	0.76
DC-STAMP	0.33	0.74	0.70	0.33	0.32	0.08	0.33	0.08	0.58	0.15	0.35	0.33
OC-STAMP	0.08	0.34	0.86	0.08	0.04	0.01	0.08	0.10	0.26	0.04	0.53	0.44
β-actin												

B

TRAP
% of TRAP/β-actin mRNAs
day0 day2 day4
Control; zoledronic acid(5μM); zoledronic acid(5μM) +GGOH; zoledronic acid(5μM) +GGPP

DC-STAMP
% of DC-STAMP/β-actin mRNAs
day0 day2 day4
Control; zoledronic acid(5μM); zoledronic acid(5μM) +GGOH; zoledronic acid(5μM) +GGPP

OC-STAMP
% of OC-STAMP/β-actin mRNAs
day0 day2 day4
Control; zoledronic acid(5μM); zoledronic acid(5μM) +GGOH; zoledronic acid(5μM) +GGPP

Fig. 3

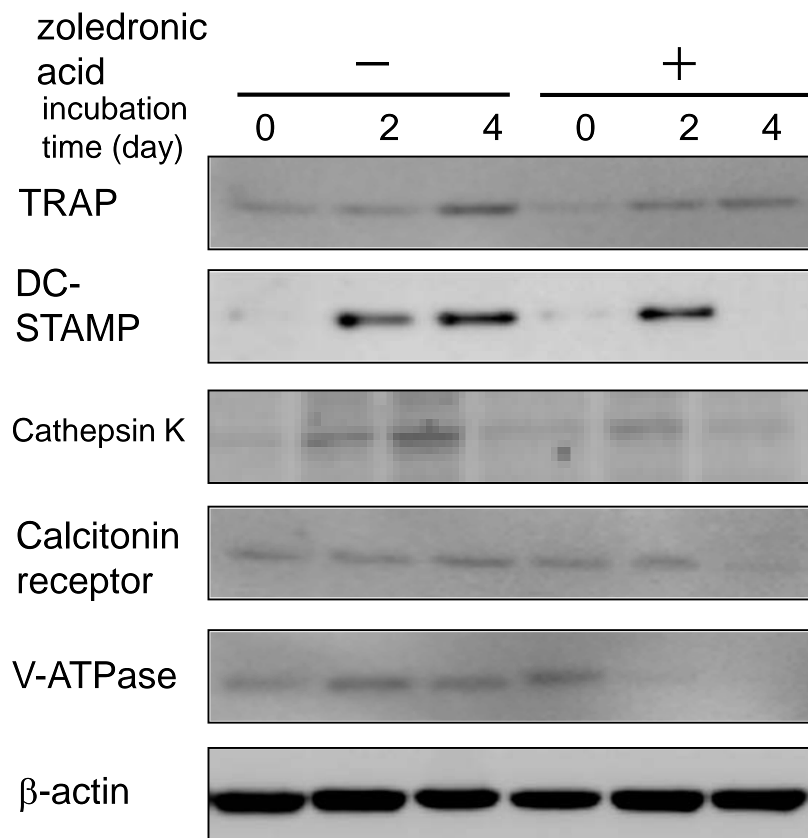


Fig. 4

A

Sagittal plane

Coronal plane

extraction

4 weeks after extraction

control

Zoledronic acid +LPS

Zoledronic acid +LPS +GGOH

Zoledronic acid +LPS +GGPP

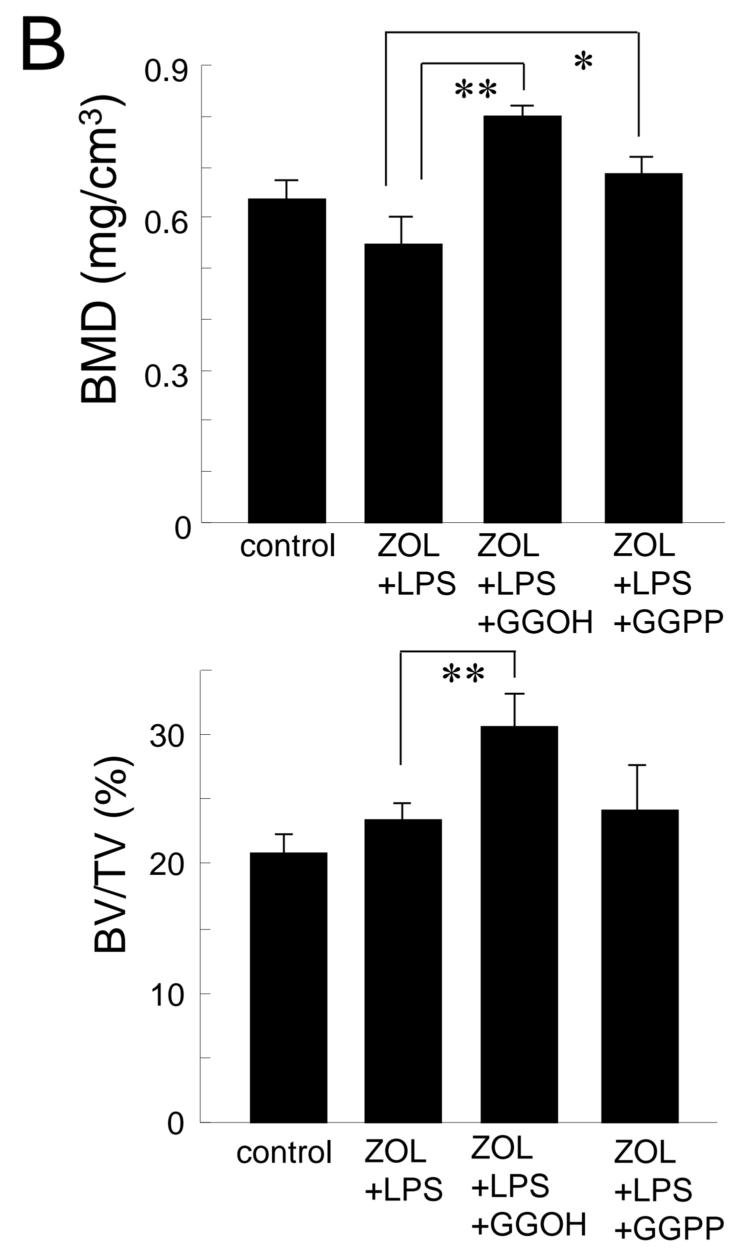


Fig. 5