Original Article

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Geranylgeraniol increased mineralization in osteoblasts treated with low concentration of alendronate: A preliminary study

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Objective: To investigate the effects of geranylgeraniol (GGOH) on alendronate (ALN)-treated osteoblast viability and bone nodule formation.

Materials and methods: MC3T3, murine osteoblast precursors, were cultured with ALN (0 and 1 μM) and GGOH (0, 10, and 50 μM). The viability of MC3T3 was determined by MTT assay at 3 and 6 days. After 3 weeks, bone nodule formation was evaluated by Alizarin Red S assay.

Results: Addition of ALN did not affect cell viability of MC3T3 at 3 and 6 days of incubation. Bone nodule formation of ALN-treated osteoblasts exhibited lower alizarin red staining compared with that of untreated control. In the presence of ALN, there was no significant difference on viability between cells treated with GGOH and those without GGOH for 3 and 6 days. On the contrary, GGOH addition significantly increased the percentage of bone nodule formation in osteoblasts treated with ALN.

Conclusion: Low concentration of ALN reduced bone nodule formation produced by osteoblasts. Exogenous GGOH could reverse the inhibitory effect of ALN. Thus, GGOH could be a potential therapy for the conditions caused by the reduction in bone formation such as bisphosphonate-related osteonecrosis of the jaw and atypical fracture of femoral diaphysis.

Keywords: alendronate, cell viability, bone nodule formation, geranylgeraniol

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Introduction

Bisphosphonates (BPs) are widely used in the management of osteoporosis and other diseases of high bone turnover. Alendronate (ALN) is a type of nitrogen-containing BP (N-BP), which acts by inhibiting enzymes in mevalonate pathway in the cells [1]. ALN has high binding affinity to calcium ion in the bone [2], especially at the sites with rapid bone turnover [3], making it the ideal candidate for treatment of osteoporosis [4]. The primary target of ALN are osteoclasts, which mediate bone resorption. ALN suppresses osteoclast resorptive activity *in vivo* and *in vitro* [5-7]. Besides the inhibition of bone resorption, long-term ALN administration appears to hinder bone formation by osteoblasts, resulting in reduction of bone turnover [7-8]. This can lead to the unwanted conditions such as bisphosphonate-related osteonecrosis of the jaw (BRONJ; also known as medication-related osteonecrosis of the jaw or MRONJ) and atypical fracture of femoral diaphysis [9-10].

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At the cellular level, osteoblasts are affected by ALN in terms of cell growth, apoptosis, differentiation, and function [8, 11-12]. High doses of ALN decrease proliferation of osteoblasts by arresting cell cycle and causing apoptosis/necrosis of the cells [11-12]. Osteoblast differentiation and bone formation are also inhibited [8, 11]. Furthermore, ALN causes reduction of protein prenylation in mevalonate pathway in osteoblasts [11].

Geranylgeraniol (GGOH) is an isoprenoid substrate that can be found in plants [13], is biochemically synthesized in yeasts [14], and is a metabolite of mevalonate pathway [5]. GGOH is converted to geranylgeranyl pyrophosphate (GGPP), which serves as a substrate for geranylgeranylation of proteins [15]. GGOH can antagonize the inhibitory effects of ALN on osteoclasts *ex vivo* and *in vitro* [5-6]. Micromolar range of GGOH is able to suppress ALN inhibition of osteoclastogenesis and restore osteoclast resorptive activity [5]. A few studies have examined the effects of GGOH on ALN-treated osteoclasts, however, the effects of GGOH on ALN-treated osteoblast viability and bone formation are still not known. In view of this, we examined the effects of ALN and GGOH on osteoblast viability and function *in vitro*. This study might grant basic knowledge and guide to the use of a potential molecule for controlling and promoting recovery of BRONJ in the future.

Materials and methods

Cell culture

MC3T3 cells, mouse osteoblast precursors, were kindly provided by Prof. Prasit Pavasant. These cells were maintained in standard culture media, which were α-minimum essential medium (α-MEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (all from Gibco, Invitrogen) at 37°C and 5% CO2 humidified atmosphere.

For MTT assay, MC3T3 cells were cultured at a density of 8 x 10^3 cells/1 cm^2 in standard culture media. Cells were treated with ALN (Sigma) at concentrations 0 and 1 μM and GGOH (Sigma) at concentration 0, 10, and 50 μM for 3 and 6 days.

For alizarin red assay, cells were cultured in standard culture media supplemented with 50 μg/ml ascorbic acid and 2mM β-glycerophosphate (osteogenic media; OM) [16]. ALN (0 and 1 μM) and GGOH (0, 10, and 50 μM) were added for 3 weeks. During the course of 3 weeks, cell confluency and distribution were monitored using a light microscope (Nikon Eclipse TS100) and photomicrographs of cell appearance were taken at the 100x magnification using a Nikon Digital sight DS-L2.

Cell viability assay

The effects of ALN and GGOH on viability of MC3T3 were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. After indicated time points, cells were incubated with 200 μl of 0.2% MTT powder dissolved in phosphate buffered saline (PBS) (w/v) per well at 37 °C for 4 hours. After that, the non-metabolized MTT solution was discarded and 200 μl DMSO and 25 μl glycine buffer were added to each well. The end product color (purple) was measured at the 490 nm absorbance using a spectophotometer (Sunrise, Tecan).

Mineralization assay

Osteoblasts were washed with PBS and fixed with ice-cold methanol for 10 min. The fixed cells were then stained with freshly prepared 1% alizarin red S (Sigma) stain solution (w/v) for 15 min and thoroughly washed with distilled water to remove the excess dye. The stained cell-matrix layers were observed using a light microscope and photographs were captured at 40x magnification. For quantification, cells stained with alizarin red S were destained with 10%

cetylpyridinium chloride (w/v) (Sigma), and then the extracted stain was measured for the absorbance at 570 nm.

Statistical analysis

All experiments were performed three times. Data were expressed as means ± SD. A statistical significance was determined by either t-test or One-way ANOVA followed by Dunnett's multiple comparison tests using GraphPad Prism version 5 (GraphPad software). Significance was assigned as *p < 0.05 when compared with control.

Results

Low concentration of ALN reduced bone nodule formation produced by osteoblasts

We first tested the effects of low dose ALN on MC3T3 cell viability and osteoblast nodule formation. Because levels of BP in human plasma and bone from patients with existing BRONJ are low (ranging from 0.12 to 4.6 μM) [17-18], we selected 1 μM ALN as a primary concentration used in this study. One μM ALN treatment did not significantly affect cell viability at day 3 and 6 compared to untreated control (Figure 1A and B). Under OM condition, treatment with ALN for 3 weeks resulted in a 70% reduction in bone nodule formation ($p < 0.0001$) (Figure 1C).

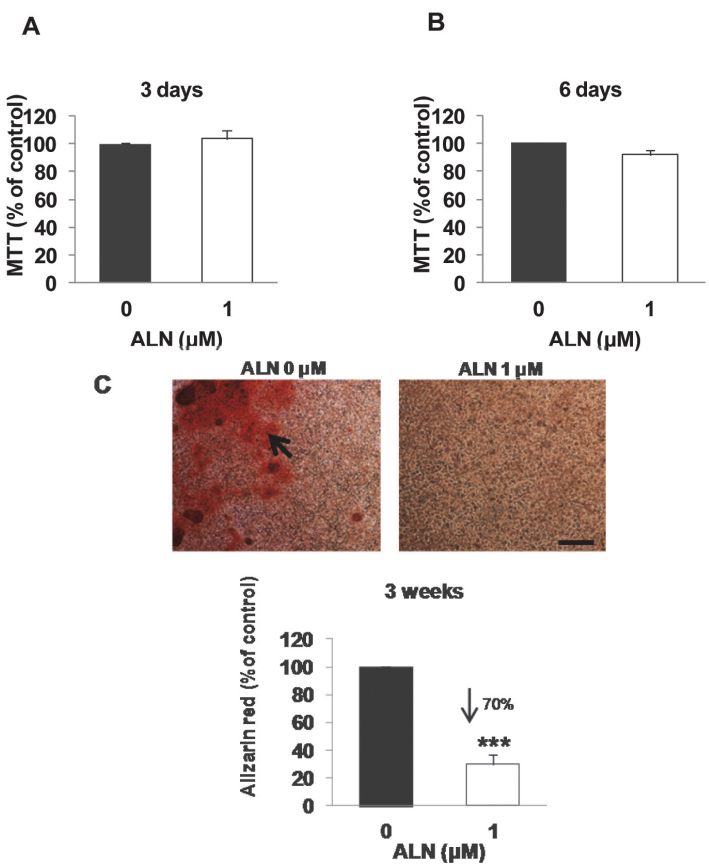


Figure 1 Effects of ALN on MC3T3 viability and bone nodule formation. (A) MTT analysis after MC3T3 cultured in the absence or presence of 1 μM ALN for 3 days. (B) MTT analysis after MC3T3 cultured in the absence or presence of 1 μM ALN for 6 days. (C) Alizarin red S staining and quantification of osteoblasts treated with ALN for 3 weeks. Cells cultured in the absence of ALN served as control. The control was presented in black bar graph, while the experimental samples were presented in white bar graphs. Scale bar = 500 μm.

GGOH enhanced bone nodule formation by ALN-treated osteoblasts

Further studies were performed to examine whether GGOH could recover the inhibitory effect of ALN. Two concentrations of GGOH were investigated. Treatment with ALN was served as control. In general, GGOH did not affect ALN-treated MC3T3 viability at 3 and 6 days (Figure 2). However, 50 μM GGOH tended to reduce cell viability by 8% at 3 days (Figure 2).

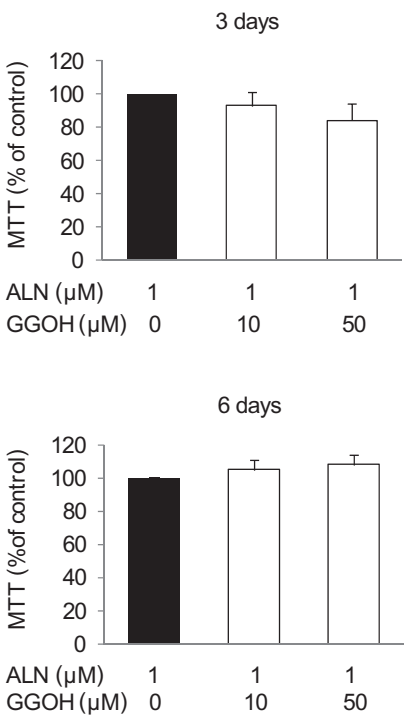


Figure 2 Effects of GGOH on MC3T3 viability under ALN treatment. MC3T3 cultured in the presence of 1 μM ALN throughout the experiments (3 and 6 days). GGOH at 10 and 50 μM were added. Cells cultured in the absence of GGOH served as control. Cell viability was evaluated by MTT assay. The control was presented in black bar graph, while the experimental samples were presented in white bar graphs.

During 3 weeks under OM condition, osteoblast appearance and distribution were observed under light microscope. At day 10-19, osteoblasts treated with ALN were 100% confluent (Figure 3A, D, and G). Cells treated with GGOH showed no difference when compared to the control cells. (Figure 3B, C, E, F, H, and I). After 3 weeks, control samples stained weakly with alizarin red S (Figure 3J). Treatment with GGOH exhibited darker red stain of mineralization as pointed by the black arrows (Figure 3K and L). Quantitatively, the addition of GGOH increased bone nodule formation up to 60% (Figure 3M). GGOH at 10 μM and 50 μM showed significantly higher values than control ($p = 0.0188$ and $p = 0.0426$, respectively).

Discussion

Although the main target of BPs is on osteoclast activities, effects on the osteoblast lineage have also been demonstrated. Some studies have reported that ALN stimulates osteoblast cell growth, differentiation [19], and mineralized bone nodule formation *in vitro* [20]. While these studies exhibit positive effects of ALN on osteoblasts, the conflicting effects of ALN on bone formation have been shown *in vitro* and *in vivo* [8, 11, 21-22]. The results of this *in vitro* study showed an inhibitory effect of low dose ALN on bone nodule formation but not on cell viability. This is in an agreement with previous studies by Idris et al, 2008 and Patntirapong et al, 2014 [8, 11]. The dissimilarity in the results could be from the different experimental systems used. This includes cell types, stages of cell differentiation, durations of treatment, and concentrations. ALN at moderate to high concentration (> 5 μM) strongly decrease cell viability and nodule formation [8, 11]. Despites the same range of concentration, different cell types and the stage of cell differentiation demonstrate different results. ALN (at 1 μM and

below) inhibits nodule formation in osteoblasts derived from mouse calvarial precursors [8, 11], while ALN (at 1 μM) does not affect nodule formation in human primary osteoblasts derived from bone marrow mesenchymal stem cells [20]. Although low dose ALN reduces osteoblast mineralization, it does not affect cell viability of osteoblast precursors [8, 11, 20]. It could be possible that the duration of ALN treatment may be the factor in the low dose condition. Treatment with 1 μM ALN shows no effect on cell viability on day 1-6 and slightly decreases cell viability after day 12 [20]. It should be noted that the concentration used in this study is within the range of concentrations found in plasma of patients and in the bone received from BRONJ patients [17-18]. A peak plasma alendronate level reaches 0.12 μM after 1 h of administration [17]. Specimens from patients naïve to BP show levels at 0 μM, while bones from patients with existing BRONJ demonstrate levels of BP ranging from 0.4 to 4.6 μM [18]. Further study should be conducted by extending the concentrations and duration of treatment up to 3 weeks.

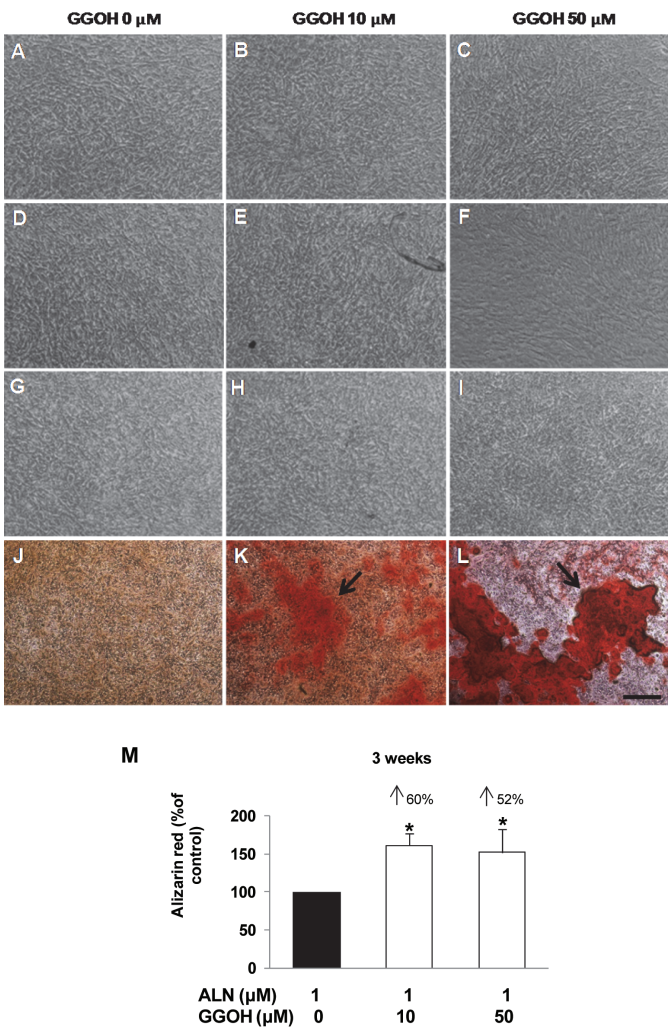


Figure 3 Enhancement of bone nodule formation by the addition of GGOH. Osteoblasts cultured in OM and in the presence of 1 μM ALN throughout the experiments (3 weeks). GGOH at 10 and 50 μM were added. Cells cultured in the absence of GGOH served as control. Nodule formation was investigated by alizarin red assay. Brightfield micrographs were taken (A-C) at day 10, (D-F) at day 14, (G-I) at day 19, and (J-L) at 3 weeks. The arrows showed nodule formation stained positive to alizarin red S. (A-I) Magnification 100x (J-L) Magnification 40x Scale bar = 500 μm. (M) Graph demonstrated alizarin red staining. The control was presented in black bar graph, while the experimental samples were presented in white bar graphs.

The molecular mechanism of action of BPs are now well discovered. In the late 1990s, studies have reported the involvement of ALN in mevalonate pathway [1, 5-6]. ALN selectively inhibits farnesyl pyrophosphate synthase (FPPS) in this pathway [1]. By inhibiting FPPS, cells are depleted of farnesyl pyrophosphate (FPP). Consequently, FPP downstream (GGPP) is reduced, resulting in disruption of all forms of protein prenylation [5]. GGPP can be synthesized from GGOH in the cells, thus supplement of GGOH increases GGPP and restores protein geranylgeranylation in the cells (Figure 4). Replenishing osteoclasts with exogenous GGOH partially overcomes the inhibitory effects of ALN on cell number and resorptive activity [5-6]. Treatment of osteoblasts with zoledronic acid (another type of N-BP) causes decrease of viable cells and bone formation. Addition of GGOH reverses the effect of zoledronic acid by increasing cell viability and, to a lesser extent, bone nodule formation [23]. Our data are consistent with the previous finding in terms of osteoblast function. GGOH treatment could partially rescue the negative effect of ALN on bone nodule formation. In contrast, GGOH did not increase viability of cell treated with low concentration ALN. In this study, we have not tested the effects of GGOH on cell viability and nodule formation in the absence of ALN. Future study should be done to clarify these effects. We propose that the addition of GGOH alone could increase bone nodule formation but could not affect cell viability.

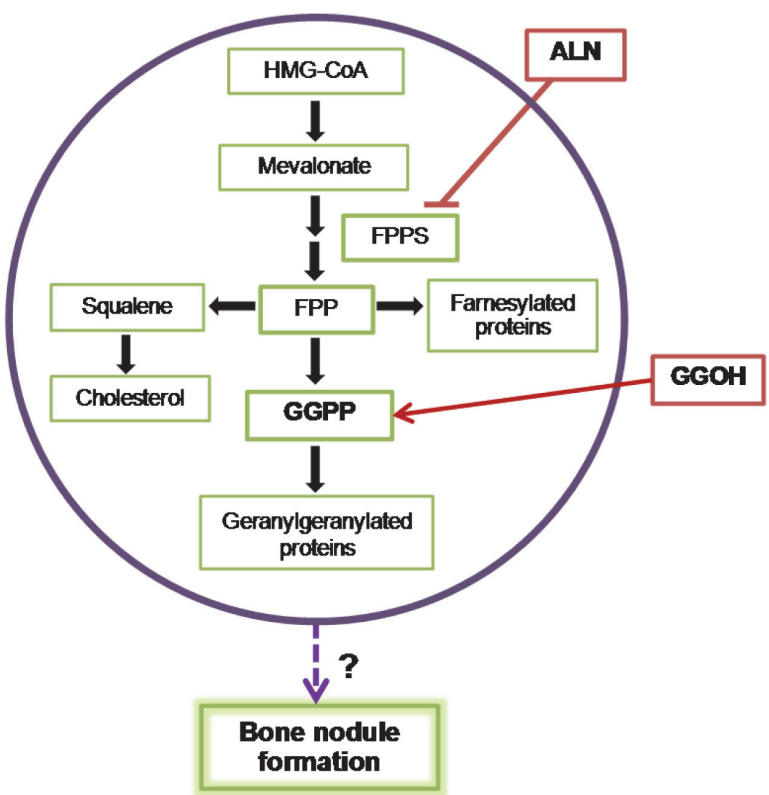


Figure 4 Proposed schematic diagram of mevalonate pathway and effects of low dose ALN and GGOH in osteoblast. ALN inhibits FPPS, leading to depleting FPP and GGPP, respectively. Exogenous GGOH can be converted to GGPP, thus, restoring the production of geranylgeranylated proteins in the cells. Addition of GGOH could drive osteoblasts to form bone nodule in the presence of ALN.

In the present study, the reversal effect of GGOH suggested the involvement of mevalonate pathway in osteoblast bone formation (Figure 4). Understanding the cellular actions of BP could lead to a new therapeutic strategy for BRONJ and atypical fracture of femoral diaphysis in the future. GGOH could be developed as a drug of choice for treating these conditions. However, the exact molecular mechanism by which BP exerts the inhibitory effects on bone formation and how GGOH restores the cell function are poorly understood. Further studies are required to explain the mechanisms underlying this effect. Perhaps, the future plan should include GGOH antagonist in order to confirm the effect and mechanism of GGOH.

Conclusion

The data presented here supported a direct action of low concentration ALN on osteoblast viability and function. GGOH could antagonize the inhibitory effect of ALN on bone formation. Therefore, GGOH could be applied as a future local therapy to BRONJ and atypical fracture of femoral diaphysis. However, more experiments are required for elucidating the exact mechanism of action, the concentration used, the duration of GGOH treatment as well as the route of administration.

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Conflict of interest: The authors declare that they have no conflict of interest.

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