*Original Article*

DOI: 110.5582/irdr.2013.v2.1.18

# Low concentrations of zoledronic acid are better at regulating bone formation and repair

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**Summary** **The purpose of this study was to investigate optimal concentrations of zoledronic acid (ZA) in terms of their effect on the proliferation, differentiation, and mineralization of primary osteoblasts (OBs) and fibroblasts (FBs). Primary OBs and FBs isolated from patients with clinical osteogenesis imperfecta (OI) and developmental dysplasia of the hip (DDH) were treated *in vitro* with serial concentrations of ZA ranging from $10^{-3}$ M to $10^{-13}$ M. An MTT ( (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) colorimetric assay, flow cytometry, alkaline phosphatase (ALP) determination activity, and alizarin red staining were used to measure the proliferation, differentiation, and mineralization of cells. The MTT assay indicated that high concentrations of ZA may be toxic to cultured cells. No obvious inhibition was observed with a ZA concentration of $10^{-7}$ M to $10^{-10}$ M. Proliferation was evident with a ZA concentration below $10^{-11}$ M ($p < 0.05$). Flow cytometry analysis revealed that cell cycle was arrested at G1/G0 stage with a ZA concentration ranging from $10^{-10}$ M to $10^{-8}$ M. ZA did not enhance ALP activity at a concentration of $10^{-8}$ M or $10^{-10}$ M. Alizarin red staining indicated the mineralization of primary OBs with a low concentration of ZA ($10^{-12}$ M). In conclusion, this *in vitro* study indicated that ZA-mediated cell proliferation was dose-dependent and that ZA did not inhibit cell proliferation at concentrations below $10^{-8}$ M. These findings suggest low concentrations of ZA have more of an effect on cell differentiation and mineralization, so low concentrations are better at regulating bone formation and repair.**

***Keywords:*** Osteogenesis imperfecta, proliferation, differentiation, mineralization

## 1. Introduction

Zoledronic acid (ZA) is the most potent bisphosphonate (BPs) in clinical use and has been used as an anti-resorptive agent to prevent bone resorption in the treatment of metabolic bone diseases like osteoporosis, Paget's disease, osteolytic disease, hypercalcemia of malignancy, and cancer-related osteolytic lesions (*1-3*). ZA regulates the bone balance by inhibiting bone resorptive activity of osteoclasts (OCs), thus effectively reducing bone loss and bone turnover (*4*,*5*). Recent evidence supports the notion that osteoblasts (OBs) could be target cells for ZA and that this action by ZA may in turn contribute to a decrease in OC formation and activity (*6*) but the mechanism for this action is less clear. Osteogenesis imperfecta (OI) is a heterogeneous group of genetic disorders characterized by low bone mass, increased bone fragility, and susceptibility to bone fractures with variable severity. Its main clinical symptoms include increased bone fragility, osteoporosis, susceptibility to fractures, and bone anisotropy, and OI is accompanied by blue sclerae, dentinogenesis imperfecta, hearing loss, excessive joint laxity, and muscle weakness (*7-10*). At present, ZA is the most promising therapy to treat OI, and this is especially true for its intravenous administration. ZA has become the accepted treatment for both adults and children who suffer from OI. Developmental dysplasia

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of the hip (DDH) is an abnormal alignment of the femoral head and acetabulum caused by unilateral or bilateral hip instability; DDH is due to genetics, breech birth, swaddling that dislocates the hips, and other factors (*11,12*). The clinical features of DDH differ vastly from those of OI (*e.g.* no osteoporosis), so in the current study DDH served as the control.

The current study used OBs and fibroblasts (FBs) from children with OI and DDH to investigate the effect of ZA on the biological function of OBs and FBs at the cellular level. This was done to determine the optimal concentration of ZA in terms of its effect on the proliferation, differentiation, and mineralization of primary OBs and FBs and to provide a theoretical basis for clinical use of ZA. This study explored the feasibility of ZA because of its role in bone formation and explored its use to treat bone-related diseases.

## 2. Materials and Methods

### 2.1. *Materials*

Bone and skin tissue were collected from children with OI and DDH. The study protocol was approved by the Ethics Committee of Shandong Medical Biotechnology Center, Ji'nan, Shandong, China and written informed consent was obtained.

### 2.2. *Cell lines and cell culture*

Primary OBs and FBs were obtained by collagenase digestion of bone and skin tissue from children with OI and DDH. Tissue was washed and placed in culture dishes with preheated phosphate buffered saline (PBS) (Gibco, NY, USA). Tissue was cultured in 5 mL serum-free Dulbecco's modified eagle medium (DMEM) (Gibco), and 12.5 U/μL collagenase type I (Sigma, NY, USA) was added for digestion at 37°C for 3 h. Cells were centrifuged and resuspended in growth medium. Cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 1× penicillin-streptomycin (Beyotime, Shanghai, China). Cells were cultured in a humidified atmosphere with 5% $CO_2$ at 37°C in 75 $cm^2$ plates. Medium was changed every 3 days until cell density reached 90%. Cells were then serially passaged and digested at 37°C for 3 min with 1 mL 0. 25% trypsin (Beyotime).

### 2.3. *Assay of alkaline phosphatase (ALP) activity (histochemistry)*

Four types of cells were cultured for 9 days in 6-well culture plates at a density of $1 \times 10^4$ cells/well. Cells were washed with 4°C precooled PBS (Gibco) and then fixed with 4% paraformaldehyde (PFA) (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) for 10 min before they were washed with PBS (Gibco) (4°C precooling). The samples were then incubated for 30 min with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate (Beyotime) at 37°C. The reaction was terminated by removing the substrate solution and washing with distilled water. ALP-positive cells appeared dark blue.

### 2.4. *Cell viability assay*

Four types of cells were cultured for 24 h in 96-well culture plates at a density of $2 \times 10^3$ cells/well and then growth medium was replaced. Cells were treated with ZA (SELLECK, Houston, USA) at concentrations of $10^{-3}$ M to $10^{-13}$ M at various times (2, 4, and 6 days). At the indicated times, a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay was performed by incubating cells with 20 μL 5 mg/mL MTT solution/well at 37°C for 4 h. The reaction was then stopped with 150 μL dimethyl sulfoxide (DMSO) (Amresco, OH, USA)/well. Color development was then analyzed by measuring absorbance at 490 nm (A490), and A490 thus corresponded to the viability of cells.

### 2.5. *Cell cycle analysis (flow cytometry)*

OBs and FBs were cultured in 6-well culture plates at a final density of $6.5 \times 10^4$ cells/well with or without $10^{-8}$ M, $10^{-9}$ M, or $10^{-10}$ M ZA for 2 days and 4 days, respectively. After culturing, cells were digested at 37°C for 3 min with 0.3 mL 0.25% trypsin. Cells were collected in 1.5 mL tubes and washed and they were then fixed with 4% PFA (Sinopharm Chemical Reagent Co. Ltd., Beijing, China) for 12 h in 4°C. Cells were washed with precooled PBS and stained with propidium (PI) (Beyotime) at 37°C for 30 min away from light. Flow cytometry was performed as described in the instructions to the Cell Cycle and Apoptosis Analysis Kit (Beyotime).

### 2.6. *Assay of ALP activity (biochemistry)*

Four types of cells were cultured as previously described in 6-well culture plates at a final density of $6.5 \times 10^4$ cells/well. They were treated with ZA for 6 days at a final concentration of $10^{-8}$ M, $10^{-10}$ M, and $10^{-8}$ M with dexamethasone (Sigma-Aldrich) serving as a positive control and growth medium without ZA serving as a normal control. Medium was replaced every 3 days. Samples were washed with PBS (Gibco) and digested and then cells were collected. One percent SDS cell lysis solution was added to each sample to lyse cells at 4°C for 2 h. Cell lysates were then obtained for analysis. The moieties of cell lysates were used to analyze protein content using the BCA Protein Assay Kit (Beyotime), with 5 μL/well cell lysates in 96-well plates. Color development was then analyzed by

measuring the optical density (OD) at 495 nm ($OD_{495}$). The moieties of cell lysates were analyzed to detect ALP activity by adding 80 μL *p*-nitrophenylphosphate (*p*-NPP) (Sigma-Aldrich) and incubating 40 μL/well cell lysates in 96-well plates at 37°C for 20 min. The reaction was then stopped with 3 N NaOH. The amount of *p*-nitrophenol (*p*-NP) product, corresponding to ALP activity, was measured at 405 nm. Activity was calculated using the formula [ALP activity = A/(X/5 × 40)].

2.7. *Mineralized matrix formation*

OBs from patients with OI were cultured in 24-well culture plates at a density of $8 \times 10^3$ cells/well. When the cells reached confluence, the medium was changed to induction medium (10% FBS (Gibco) and 1× penicillin-streptomycin (Beyotime), 500 μg/mL L-ascorbic acid (Sigma-Aldrich), and $10^{-2}$ mol/L β-glycerophosphate disodium salt hydrate (Sigma-Aldrich) for control cells. For treated cells, the medium was changed to induction medium with ZA at a final concentration of $10^{-6}$ M, $10^{-8}$ M, $10^{-10}$ M, and $10^{-12}$ M. Medium was replaced every 3 days. Matrix formation was detected at 18 and 21 days by washing cell matrix layers three times with PBS, fixing them with ice-cold 4% PFA (Sinopharm Chemical Reagent Co. Ltd.) for 10 min, and then washing them with distilled water. Matrix layers were then thoroughly stained with 1% alizarin red (Sinopharm Chemical Reagent Co. Ltd.) for 10 min, and excess stain was removed with distilled water. Mineralized matrix formation appeared in red.

2.8. *Statistical analysis*

Results are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA was used to determine the statistical significance of differences between the means of experiments if data had a normal distribution. A Wilcoxon test was used for non-parametric data, and $p < 0.05$ was considered to be statistically significant.

## 3. Results

3.1. *Assay of ALP activity (histochemistry)*

After plates were stained, microscopic images revealed OBs with a long, fusiform shape, elongation, abundant cytoplasm, and a clear nucleus. Cells grew in clumps and overlapped. FBs also had a long, fusiform shape with a round or elliptical nucleus, and FBs were larger than OBs. All of the four cell types were dyed dark bluish-purple, consistent with the characteristics of OBs and FBs. More OBs were stained and they had a deeper color than FBs. OBs had greater ALP activity than did FBs (Figure 1).

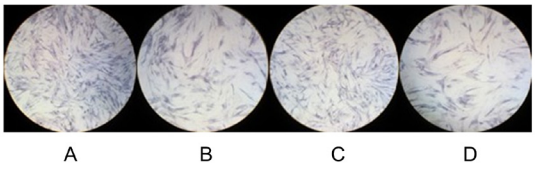


**Figure 1. Microscopic images of four types of cells after histochemical staining. (A)**, OBs from patients with DDH; **(B)**, FBs from patients with DDH; **(C)**, OBs from patients with OI; **(D)**, FBs from patients with OI. All four types of cells were stained deep bluish-purple.

3.2. *Cell viability assay*

Action of ZA was detected at three times (2, 4, and 6 days). ZA at a concentration of $10^{-6}$ M and higher inhibited the proliferation of OBs from patients with OI and DDH ($p < 0.05$). A ZA concentration between $10^{-7}$ M to $10^{-10}$ M slightly inhibited the proliferation of cells. ZA concentrations below $10^{-11}$ M tended to promote cell proliferation ($p < 0.05$). All of the ZA concentrations tested had little effect on FBs viability at the observed times. In addition, cell proliferation decreased most with ZA concentrations greater than $10^{-6}$ M (cell viability was approximately 90% of the control), but the rate of inhibition increased over time. There was a dose-dependent change in cell viability with a ZA concentration of $10^{-6}$ M or lower. Inhibition diminished with a lower ZA concentration as time passed. A ZA concentration below $10^{-11}$ M tended to promote cell proliferation. Proliferation of cells from patients with OI was promoted more than was the proliferation of cells from patients with DDH (Figure 2). *Note*: cell proliferation rate (%) = (OD of treated cells – OD of control cells)/OD of control cells × 100%.

3.3. *Cell cycle analysis*

Treatment with ZA concentrations of $10^{-8}$ M, $10^{-9}$ M, or $10^{-10}$ M arrested both OBs and FBs from patients with OI and DDH in the G1/G0 phase. Cell cycle arrest was more obvious in OBs than in FBs. ZA had more of an effect after 4 days than it did after 2 days, and it had more of an effect on cells from patients with DDH than it did on cells from patients with OI (Figure 3). *Note*: cell arrest rate = (percentage of ZA-treated cells in G1 phase – percentage of control cells in G1 phase)/percentage of control cells in G1 phase × 100%.

3.4. *Assay of ALP activity (biochemistry)*

ALP activity is a well-known marker of OBs differentiation. After 6 days of culturing, ALP activity was measured. Compared to the positive control (with $10^{-8}$ M dexamethasone) and normal control, $10^{-8}$ M and $10^{-10}$ M had no effect on ALP (Figure 4).

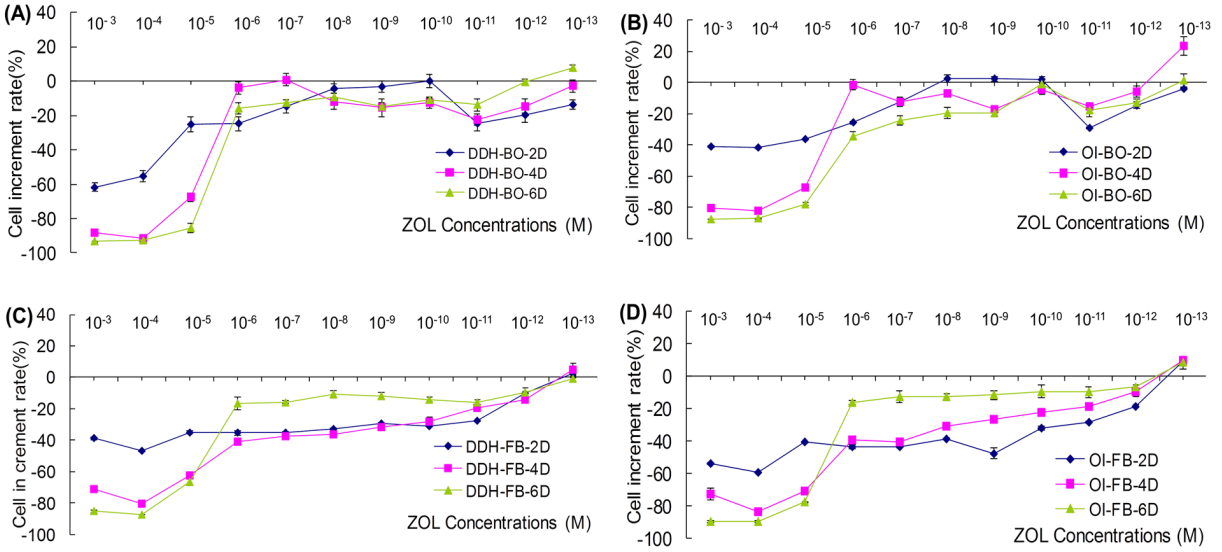


**Figure 2. OBs and FBs from patients with OI and DDH cultured in decreasing concentrations of ZA and assessment of the cell count and viability at 2, 4, and 6 days using MTT. (A)**, Effect on the proliferation of OBs from patients with DDH. At a concentration of $10^{-6}$ M and higher, ZA inhibited the proliferation of OBs from patients with OI and DDH ($p < 0.05$) after 2, 4, and 6 days of treatment. A ZA concentration between $10^{-7}$ M to $10^{-10}$ M slightly inhibited the proliferation of cells. At concentrations below $10^{-11}$ M, ZA tended to promote cell proliferation ($p < 0.05$), and this trend was more apparent with longer treatment; **(B)**, Effect on the proliferation of OBs from patients with OI (same as in **(A)**); **(C)**, Effect on the proliferation of FBs from patients with DDH. Generally, ZA inhibits FBs. ZA at concentrations greater than $10^{-6}$ M ($p < 0.05$) resulted in a significant decrease in the cell count compared to untreated control cells as time passed. At concentrations greater than $10^{-6}$ M, inhibition of cell proliferation diminished as time passed ($p < 0.05$). At concentrations below $10^{-11}$ M, cell proliferation tended to be promoted ($p < 0.05$); **(D)**, Effect on the proliferation of FBs from patients with OI (same as in **(C)**). Clearly, ZA acted on cells from patients with OI more than it did on cells from patients with DDH.

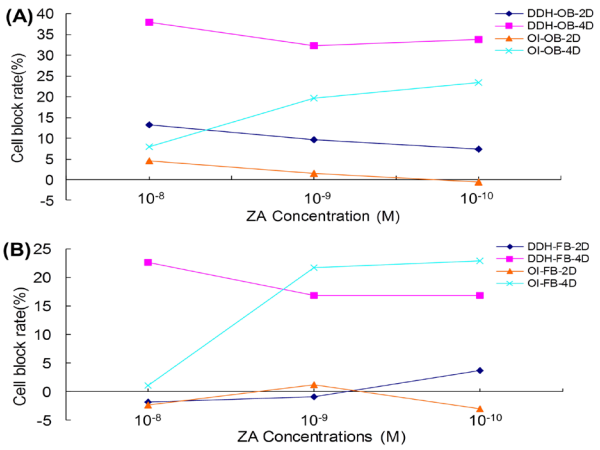


**Figure 3. Effect of ZA on cell cycle arrest. (A)**, ZA arrested the cell cycle of OBs in 2 days. This was true for OBs from patients with OI and those with DDH. After 4 days, the percentage of cells with a cycle arrested at G1/G0 decreased to 38%. A greater percentage of cells from patients with DDH than from patients with OI had their cycle arrested; **(B)**, After 2 days, ZA had no effect on the cell cycle of FBs in the G1/G0 phase. After 4 days, a $10^{-8}$ M concentration of ZA inhibited cells from patients with DDH at a rate of 23%; this was higher than the rate of inhibition of cells from patients with OI.

### 3.5. *Mineralized matrix formation*

In the absence of ZA, OBs stained positive with alizarin red in induction medium after 18 days, as did cells treated with $10^{-12}$ M ZA. A ZA concentration of $10^{-12}$ M promoted mineralization (Figure 5).

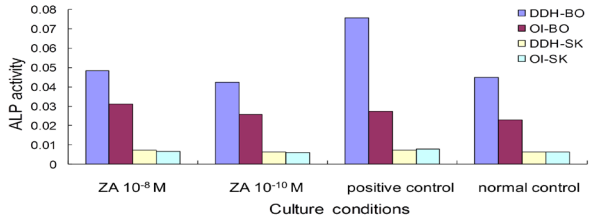


**Figure 4. Absence of ZA's effect on ALP activity.** Two concentrations of ZA had no effect on ALP, and OBs had greater ALP activity than did FBs.

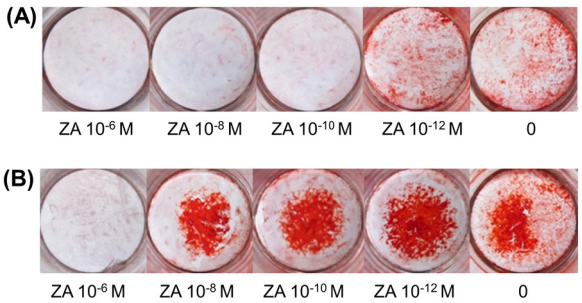


**Figure 5. Mineralization staining. (A)**, After 18 days of culturing, bone nodules were stained with alizarin red. Cultured control cells and cultured cells treated with $10^{-12}$ M ZA had mineralized matrix deposition in red; staining was more evident in cells treated with $10^{-12}$ M ZA than in control cells; **(B)**, After 21 days of culturing, all plates had mineralized matrix deposition in red except cells treated with $10^{-6}$ M ZA. Staining was more evident at a lower ZA concentration, and cells treated with $10^{-12}$ M ZA had more evident staining than control cells did.

## 4. Discussion

The first BP was approved in the US in 1977, and BPs are now widely used for the treatment of osteoporosis, hypercalcemia of malignancy, and Paget's disease. Clinical studies have revealed that third-generation BPs have greater efficacy and are more effective at preventing bone-related events. If treatment with other BPs fails, ZA is available. ZA is currently the only BP approved for use in treating a variety of bone diseases. Numerous reports have described the action of BPs on OCs. BPs can have a negative impact on OCs, inhibiting their formation and recruitment (*13,14*), inhibiting the activation of OCs by OBs (*15-18*), inhibiting the maturation and activity of OCs (*19,20*), and promoting the apoptosis of OCs (*21,22*). However, there is no experimental evidence that BPs can reduce the number of mature OCs (*23*). Nitrogen-containing BPs can act by inhibiting the mevalonate pathway and nitrogen-free BPs can act by interfering with the process of energy conversion inside a cell (*24,25*).

Numerous reports have described the role of BPs and their action on OBs in terms of their effect on the proliferation, differentiation, and mineralization of OBs, but the conclusions of these reports differ (*26-29*). The current study was an *in vitro* study. Findings indicated that ZA affected the proliferation of primary OBs in a dose-dependent manner. ZA is also toxic to human OBs at concentrations below $10^{-6}$ M, since ZA inhibited OBs and FBs proliferation. This inhibition was more evident over time, and the rate of inhibition was about 90% after 6 days of culturing. ZA concentrations from $10^{-7}$ M to $10^{-10}$ M slightly inhibited cell proliferation. ZA concentrations greater than $10^{-10}$ M tended to promote cell proliferation, and this trend was more evident over time. Second-generation BPs can promote the proliferation, differentiation, and mineralization of OBs, and a concentration of $10^{-8}$ M is optimal (*30,31*). ZA has little effect on cell proliferation (*32*), which is true according to the current results as well. The current results agree with the conclusions of Orriss *et al.* (*33*) and the hypothesis of Maruotti *et al*. (*34*).

ALP is an essential enzyme for bone formation, and ALP is an early indicator to identify and evaluate the degree of differentiation of OBs (*35*). ALP also reflects the activity of OBs. In the current study, treatment with ZA ($10^{-8}$ M and $10^{-10}$ M) led to changes in ALP activity. Alizarin red staining showed that mineralization/nodule formation increased as the ZA concentration decreased, which is consistent with the findings regarding cell proliferation.

In this study, different concentrations of ZA affected OBs proliferation differently. ZA may affect OI more during mineralization rather than during differentiation. Lower concentrations of ZA may be able to inhibit OCs but also promote OBs and FBs proliferation and differentiation. Selecting an appropriate dose and dosing regimen of ZA may help facilitate bone formation.

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*(Received December 26, 2012; Revised January 20,2013; Accepted January 25,2013)*