ARTICLE

Journal of Cellular Biochemistry 106:109–118 (2009)

Journal of Cellular Biochemistry

# Inhibition of Osteoblast Function In Vitro by Aminobisphosphonates

Isabel R. Orriss,[1] Michelle L. Key,[1] Kay W. Colston,[2] and Timothy R. Arnett[1]*

[1] *Department of Cell and Developmental Biology, University College London, London, UK*

[2] *Department of Cellular and Molecular Medicine, St. George's, University of London, London, UK*

## ABSTRACT

Bisphosphonates are analogues of pyrophosphate, a key physicochemical inhibitor of mineralisation. We examined the direct actions of bisphosphonates on the function of cultured osteoblasts derived from rat calvariae. Treatment with zoledronate, the most potent bisphosphonate studied, reduced osteoblast number at concentrations ≥100 nM and was strongly toxic at 10 μM, causing a threefold decrease in osteoblast viability after 2 days and a 90% decrease in cell numbers after 14 days. In control osteoblast cultures on plastic, abundant formation of 'trabecular' mineralised bone matrix nodules began after 10 days. Continuous exposure to zoledronate inhibited bone mineralisation at concentrations as low as 10 nM. Pamidronate and clodronate exerted similar effects but at higher doses (≥1 and ≥10 μM, respectively). Short-term or intermittent exposure of osteoblasts to zoledronate and pamidronate (1–10 μM) was sufficient to inhibit bone mineralisation by ≥85%. Zoledronate but not pamidronate or clodronate also strongly inhibited osteoblast alkaline phosphatase activity at concentrations ≥100 nM and soluble collagen production at concentrations ≥1 μM. We additionally studied the effects of zoledronate on osteoblasts cultured on dentine, a bone-like mineralised substrate, observing similar inhibitory effects, although at concentrations 10–100-fold higher; this shift presumably reflected adsorption of zoledronate to dentine mineral. Thus, zoledronate blocked bone formation in two ways: first, a relatively non-toxic, selective inhibition of mineralisation at concentrations in the low nanomolar range and second, a cytotoxic inhibition of osteoblast growth and function at concentrations ≥1 μM. Although no data are available on the bisphosphonate concentrations that osteoblasts could be exposed to in vivo, our results are consistent with earlier observations that bisphosphonates may inhibit bone formation. J. Cell. Biochem. 106: 109–118, 2009. © 2008 Wiley-Liss, Inc.

**KEY WORDS:** OSTEOBLASTS; BISPHOSPHONATES; ZOLEDRONATE; MINERALISATION; BONE FORMATION

The bisphosphonates are potent inhibitors of osteoclast activity and are widely used clinically to prevent the bone loss associated with conditions such as osteoporosis, Paget's disease and metastatic bone disease [Russell, 2006].

Bisphosphonates are chemically stable analogues of the mineralisation inhibitor inorganic pyrophosphate (PPi) in which the central oxygen atom is replaced by a carbon atom to form a P-C-P moiety; variations in the R1 and R2 side chains off the central carbon produce the individual bisphosphonates. Like PPi, bisphosphonates bind bone mineral with a high affinity and inhibit the formation and propagation of hydroxyapatite crystals [Jung et al., 1973]. The binding affinity of the different bisphosphonates for hydroxyapatite, and hence their uptake and persistence, is influenced by their R1 and R2 groups [Russell, 2006]; this is thought to explain the differences in their duration of action [Nancollas et al., 2006].

There are two major classes of bisphosphonates which have differing molecular modes of action. The first group comprises of the non-nitrogen containing bisphosphonates, such as clodronate and etidronate. These compounds are internalised, metabolised and incorporated into non-hydrolysable analogues of adenosine triphosphate (ATP), accumulation of which will inhibit cellular metabolism and lead to osteoclast cell death [Frith et al., 2001]. Nitrogen-containing bisphosphonates, such as zoledronate, pamidronate, alendronate and risedronate, make up the second, more potent class of bisphosphonate. These agents reduce osteoclast activity by inhibiting an important enzyme in the mevalonate pathway, farnesyl diphosphate synthase (FPPS), and blocking protein prenylation of small GTPases [Luckman et al., 1998; van Beek et al., 1999].

The ability of bisphosphonates to inhibit osteoclast formation and activity in vitro and in vivo is well documented [see reviews by

*Correspondence to: Timothy R. Arnett, Department of Cell and Developmental Biology, University College London, London WC1E 6BT, UK. E-mail: t.arnett@ucl.ac.uk

Received 20 June 2008; Accepted 7 October 2008 • DOI 10.1002/jcb.21983 • 2008 Wiley-Liss, Inc.

Published online 11 November 2008 in Wiley InterScience (www.interscience.wiley.com).

Russell, 2007; Silverman and Maricic, 2007]. However, their effects on osteoblast differentiation and function are less clear. Some in vivo studies have indicated that bisphosphonate treatment does not adversely effect osteoblast differentiation or bone formation [Chavassieux et al., 1997; Glorieux et al., 1998]. Furthermore, Recker et al. [2008] demonstrated that patients receiving yearly zoledronate infusions have normal osteoblast function and matrix mineralisation, despite having reduced bone turnover [Recker et al., 2008]. In contrast, others have reported inhibitory effects of bisphosphonates on osteoblast function and mineral apposition rate in vivo [Tobias et al., 1993; Iwata et al., 2006].

The in vitro data available are also conflicting and involve studies in a number of different experimental systems including mesenchymal stem cells, 'osteoblast-like' cell lines, and primary osteoblasts. Short-term treatment with micromolar concentrations of zoledronate has been reported to cause decreased proliferation, mineralisation, viability and alkaline phosphatase (ALP) activity in MC3T3-E1 and MG-63 cells [Schindeler and Little, 2005; Peter et al., 2005b; Tenta et al., 2006]. Recently, long-term, intermittent exposure to nanomolar concentrations of pamidronate and alendronate has been shown to inhibit bone nodule formation by mouse calvarial osteoblasts in vitro [Idris et al., 2008]. In contrast, a study by Pan et al. [2004] [Pan et al., 2004] reported that treatment of human bone-derived osteoblast-like cells with high concentrations of zoledronate (5–25 $\mu$M) increased mineral deposition, albeit accompanied by cytotoxic reductions in cell numbers and cell death. It has also been reported that zoledronate (10 nM) can promote the proliferation and differentiation of human mesenchymal stem cells [von Knoch et al., 2005]. Furthermore, in murine bone marrow cultures, the formation of mineralised nodules was reportedly stimulated by nanomolar concentrations of etidronate and alendronate but inhibited by micromolar concentrations [Giuliani et al., 1998]. Additionally, Duque and Rivas [2007] recently reported that nanomolar concentrations of alendronate promote the differentiation of human mesenchymal stem cells towards the osteoblastic lineage.

The objective of this investigation was to determine the direct effects of three different bisphosphonates (zoledronate, pamidronate and clodronate) on the growth, function and survival of cells derived from rat calvaria as they proliferate and differentiate from precursors into mature bone-forming osteoblasts.

## MATERIALS AND METHODS

### REAGENTS

Reagents were purchased from Sigma–Aldrich (Poole, UK) unless otherwise stated. Elephant ivory was kindly donated by HM Customs and Excise (Heathrow, London, UK). In all tissue culture experiments, medium pH, $pCO_2$ and $pO_2$ were monitored throughout using a blood gas analyser (ABL-705, Radiometer, Crawley, UK).

### OSTEOBLAST CELL CULTURE

Primary rat osteoblast cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague-Dawley rats using a 3-step process (1% trypsin in PBS for 10 min; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 min; 0.2% collagenase type II in HBSS for 60 min). The first two digests were discarded and the cells resuspended in Dulbecco's Modified Essential Medium (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 0.25 $\mu$g/ml amphotericin (complete mixture abbreviated to 'DMEM'). Cells were cultured for 2–4 days in a humidified atmosphere of 5% $CO_2$–95% air at 37°C in 75 $cm^2$ flasks until confluent. Upon confluence, cells were sub-cultured into 24-well plates or onto 5 mm diameter dentine discs in DMEM supplemented with 2 mM $\beta$-glycerophosphate, 50 $\mu$g/ml ascorbic acid and 10 nM dexamethasone (mixture abbreviated to 'supplemented DMEM'), with half medium changes every 3 days. Osteoblasts were cultured in the presence of different bisphosphonates (zoledronate, pamidronate or clodronate), either continuously or intermittently, at concentrations between 1 nM and 10 $\mu$M. For continuous exposure, fresh bisphosphonates were added at every medium change. Intermittent dosing was for 24 h every 3 days, with two PBS washes on removal of the bisphosphonate.

Bone nodule formation by osteoblasts cultured in 24-well plates was measured using an assay described previously [Brandao-Burch et al., 2005; Utting et al., 2006; Orriss et al., 2007]. This method enables organic (collagenous) matrix deposition and mineralisation to be studied independently. Briefly, experiments were terminated by fixing cell layers in 2% glutaraldehyde for 5 min; mineralised bone nodules were visualised by staining with alizarin red (1% solution in water) for 5 min, rinsed with 50% ethanol to remove excess stain, then air-dried. The plates were imaged at 2,000 dots/$cm^2$ using a high-resolution, flat-bed scanner (Epson Perfection Photo 3200). Binary images of each individual well were then subjected to automated analysis (Scion Image software, Scion Corporation; http://www.scioncorp.com), using constant 'threshold' and 'minimum particle' levels, to determine the number and plan surface area of mineralised bone nodules.

### CELL PROLIFERATION ASSAY

Osteoblasts were cultured in 24-well trays or on dentine discs and cell number was measured at 4, 7 and 14 days of culture using the CytoTox 96® non-radioactive cytotoxicity assay (Promega UK, Southampton, UK). This assay quantitatively measures cellular lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released on cell lysis. LDH oxidises lactate into pyruvate, generating NADH, which is then used to convert a tetrazolium salt into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. One hour prior to assaying, osteoblasts were switched to serum free DMEM containing lysis buffer (1% Triton X-100 in water); following sample collection the assay was performed as per manufacturer's instructions. A standard curve for determination of cell numbers was constructed using cells seeded at $10^2$ to $10^6$/well. Manual cell counts were performed in parallel for assay validation.

### DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY AND EXPRESSION

The ALP activity of cell lysates was determined colorimetrically (Bio-Tek $EL_X800$ plate reader, Fisher Scientific, Loughborough, UK) using a commercially available kit (Biotron Diagnostics, CA); this

assay uses $p$-nitrophenyl phosphate as a substrate, which in the presence of ALP, is converted to the yellow chromogen $p$-nitrophenyl. Osteoblasts were cultured in 24-well trays or on dentine discs and enzyme activity measured at progressive stages of differentiation (7 and 14 days). To assay ALP activity, cell layers were washed and cells harvested using a scraper ($n = 6$) followed by sonication at 4°C and centrifugation at 500$g$. The supernatant was collected and stored at 4°C until assaying at pH 9.8. Total protein in cell lysates was determined using the Bradford assay (Sigma–Aldrich).

### MEASUREMENT OF COLLAGEN PRODUCTION

Collagen production was determined in osteoblasts after 7 and 14 days of culture; total protein concentration in lysates was determined using the Bradford assay. To measure soluble collagen production, osteoblasts were transferred to medium containing 5% FCS, 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid, 10 nM dexamethasone and the lysyl oxidase inhibitor β-aminoproponitrile (BAPN, 50 μg/ml) for the final 24 h of culture. The concentration of collagen accumulated in the tissue culture medium was assayed using a Sirius red dye-based kit (Sircol™ collagen assay, Biocolor

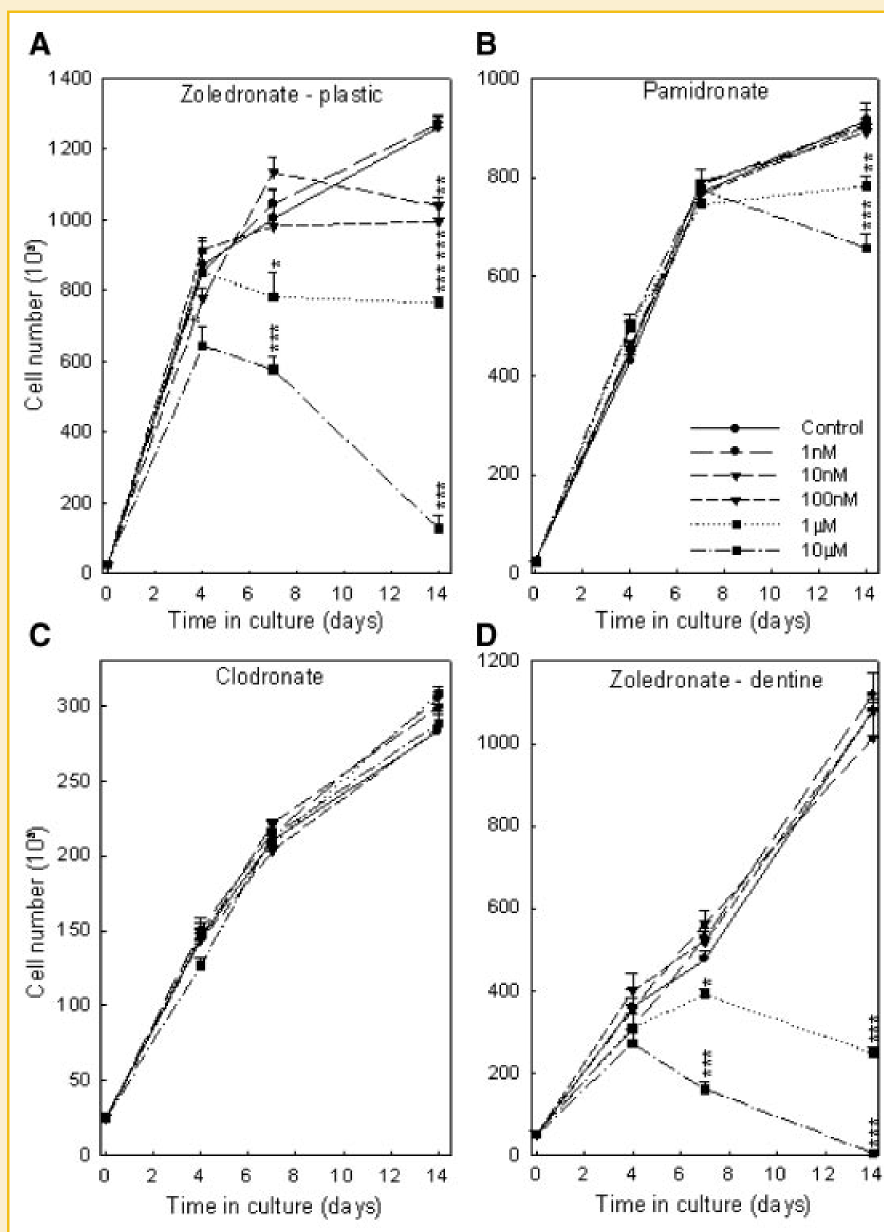


Fig. 1. Zoledronate and pamidronate inhibit osteoblast growth. Time- and dose-dependent inhibition of osteoblast growth on plastic by zoledronate (A) and pamidronate (B) but not clodronate (C). Zoledronate also strongly inhibited the growth of osteoblasts cultured on dentine discs (D), albeit from a higher threshold concentration (≥1 μM). Significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; values are means ± SEM (n = 6).

Ltd, Newtownabbey, UK) according to the manufacturer's instructions. This assay measures collagen Types I–IV but does not discriminate between collagen types.

### ASSESSMENT OF CELL VIABILITY

Cell viability was investigated using the LDH-based CytoTox 96® cytotoxicity assay (Promega UK), after a single dose of bisphosphonate. To measure cellular LDH, osteoblasts were transferred to medium containing 5% FCS and 1 nM–10 μM bisphosphonate. Samples were taken at 6, 24 and 48 h post treatment (24 h only for cells on dentine): all test samples were kept at 4°C until assaying. To establish total cellular LDH levels, osteoblasts were switched to DMEM containing 5% FCS and lysis buffer (1% Triton X-100 in water) for 1 h before sample collection. The LDH content of the supernatants and cell lysates was assayed as per manufacturer's instructions. Cell viability was estimated by expressing released LDH as a percentage of the total cellular LDH.

### STATISTICAL ANALYSIS

Statistical comparisons were made by one-way analysis of variance and adjusted using the Bonferroni method. Results shown are for representative experiments that were each repeated at least three times.

## RESULTS

### ZOLEDRONATE AND PAMIDRONATE INHIBIT OSTEOBLAST GROWTH

Osteoblast number was measured at 4, 7 and 14 days of culture. Zoledronate had the most potent effect on cell number, with inhibitory effects evident at all time points (Fig. 1A); by day 14 of culture, treatment with 10 nM, 100 nM, 1 μM and 10 μM zoledronate caused 20%, 30%, 40% and 90% decreases in osteoblast number, respectively. Treatment with pamidronate had no effect at 4 and 7 days of culture; by day 14, 1 and 10 μM pamidronate resulted in a 15% and 30% decrease in osteoblast number, respectively (Fig. 1B). Clodronate did not affect cell number at any time point (Fig. 1C). Significant reductions in cell number were also seen in osteoblasts grown on dentine, with inhibitory effects apparent from day 7; 1 and 10 μM zoledronate decreased cell number at day 14 by 80% and 99%, respectively (Fig. 1D). Pamidronate and clodronate did not affect osteoblast proliferation when cells were cultured on dentine (not shown).

### BISPHOSPHONATES INHIBIT BONE FORMATION BY RAT OSTEOBLASTS IN VITRO

Primary rat osteoblasts cultured in osteogenic medium for 14 days on plastic formed abundant mineralised bone nodules that typically display a 'trabecular-shaped' morphology (Fig. 2A). Bisphosphonate treatment significantly inhibited formation of alizarin red-stained bone nodules. Zoledronate, the most potent bisphosphonate tested, selectively inhibited nodule mineralisation at low concentrations ($IC_{50} \approx 10$ nM) in a relatively non-toxic manner, resulting in the formation of partially mineralised collagenous matrix (Fig. 2B). At higher concentrations ($\geq 1$ μM), zoledronate completely inhibited bone nodule formation (Figs. 2C and 3A). In cultures treated with 1 and 10 μM pamidronate, some deposition of collagenous matrix

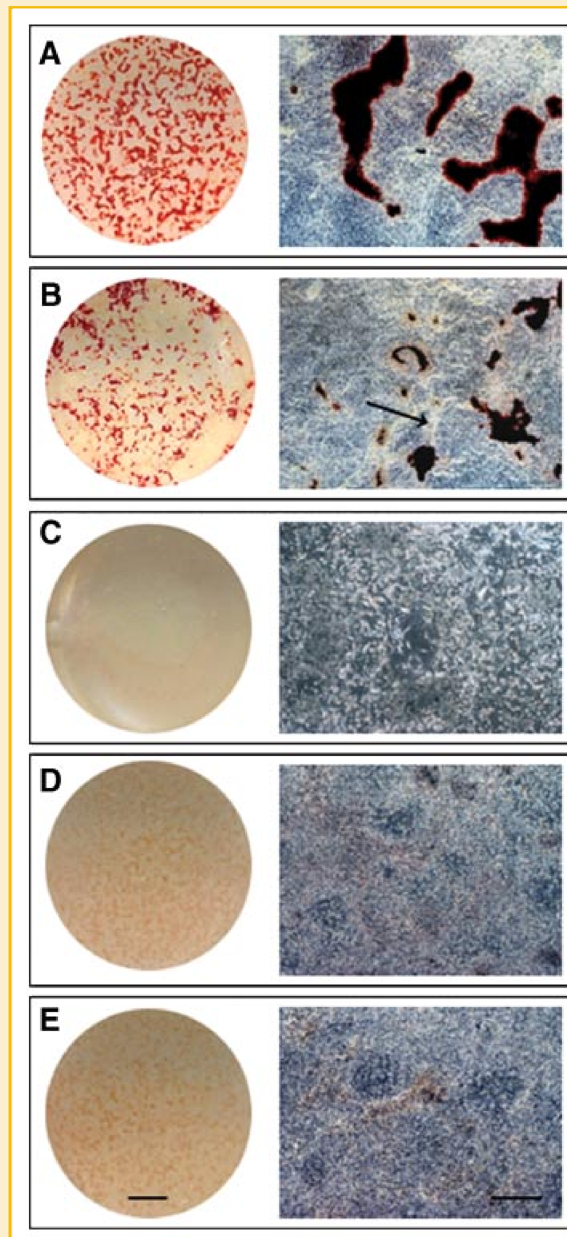


Fig. 2. Effect of bisphosphonate treatment on bone nodule formation by rat osteoblasts. Representative images of 14-day primary rat osteoblast cultures, stained with alizarin red to demonstrate mineralisation. Images on the left are low power scans of a tissue culture well (scale bar = 0.2 cm); images on the right are higher power details, viewed by phase-contrast microscopy (scale bar = 50 μm). A: Formation of typical mineralised 'trabecular' bone matrix structures in control cultures. B: 10 nM zoledronate decreased bone nodule mineralization (evidenced by the reduction in alizarin red staining); however, organic matrix deposition appeared unaffected (as highlighted by the arrow in the phase contrast micrograph). C: Treatment with 1 μM zoledronate inhibited both mineralisation and collagen deposition, with widespread cell death. Treatment with both 10 μM pamidronate (D) and 10 μM clodronate (E) inhibited bone nodule mineralisation, without affecting organic matrix formation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

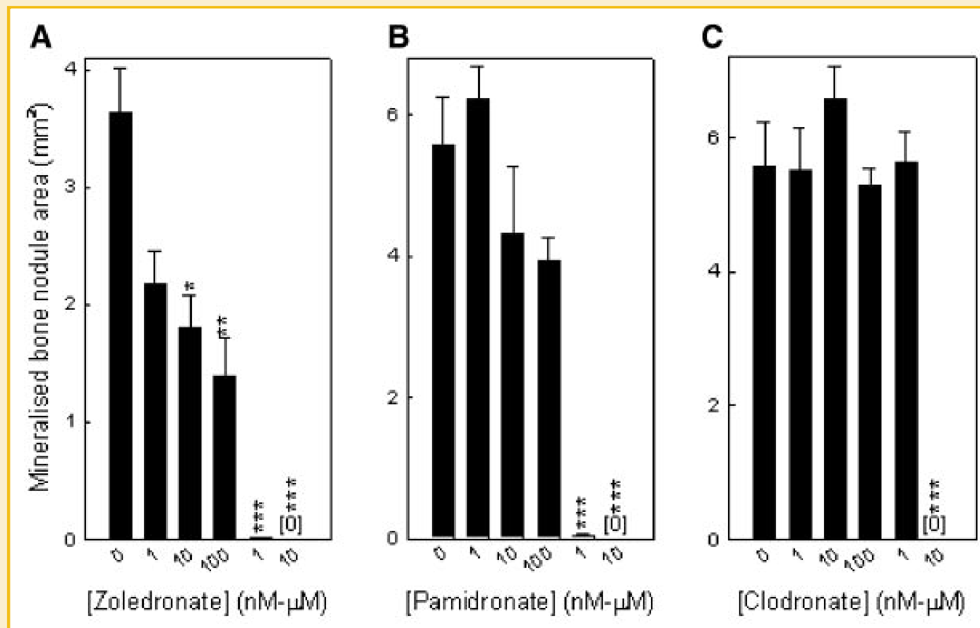


Fig. 3. Bisphosphonates inhibit bone nodule formation. A: Continuous exposure to zoledronate inhibited or abolished formation of mineralised bone nodules by osteoblasts in 14-day cultures at concentrations of ≥10 nM or ≥1 μM, respectively. Pamidronate (B) and clodronate (C) were less potent, blocking bone formation at concentrations ≥1 and ≥10 μM, respectively. Significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; values are means ± SEM (n = 6).

was still evident, although mineralisation was completely blocked; at lower concentrations (10–100 nM), no significant effects of pamidronate on mineralised nodule formation were observed, although a downward trend was noted (Figs. 2D and 3B). Clodronate was the least potent of the three bisphosphonates tested blocking bone nodule mineralisation, but not collagenous matrix deposition, at the highest concentration tested (10 μM) (Figs. 2E and 3C).

We also tested the effects of varying the duration of exposure of osteoblasts in 14-day cultures to bisphosphonates. Cells were either cultured until the onset of bone formation (day 10) and exposed to a single dose of bisphosphonate for the final 4 days or treated intermittently with bisphosphonate for 24 h every 3 days for the duration of the culture. A single dose of 1 and 10 μM zoledronate was sufficient to inhibit bone formation by 85% and 95%, respectively, as assessed by alizarin red staining (Fig. 4A); treatment with 10 μM pamidronate for 4 days also inhibited bone formation by 95%, whilst clodronate was without effect (not shown). Intermittent exposure to both 1 μM zoledronate (Fig. 4B) and 1 μM pamidronate (Fig. 4C) inhibited bone formation by 97% and 80%, respectively; treatment with 10 μM zoledronate or pamidronate caused complete abolition of bone formation. Intermittent exposure to clodronate, even at the highest doses, did not effect bone formation (Fig. 4D).

## ZOLEDRONATE INHIBITS COLLAGEN PRODUCTION BY OSTEOBLASTS

The effect of continuous bisphosphonate treatment on collagen formation by calvarial osteoblasts was investigated at 7 and 14 days of culture. Exposure to 1 and 10 μM zoledronate caused ~80% and 100% inhibition of soluble collagen production, respectively; whilst concentrations ≤100 nM were without effect (Fig. 5A). Treatment with pamidronate (Fig. 5B) and clodronate (data not shown), at concentrations up to 10 μM, had no effect on soluble collagen levels at either time point.

## THE EFFECT OF BISPHOSPHONATES ON OSTEOBLAST ALKALINE PHOSPHATASE ACTIVITY

The effect of continuous bisphosphonate treatment on ALP activity of osteoblasts was investigated at 7 and 14 days of culture on plastic. Zoledronate caused up to 78%, 98% and >99% inhibition of ALP activity at concentrations of 100 nM, 1 μM and 10 μM, respectively (Fig. 6A). Zoledronate also caused dose-dependent inhibition of the ALP activity of osteoblasts cultured on dentine but at concentrations that were ≥10-fold higher (Fig. 6B). In contrast, neither pamidronate nor clodronate, at concentrations up to 10 μM, inhibited ALP activity of osteoblasts cultured on plastic or dentine (not shown).

## ZOLEDRONATE DECREASES OSTEOBLAST VIABILITY

Since bisphosphonate treatment, particularly zoledronate, had a significant inhibitory effect on cell number, osteoblast viability was measured at 6, 24 and 48 h after a single dose of 1 nM–10 μM bisphosphonate. Cellular LDH levels in the medium were increased two- and threefold following treatment with 1 and 10 μM zoledronate, respectively, at each time point (Fig. 7A). Culture on dentine did not protect osteoblasts from the negative effects of zoledronate on cell survival; osteoblast viability was decreased 60% and 70% 24 h after treatment with 1 and 10 μM zoledronate, respectively (Fig. 7B). Pamidronate did not affect osteoblast viability when cells were cultured on plastic (Fig. 7C) but caused small (≤40%) decreases

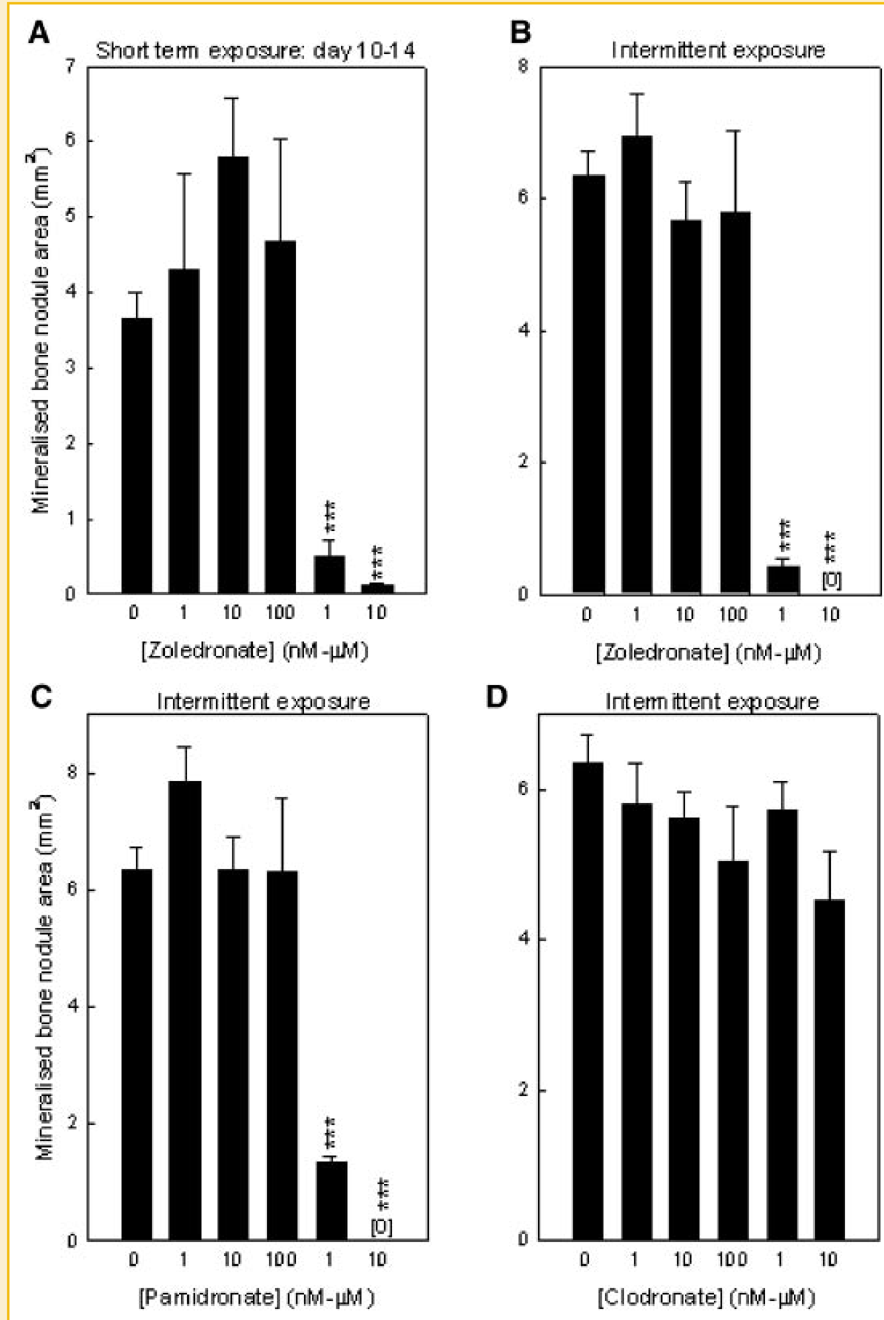


Fig. 4. Short term or intermittent exposure to bisphosphonates inhibits bone formation. A: Single doses of 1 and 10 μM zoledronate inhibited bone formation by cultured osteoblasts by 85% and 95%, respectively. B: Intermittent zoledronate treatment (see details in Materials and Methods Section) blocked bone formation at concentrations of ≥1 μM. C: Intermittent treatment with 1 and 10 μM pamidronate inhibited mineralised nodule formation by 80% and 100%, respectively. D: Intermittent treatment with clodronate did not affect bone formation. Significantly different from control: $^{***}P < 0.001$; values are means ± SEM (n = 6).

in viability when cultured on dentine (not shown). Clodronate did not affect osteoblast viability on either plastic or dentine.

## DISCUSSION

Bisphosphonates are widely used clinically to treat bone disorders, such as osteoporosis, Paget's disease and metastatic bone disease [Russell, 2006] which involve increased osteoclast resorption. Although the inhibitory effects of bisphosphonates on osteoclast formation and activity are well documented, both in vivo and in vitro, their effects on osteoblast function have been less well-studied. However, there is evidence from in vivo studies to suggest that bisphosphonates may suppress bone formation in rats [Tobias et al., 1993; Iwata et al., 2006] and impair the anabolic

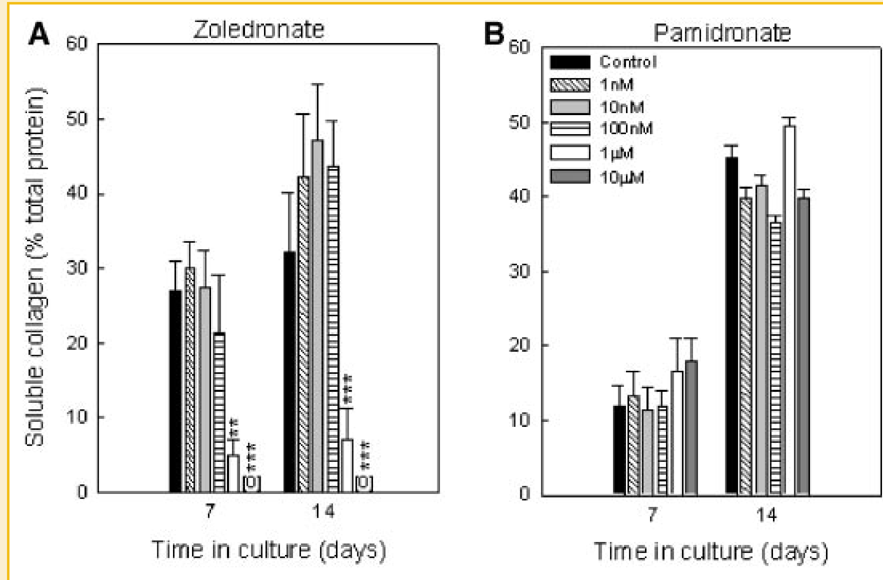


Fig. 5. Zoledronate inhibits collagen production by osteoblasts. A: In osteoblast cultures treated with 1 and 10 μM zoledronate, soluble collagen in culture medium was decreased by 80% and 100%, respectively. B: Pamidronate (and clodronate; not shown) did not affect collagen production. Significantly different from control: $^{**}P < 0.01$, $^{***}P < 0.001$; values are means ± SEM (n = 6).

response of bone to parathyroid hormone in sheep, humans, and mice [Delmas et al., 1995; Black et al., 2003; Samadfam et al., 2007].

The primary calvarial osteoblast culture system used here provides the opportunity to study separately the processes of bone matrix deposition and mineralisation; furthermore, it enables cell proliferation and survival to be investigated as the cells differentiate from precursors into mature bone-forming osteoblasts [Orriss et al., 2007]. We found that all three bisphosphonates tested significantly

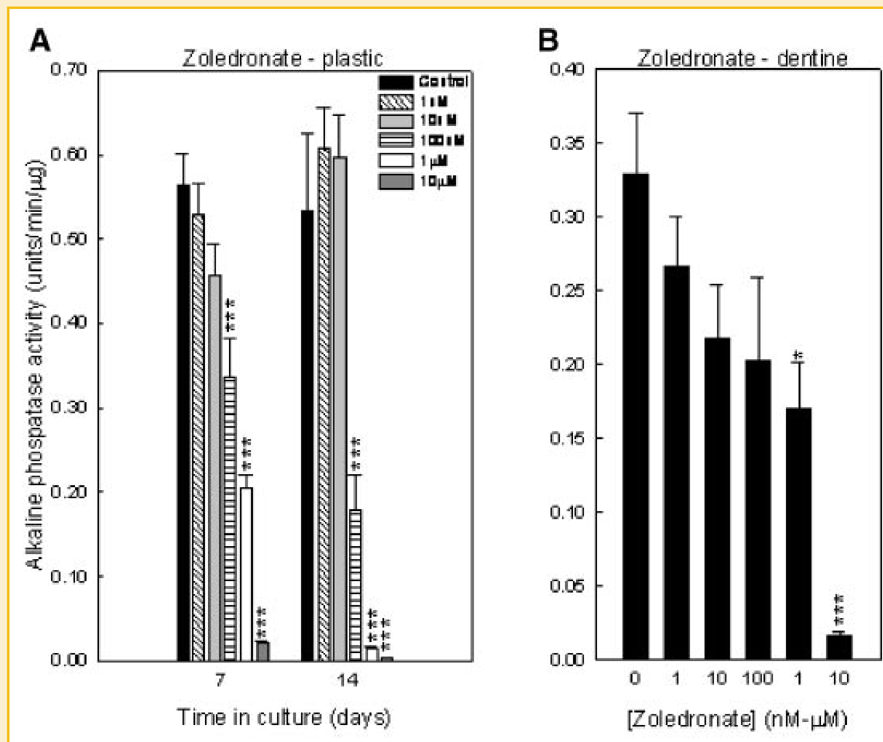


Fig. 6. The effect of zoledronate on alkaline phosphatase (ALP) activity. A: Zoledronate caused strong, dose-dependent inhibition of osteoblast ALP activity at concentrations ≥100 nM. B: Zoledronate, at concentrations ≥1 μM, also inhibited the ALP activity of osteoblasts cultured on dentine for 14 days. Significantly different from control: $^{*}P < 0.05$, $^{***}P < 0.001$; values are means ± SEM (n = 6).

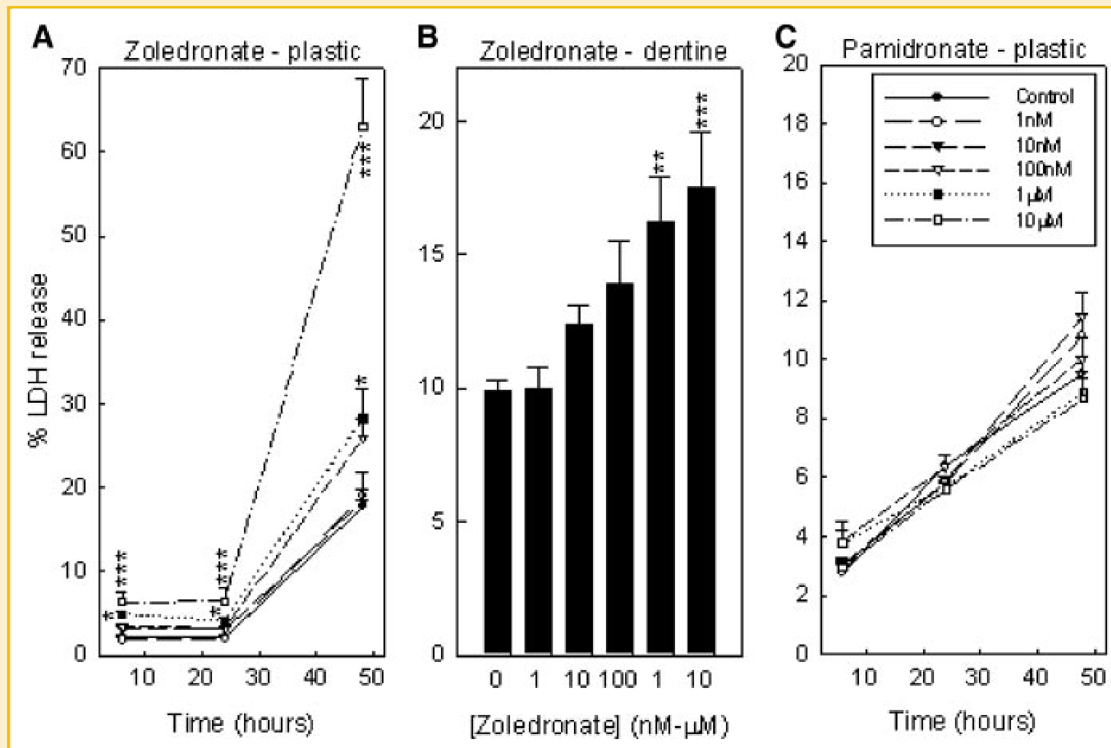


Fig. 7. Zoledronate treatment decreases osteoblast viability. A: Treatment with 1 μM or 10 μM zoledronate caused two- and threefold increases, respectively, in medium lactate dehydrogenase (LDH), a negative index of cell viability, at all time points. B: Zoledronate treatment for 24 h also dose-dependently increased LDH release from osteoblasts cultured on dentine. C: Pamidronate did not affect osteoblast viability. Significantly different from control: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; values are means ± SEM (n = 6).

inhibited bone nodule formation, the order of potency being zoledronate > pamidronate > clodronate. Chronic exposure to zoledronate appeared to have two distinct effects: first, a selective inhibition of mineralised bone nodule formation (at nanomolar concentrations) and second, a cytotoxic inhibition of osteoblast proliferation, collagen production and the deposition of 'trabecular-shaped' bone nodules (at concentrations ≥1 μM). The cytotoxic action of higher concentrations of zoledronate on osteoblasts was confirmed by decreased cell viability (measured as LDH release) after 6, 24 and 48 h of treatment. These data are consistent with earlier studies which demonstrated that a single dose of zoledronate inhibited cell proliferation in MG-63 cells [Tenta et al., 2006] and human osteoblasts [Greiner et al., 2007]. However, our results differ from those of von Knoch et al. [2005], who demonstrated increased proliferation and differentiation in mesenchymal stem cells treated with zoledronate (10 nM). In contrast, pamidronate and clodronate exerted little or no inhibitory effect on osteoblast numbers and viability or collagen production; however, both agents significantly inhibited bone mineralisation at micromolar concentrations. Treatment with zoledronate, pamidronate or clodronate has also been shown to cause decreased mineral deposition, without effecting differentiation, in cultures of human mesenchymal stem cells [Kellinsalmi et al., 2005].

Since there are distinct structural similarities between the bisphosphonates and the mineralisation inhibitor, pyrophosphate, it is likely that the inhibition of bone matrix mineralisation by low concentrations (≥10 nM) of zoledronate (and by higher concentrations of pamidronate and clodronate) involves direct physiochemical effects on hydroxyapatite crystal propagation. In broad agreement with our observations, a recent study has shown that ~250 nM zoledronate causes half-maximal inhibition of hydroxyapatite crystal growth in a cell-free system [Nancollas et al., 2006]. However, our results suggest that decreased osteoblast ALP activity could also contribute to the inhibition of mineralisation in zoledronate-treated cultures. Other investigators have also reported an inhibitory effect of zoledronate on osteoblast ALP activity in vitro, albeit at higher concentrations (50 μM) [Schindeler and Little, 2005]. Conversely, a recent in vivo study [Recker et al., 2008] demonstrated that whilst yearly infusions of zoledronate led to a suppression of bone formation markers, the mineral apposition rate was increased. These contrasting findings may reflect, inter alia, major differences in dosing regimens and the adsorption of zoledronate onto bone surfaces in vivo.

Following administration in vivo, bisphosphonates appear to be rapidly adsorbed onto mineralised bone surfaces from the microcirculation [Russell, 2007]. Thus, osteoblasts in situ are unlikely to be exposed to bisphosphonates in free solution for long periods, although they could be in close proximity to adsorbed bisphosphonates for months or even years. To model the in vivo pharmacokinetics of bisphosphonates more closely, we performed

experiments in which osteoblasts were cultured on discs of dentine, a relatively homogeneous mineralised tissue with a similar composition to bone. Overall, the inhibitory actions of bisphosphonates in this system were about 10–100-fold less potent than when osteoblasts were cultured on plastic. At concentrations $\geq$1 μM, zoledronate caused significant reductions in cell number, viability and ALP activity, whereas pamidronate and clodronate were without effect. These results may go some way towards explaining the apparent lack of inhibitory effect of bisphosphonates on osteoblast function in vivo in some studies [Chavassieux et al., 1997; Glorieux et al., 1998].

We also investigated the effects of different dosing regimens on bone formation in vitro. Both short term (final 4 days of culture) and intermittent (24 h every 3 days) exposure of osteoblasts cultured on plastic to zoledronate and pamidronate caused significant reductions in bone mineralisation. Our observations are highly consistent with those of Idris et al. [2008], who recently reported that intermittent treatment with pamidronate or alendronate in the high nanomolar range inhibited bone nodule formation by mouse calvarial osteoblasts in vitro. However, our results appear to be at variance with those of Pan et al. [2004], who reported that, despite decreased osteoblast number and increased cell death, treatment of human bone-derived osteoblasts with high concentrations of zoledronate (5–25 μM) increased mineralised matrix formation. Furthermore, our results also differ somewhat from those of Peter et al. [2005a], who found that higher concentrations of zoledronate ($\geq$10 μM) were required to inhibit the proliferation of and osteoblast-like cell lines. It is possible that immortalised cell lines may be less sensitive than primary cells to the cytotoxic action of zoledronate and other bisphosphonates. Consistent with this notion, we previously showed that relatively high concentrations of zoledronate ($\geq$10 μM) are needed to inhibit the viability of breast cancer cell lines [Senaratne et al., 2000].

The present results must be interpreted with some caution. Bisphosphonates adsorb to bone mineral and the concentrations of bisphosphonates to which bone cells are exposed in vivo are not known. Osteoblasts are likely to experience lower intracellular levels of bisphosphonates than actively resorbing osteoclasts. In support of this notion, we found that osteoclast-forming human peripheral blood mononuclear cell and mouse marrow cultures on dentine were considerably more sensitive to the inhibitory effects of bisphosphonates than osteoblasts. Surprisingly, we observed consistently that clodronate caused graded inhibitions of osteoclast numbers and resorption from the lowest dose tested (1 nM); the response to pamidronate was also graded, but with clear evidence of cytotoxicity at concentrations $\geq$1 μM. In contrast, the response to zoledronate was more 'quantal' in nature, with a sharp cytotoxic inhibition of osteoclast numbers and function evident at concentrations $\geq$10 nM [Orriss et al., unpublished work].

Although osteoblasts, including bone lining cells, might experience lower concentrations of bisphosphonates than osteoclasts, they may be exposed for longer periods. Moreover, osteocytes, owing to their fixed location and the high surface area presented by their canalicular projections through the bone matrix, may be at particular risk of exposure to any long-term effects of low-level bisphosphonates.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of the Arthritis Research Campaign (ARC), Biotechnology and Biological Sciences Research Council (BBSRC) and the European Union (EU; FP7 Programme).

## REFERENCES

Black DM, Greenspan SL, Ensrud KE, Palermo L, McGowan JA, Lang TF, Garnero P, Bouxsein ML, Bilezikian JP, Rosen CJ. 2003. The effects of parathyroid hormone and alendronate alone or in combination in postmenopausal osteoporosis. N Engl J Med 349:1207–1215.

Brandao-Burch A, Utting JC, Orriss IR, Arnett TR. 2005. Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcif Tissue Int 77:167–174.

Chavassieux PM, Arlot ME, Reda C, Wei L, Yates AJ, Meunier PJ. 1997. Histomorphometric assessment of the long-term effects of alendronate on bone quality and remodeling in patients with osteoporosis. J Clin Invest 100: 1475–1480.

Delmas PD, Vergnaud P, Arlot ME, Pastoureau P, Meunier PJ, Nilssen MH. 1995. The anabolic effect of human PTH (1-34) on bone formation is blunted when bone resorption is inhibited by the bisphosphonate tiludronate—Is activated resorption a prerequisite for the in vivo effect of PTH on formation in a remodeling system? Bone 16:603–610.

Duque G, Rivas D. 2007. Alendronate has an anabolic effect on bone through the differentiation of mesenchymal stem cells. J Bone Miner Res 22:1603–1611.

Frith JC, Monkkonen J, Auriola S, Monkkonen H, Rogers MJ. 2001. The molecular mechanism of action of the antiresorptive and anti-inflammatory drug clodronate: Evidence for the formation in vivo of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis. Arthritis Rheum 44:2201–2210.

Giuliani N, Pedrazzoni M, Negri G, Passeri G, Impicciatore M, Girasole G. 1998. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures in vitro and promote early osteoblastogenesis in young and aged mice in vivo. Bone 22:455–461.

Glorieux FH, Bishop NJ, Plotkin H, Chabot G, Lanoue G, Travers R. 1998. Cyclic administration of pamidronate in children with severe osteogenesis imperfecta. N Engl J Med 339:947–952.

Greiner S, Kadow-Romacker A, Lubberstedt M, Schmidmaier G, Wildemann B. 2007. The effect of zoledronic acid incorporated in a poly(D,L-lactide) implant coating on osteoblasts in vitro. J Biomed Mater Res A 80:769–775.

Idris AI, Rojas J, Greig IR, van't Hof RJ, Ralston SH. 2008. Aminobisphosphonates cause osteoblast apoptosis and inhibit bone nodule formation in vitro. Calcif Tissue Int 82:191–201.

Iwata K, Li J, Follet H, Phipps RJ, Burr DB. 2006. Bisphosphonates suppress periosteal osteoblast activity independently of resorption in rat femur and tibia. Bone 39:1053–1058.

Jung A, Bisaz S, Fleisch H. 1973. The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. Calcif Tissue Res 11:269–280.

Kellinsalmi M, Monkkonen H, Monkkonen J, Leskela HV, Parikka V, Hamalainen M, Lehenkari P. 2005. In vitro comparison of clodronate, pamidronate and zoledronic acid effects on rat osteoclasts and human stem cell-derived osteoblasts. Basic Clin Pharmacol Toxicol 97:382–391.

Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ. 1998. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res 13:581–589.

Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, Mangood A, Russell RG, Ebetino FH. 2006. Novel insights into actions of bisphosphonates on bone: Differences in interactions with hydroxyapatite. Bone 38:617–627.

Orriss IR, Utting JC, Brandao-Burch A, Colston K, Grubb BR, Burnstock G, Arnett TR. 2007. Extracellular nucleotides block bone mineralization in vitro: Evidence for dual inhibitory mechanisms involving both $P2Y_2$ receptors and pyrophosphate. Endocrinology 148:4208–4216.

Pan B, To LB, Farrugia AN, Findlay DM, Green J, Gronthos S, Evdokiou A, Lynch K, Atkins GJ, Zannettino AC. 2004. The nitrogen-containing bisphosphonate, zoledronic acid, increases mineralisation of human bone-derived cells in vitro. Bone 34:112–123.

Peter B, Pioletti DP, Laib S, Bujoli B, Pilet P, Janvier P, Guicheux J, Zambelli PY, Bouler JM, Gauthier O. 2005a. Calcium phosphate drug delivery system: Influence of local zoledronate release on bone implant osteointegration. Bone 36:52–60.

Peter B, Zambelli PY, Guicheux J, Pioletti DP. 2005b. The effect of bisphosphonates and titanium particles on osteoblasts: An in vitro study. J Bone Joint Surg Br 87:1157–1163.

Recker RR, Delmas PD, Halse J, Reid IR, Boonen S, Garcia-Hernandez PA, Supronik J, Lewiecki EM, Ochoa L, Miller P, Hu H, Mesenbrink P, Hartl F, Gasser J, Eriksen EF. 2008. Effects of intravenous zoledronic acid once yearly on bone remodeling and bone structure. J Bone Miner Res 23:6–16.

Russell RG. 2006. Bisphosphonates: From bench to bedside. Ann NY Acad Sci 1068:367–401.

Russell RG. 2007. Bisphosphonates: Mode of action and pharmacology. Pediatrics 119 (Suppl 2): S150–S162.

Samadfam R, Xia Q, Goltzman D. 2007. Pretreatment with anticatabolic agents blunts but does not eliminate the skeletal anabolic response to parathyroid hormone in oophorectomized mice. Endocrinology 148:2778–2787.

Schindeler A, Little DG. 2005. Osteoclasts but not osteoblasts are affected by a calcified surface treated with zoledronic acid in vitro. Biochem Biophys Res Commun 338:710–716.

Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. 2000. Bisphosphonates induce apoptosis in human breast cancer cell lines. Br J Cancer 82:1459–1468.

Silverman SL, Maricic M. 2007. Recent developments in bisphosphonate therapy. Semin Arthritis Rheum 37:1–12.

Tenta R, Sourla A, Lembessis P, Koutsilieris M. 2006. Bone-related growth factors and zoledronic acid regulate the PTHrP/PTH.1 receptor bioregulation systems in MG-63 human osteosarcoma cells. Anticancer Res 26:283–291.

Tobias JH, Chow JW, Chambers TJ. 1993. 3-Amino-1-hydroxypropylidine-1-bisphosphonate (AHPrBP) suppresses not only the induction of new, but also the persistence of existing bone-forming surfaces in rat cancellous bone. Bone 14:619–623.

Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J, Arnett TR. 2006. Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. Exp Cell Res 312:1693–1702.

van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. 1999. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem Biophys Res Commun 264:108–111.

von Knoch F, Jaquiery C, Kowalsky M, Schaeren S, Alabre C, Martin I, Rubash HE, Shanbhag AS. 2005. Effects of bisphosphonates on proliferation and osteoblast differentiation of human bone marrow stromal cells. Biomaterials 26:6941–6949.