Dose-dependent metabolic effect of zoledronate on primary human osteoblastic cell cultures

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Abstract

Objectives

To evaluate the in vitro effect of the bisphosponate zoledronate on metabolic activity, proliferation and viability of human osteoblasts.

Methods

Primary human osteoblasts cultures were obtained from cancellous bone of healthy subjects undergoing bone marrow biopsy. Cell cultures were treated with crescent concentrations of zoledronate ($10^{-10}$to 10^{-3}), with and without 1,25$(OH)_2$ vitamin D_3. In these experimental conditions we evaluated cells viability and proliferation using the MMT colorimetric test, cell apoptosis by measurement of Caspase 3 activity and metabolic cell activity through alkaline phosphatase activity and osteocalcin production.

Results

Osteocalcin and alkaline phosphatase synthesis was significantly enhanced by 10^{-10}M to 10^{-5}M zoledronate concentrations, whereas was dramatically decreased by higher drug concentrations. Vitamin D_3 enhanced the positive metabolic effect of zoledronate. The effect of zoledronate on cell proliferation was variable and dose-dependent. While no effect was observed with lower drug concentrations (10^{-10}M to 10^{-8}M), zoledronate 10^{-7}M increased cell proliferation. Conversely, concentrations higher than 10^{-7}M significantly reduced cell proliferation, in a dose-dependent manner. Osteoblast apoptosis was enhanced after treatment with the highest zoledronate concentrations. The maximum positive effect on osteoblasts metabolic activity and proliferation was observed with the zoledronate concentrations corresponding to those theoretically reached in bone microenvironment when zoledronate is used in clinical practice for post-menopausal osteoporosis treatment.

Conclusion

The results of this study confirm that bisphosphonates exert different cellular biochemical effects depending on dosage and support the hypothesis that their positive effect on bone mineral density could be partially due to an anabolic action on bone forming cells.

Key words

osteoblasts, bisphosphonates

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Introduction

Bisphosphonates (BPs) are pyrophosphate analogues in which the oxygen atom linked to the phosphates is replaced by a carbon atom. Due to their chemical structure, bisphosphonates are resistant to pyrophosphatase hydrolysis while still maintaining the chemical-physical properties of pyrophosphate (1). Like pyrophosphate, BPs have a high affinity for bone mineralised matrix (2) and inhibit the formation, aggregation and dissolution of calcium phosphate crystals (3, 4). Their most important biological effect is the strong ability to reduce the bone resorption processes through the inhibition of recruitment, maturation and lifespan of osteoclasts, the mechanisms of which have been well clarified to date (5). BPs, therefore, are widely used to treat bone diseases characterised by increased bone remodelling, such as Paget's disease of bone (6), post-menopausal osteoporosis (7), bone fractures related to osteoporosis (8) and lytic bone metastasis (9). Nevertheless, BPs have shown to be effective in improving clinical manifestations and disease biological parameters in various chronic inflammatory disease, such as rheumatoid arthritis, spondyloarthritis, SAPHO syndrome (10-13). Thus, there is much clinical and experimental evidence to suggest that the mechanism of action of BPs is more complex and involves cellular targets other than osteoclasts. In particular, there is increasing evidence that BPs can affect osteoblast metabolism, but the exact mechanisms involved are not clearly understood and vary depending on cellular experimental model, on the chemical structure of the compound and the concentration used and on the type of cells on which the compound is experimented. Important differences exist between amino and non amino-bisphosphonates, probably due to the different molecular mechanism of action existing between the two classes. The non-amino BPs are chemically more similar to pyrophosphate (*e.g.* clodronate and etidronate) and can be incorporated into non-hydrolysable analogues of adenosine triphosphate (ATP) thus inhibiting ATP-dependent intracellular enzymes (14). Conversely, the nitrogen-containing bisphosphonates (*e.g.* alendronate, pamidronate, zoledronate) alter the osteoclast metabolic functions by inhibiting the enzymes of the mevalonate pathway that are essential for the post-translational changes (prenylation) of small GPTases of the Tho/Rac/Rab family, crucial to several essential cellular processes (14, 15).

Zoledronate is a third generation nitrogen-containing heterocyclic imidazole BP which, to date, represents the most potent inhibitor of bone resorption (16).

The aim of this study was to detail the effect of zoledronate on the proliferation, viability and metabolic activity of primary cultures of adult human osteoblasts.

Patients and methods

Normal human osteoblasts

Normal bone specimens were obtained from eight healthy subjects (5 men, 3 women) aged 55, 12±9,6 years (mean±SD, range: 39–67) undergoing to bone marrow biopsy for piastrinosis of unknown origin and for which a following diagnosis of essential piastrinosis was made. On histological analysis the bone tissue fragments showed good trabecular architecture and normal trabecular thickness, consistent with a normal bone structure. None of these subjects was affected by metabolic bone diseases and none received medication, including corticosteroids, which could interfere with bone metabolism for 6 months prior to bone biopsy.

Appropriate informed consent was obtained from each patients and the study was approved by the Institutional Ethics Committee.

Primary subchondral bone osteoblast cell cultures

Bone marrow biopsies were performed using a bone biopsy needle to obtain very small fragments of cancellous bone. Each fragment was washed using sterile polysaline buffer solution to eliminate bone marrow cells, then digested with 0.5mg/ml type II collagenase (PAA, Austria) with Dulbecco's modified Eagle's medium (DMEM) without serum and supplemented with

antibiotics (penicillin 100IU/ml and streptomycin 100mg/ml) for 1h at 37°C to remove all fibroblasts and residual blood cells. Bone fragments were subsequently washed in DMEM supplemented with antibiotics and containing 20% foetal calf serum (FCS) to remove collagenase and then cultured in sterile flasks in the same medium at 37°C in a water-saturated atmosphere containing 5% CO_2. When cells were observed in the flasks, the culture medium was replaced every 3 days with a fresh medium containing 10% FCS. Osteoblasts began to grow out from the bone specimens after approximately 1 week and proliferated on the flask surface, reaching confluence within 3–4 weeks.

Zoledronate treatment

Zoledronate (1-hydroxy-(1H-minidazol-1-yl))-phosphonoethyl-phosphonic acid monohydrate, was provided by Novartis Pharma AG (Basel, Switzerland) as hydrated disodium salt. A stock solution was prepared in phosphate-buffered saline and stored at -20°C until use.

When reaching confluence, osteoblasts were isolated using trypsin 1% for 5 min, with subsequent temperature inactivation at 37°C, and then seeded in 24-well plates in DMEM containing 10% FCS and antibiotics at $60x10^3$ cells for every plate until semi-confluence was reached. Cells were then treated either with zoledronate at concentrations ranging from 10^{-10}M to 10^{-3}M, either alone or combined with 1,25 dihydroxy-vitamin D_3 (1,25 OH vitamin D_3, Roche) 10^{-8}M for 48 hours.

The calculation of zoledronate concentration to be used in culture media was based on therapeutic dosage usually administered to patients for treatment of post-menopausal osteoporosis: zoledronate 5mg/year intravenously. Since 100% of intravenously administered zoledronate is actually absorbed, and this quantity then becomes diluted in 5 l of blood, the haematic concentration of zoledronate is approximately 1μg/ml (3 X 10^{-6}M). Because at bone level drug concentration seems to be different than at haematic level, experiments were performed testing a large range of zoledronate concentrations (10^{-10}M to 10^{-3}M) obtained performing serial dilutions of the stock solution in the culture media.

After 48h drug exposure, osteocalcin levels, alkaline phosphatase activity, apoptosis and cell proliferation were determined

All experiments were performed in triplicate for each patient sample.

Osteocalcin production and alkaline phosphatase activity

Osteocalcin production and alkaline phosphatase (ALP) activity were assessed in osteoblast cultures after 48h incubation with zoledronate at different concentrations (10^{-10}M to 10^{-3}M) with and without vitamin D_3.

ALP activity was evaluated in cell lysate, obtained through the solubilization of cell monolayer with 0.1(vol/vol) Triton X-100. ALP activity was determined by colorimetric assay using p-nitrophenylphosphate (pNPP) as a substrate (MetraBap EIA kit, San Diego, CA) and measured as the release of p-nitrophenol derived from hydrolysis of p-nitrophenyl phosphate by reading the OD at 405nm according to the manufacturer's recommendations in the 96-well plates provided.

The results were expressed as UI and were normalised per mg/intracellular proteins. In parallel, the cell medium was removed and assayed for osteocalcin. Osteocalcin release into the culture medium was measured using an intact human osteocalcin ELISA (MetraOsteocalcin EIA kit, San Diego, CA). Osteocalcin secretion rates are expressed as nanogram/mg of intracellular proteins. The intracellular protein content of each well was determined by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Richmond, CA).

Cell proliferation

After 48h incubation with zoledronate at different concentrations (10^{-10}M to 10^{-3}M) with and without vitamin D_3, the medium was aspirated. Three hundred micro-litres of MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (stock solution 50mg/ml, Sigma, St. Louis, MO) was added to each well and cultures were continued for 2 h at 37°C. This time period permitted the cellular conversion of MTT to insoluble formazan. The cells were then lysed and the formazan solubilised with acidic propanol at room temperature for 24h. Two hundred microlitres of supernatant were transferred to microplate wells and colorimetric changes were quantified in a microplate reader at an OD of 540nm (Multiskan EX, Thermo Electron Corporation, Finland). The percentage of viable cells were expressed as percent ratio between the OD of samples and OD of control (untreated cells) (17).

Apoptosis

Cell apoptosis was assessed by measurement of caspase-3 activity using a colorimetric assay kit (APOCYTO Caspase-3 Colorimetric Assay Kit, MBL, Woburn, MA) according to the manufacturer's protocol. The sub-confluent cells were lysed after 48h in the different experimental conditions and subjected to Caspase-3 activity detection. Caspase 3 labelled substrate (DVED-p-NA) was added to cell lysate and the mixture was incubated at 37°C for 1-2 hours. The concentration of p-NA released from the Caspase 3 substrate was measured using a microplate reader (Multiskan EX, Thermo Electron Corporation, Finland) reading the OD at 405nm according to the manufacturer's recommendations. The results were expressed as nmoles/ml *p*NA production.

Results

Osteocalcin and alkaline phosphatase production

Dose-response experiments were performed to determine the effect of zoledronate on the metabolism, via-bility and proliferation of normal human osteoblasts. Figures 1 and 2 show osteocalcin and alkaline phosphatase production by human osteoblast cultures treated with variable zoledronate concentrations.

The osteocalcin synthesis was significantly enhanced by zoledronate treatment for all zoledronate concentrations used to perform the experiments (range 10^{-10}M to 10^{-5}M), except for the higher concentrations (10^{-4}M and 10^{-3}M).

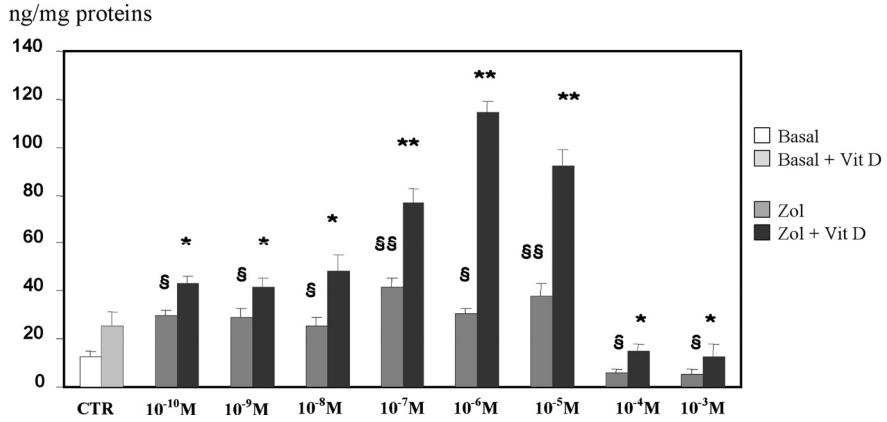

Fig. 1. Osteocalcin production in human osteoblast cell cultures derived from bone biopsies of healthy subjects in basal condition and after stimulation with increasing concentration of zoledronate, with and without vitamin D_3. Osteocalcin synthesis is significantly enhanced by zoledronate concentration ranging from 10^{-10}M to 10^{-5}M. Conversely, higher zoledronate concentrations (10^{-4}M and 10^{-3}M) significantly inhibit osteocalcin synthesis. In all experimental conditions, vitamin D_3 enhances osteocalcin synthesis Osteocalcin production is expressed as ng/mg intracellular proteins/48.
Zol: zoledronate.
$^{*}p<0.05$; $^{**}p<0.001$ (Untreated cells versus zoledronate treated cells); $^{\S}p<0.05$; $^{\S\S}p<0.001$ (vitamin D_3 treated cells *versus* zoledronate and vitamin D_3 treated cells).

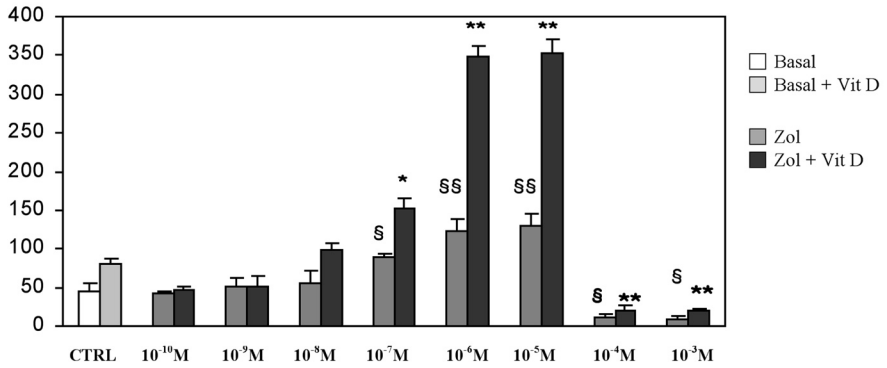

Fig. 2. Alkaline phosphatase (ALP) activity in human osteoblast cell cultures derived from healthy subjects in basal condition and after stimulation with increasing concentration of zoledronate, with and without vitamin D_3. Similarly to osteocalcin, zoledronate induces a significant increase in ALP activity, starting from 10^{-7}M to 10^{-5}M whereas, with lower drug concentrations, alkaline phosphatase activity is not affected. Conversely, higher zoledronate concentrations (10^{-4}M and 10^{-3}M) significantly inhibit ALP activity. ALP levels are expressed as UI/mg intracellular proteins/48.
Zol: zoledronate. $^{*}p<0.05$; $^{**}p<0.001$ (Untreated cells versus zoledronate treated cells); $^{\S}p<0.05$; $^{\S\S}p<0.001$ (vitamin D_3 treated cells versus zoledronate + vitamin D_3 treated cells.)

zoledronate induced a significant increase in phosphatase alkaline activity, starting from 10^{-7}M to 10^{-5}M whereas, at lower concentrations, alkaline phosphatase activity showed no difference compared to untreated osteoblast cultures.

Conversely, compared to control sample osteoblast cultures, the higher zoledronate concentrations (10^{-4}M and 10^{-3}M), induced a dramatic decrease in both osteocalcin production and alkaline phospatase activity, consistent with a reduced metabolic activity.

Osteocalcin synthesis significantly increased after vitamin D stimulation in all experimental conditions, regardless of zoledronate concentration. Interestingly, however, with zoledronate concentrations ranging from 10^{-7}M to 10^{-5}M, the vitamin D-induced increase in osteocalcin synthesis was significantly higher when compared to both greater and lower zoledronate doses, with a maximum effect observed at 10^{-6}M. The same effect was observed for alkaline phosphatase synthesis with a zoledronate concentration of 10^{-6}M and 10^{-7}M. The "fold increase" of osteocalcin production and alkaline phosphatase activity after vitamin D_3 treatment is significantly higher with zoledronate concentration of 10^{-5}M, 10^{-6}M, 10^{-7}M and 10^{-6}M, 10^{-7}M respectively (data not shown).

Cell proliferation

The effect of zoledronate treatment on osteoblast proliferation was variable and strictly dependent on the concentrations used, with an apparent dose-dependent effect. While lower zoledronate concentration ranging between 10^{-10}M and 10^{-8}M did not modify cell proliferation, this increased significantly at 10^{-7}M. Conversely, from 10^{-6}M to 10^{-3}M, zoledronate induced a significant and progressively marked decrease in cell proliferation, strictly dose-dependent, with the maximum effect observed with concentrations of 10^{-4}M and 10^{-3}M. Cell proliferation was increased by vitamin D_3 stimulation in all experimental conditions, except in the highest zoledronate concentration (10^{-3}M). Figure 3 illustrates the changes in cell proliferation induced by the different concentrations of zoledronate with and without vitamin D_3.

Apoptosis

Caspase 3 activity was not affected by zoledronate concentrations ranging from 10^{-10}M to 10^{-5}M. However, a significant increase was observed in osteoblast cultures after treatment with zoledronate 10^{-4}M and 10^{-3}M compared to untreated osteoblasts (Fig. 4)

Discussion

BPs are usually thought to act selectively on osteoclasts in areas of high bone turnover, resulting in an antiresorptive effect (18). However, clinical and experimental evidences suggest that bisphosphonates can act on various cellular targets, indicating that these drugs are not always selective for osteoclasts; particularly, many experimental studies have focused on the metabolic effects of BPs on osteoblasts, although results are extremely variable and often discordant.

Zoledronate is a third generation aminobisphosphonate which greatly inhibits

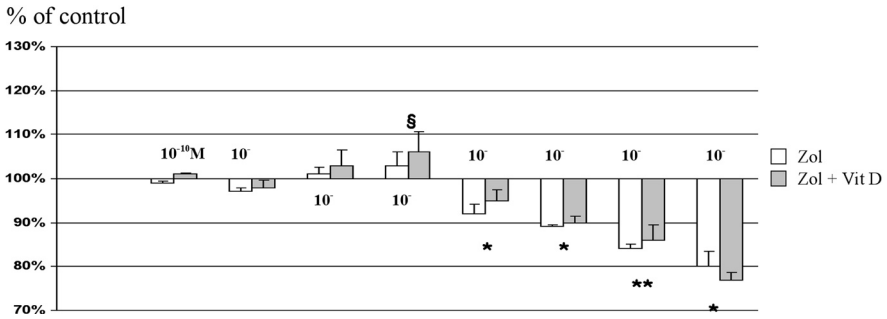

Fig. 3. Proliferation of osteoblasts treated with increasing concentration of zoledronate ranging from 10^{-10}M to 10^{-3}M. Proliferation was determined using the MTT colorimetric assay and expressed as mean±SD percent of control (untreated osteoblasts). Zoledronate concentrations ranging between 10^{-10}M and 10^{-8}M do not modify cell proliferation. Cell proliferation is significantly increased with zoledronate 10^{-7}M, whereas starting from 10^{-6}M until 10^{-3}M zoledronate induces a significant and progressively greater decrease in cell proliferation.
$^{\S}p<0.05$ significantly greater than untreated control; $^{*}p<0.01$; $^{**}p<0.0001$ significantly lower than untreated control.

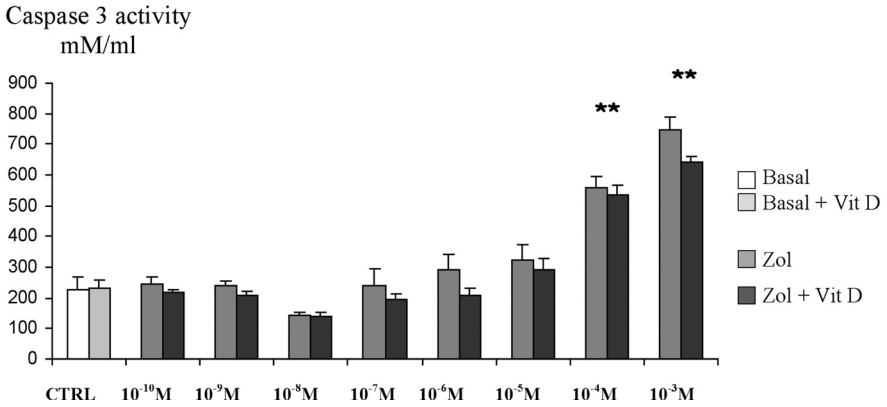

Fig. 4. Osteoblast apoptosis determination using direct Caspase-3 activity measurements by APOCYTO Caspase-3 Colorimetric Assay Kit in osteoblasts treated with increasing concentration of zoledronate. Treatment with the highest zoledronate concentrations (10^{-4}M and 10^{-3}M) significantly enhances cell apoptosis.
$^{**}p<0.0001$.

farnesyl diphosphate synthase and has a higher affinity for hydroxyapatite crystals. Zoledronate can be administered intravenously once a year for treatment of postmenopausal osteoporosis (19), as it is able to reduce bone turnover and to increase spine and hip BMD with a consequent reduction in vertebral, peripheral and hip fracture risk. Preliminary data based on histomorphometric analysis have recently shown that zoledronate treatment is associated with an increase in mineral apposition rate (MAR) (20), in contrast with several previous animal studies which showed that other bisphosphonates (alendronate and risedronate) reduced MAR (21-24). Other data from human bone biopsy studies have not shown any significant effect of other aminobisphosphonates on MAR. An increased MAR is generally interpreted as a consequence of the enhanced activity of individual osteoblasts at the basic multicellular unit (BMU) level (25). These data, if confirmed, could suggest that the mechanism of action of zoledronate, besides a strong inhibition of osteoclasts, may implicate a stimulatory effect on osteoblast activity, but more detailed studies based on histomorphometric analysis are not available yet.

Reinholz *et al*. (26) demonstrated that zoledronate inhibits osteoblast proliferation, but the concentrations of the drug used in their study were higher than 4 x 10^{-5}M and the effect with lower doses was not evaluated. On the other hand, von Knoch *et al*. (27) found that, at a lower concentration (10^{-8}M), zoledronate strongly enhanced both proliferation and osteoblastic differentiation of human bone marrow stromal cells.

The actual concentration range of BPs to which osteoblasts would be exposed *in vivo* is unknown, therefore we evaluated a wide range of zoledronate concentrations based on the theoretical concentrations reached in the bone microenvironment with the doses used in clinical practice for the treatment of postmenopausal osteoporosis.

In vivo, osteoblasts are probably exposed to different concentrations of the drug depending on various systemic and local factors. The lower concentrations we used were due to the fact that, at bone level, the drug concentrations may probably be much lower compared to the haematic level, as about 60% of the BP dose is excreted into the urine within 24 hours from administration, whereas the greater part of the remainder is rapidly taken up in the bone. We also used higher zoledronate concentrations as some experimental data on bone incorporation of alendronate suggest that osteoblasts could be transiently exposed to local, higher concentrations of the drug, up to 10^{-3}M, especially in the resorption space (28) due to bisphosphonate release from the bone by osteoclasts. Further, relevant variations in drug concentrations can be due to protein binding which might be different *in vitro* and in *vivo*.

This study has shown that zoledronate induces a significant increase in osteocalcin synthesis compared to untreated osteoblasts at all concentrations tested, except for the higher concentrations of 10^{-4}M and 10^{-3}M. Alkaline phosphatase synthesis is not affected by lower zoledronate concentration but is significantly enhanced with drug concentrations ranging between 10^{-7}M and 10^{-5}M and, similarly to osteocalcin, is dramatically reduced with higher zoledronate doses. In addition, the combined treatment with zoledronate and vitamin D_3 appears to act synergically in determining the positive metabolic effect on alkaline phosphatase and osteocalcin synthesis, with a maximum effect with zoledronate concentrations ranging from 10^{-7}M to 10^{-5}M. In agreement with these data, it has been previously

demonstrated in an *in vivo* animal study that differences in vitamin D status can affect the anti-catabolic response of bone tissue to bisphosphonate treatment (29). This underlying the relevance of vitamin D supplementation and/or vitamin D dietary intake in routine clinical practice in patients treated with antiresorptive drugs, and confirm that an optimal vitamin D repletion is a prerequisite for maximising the response to antiresorptive treatment in terms of both BMD changes and anti-fracture efficacy (30, 31) Further, these data are in agrrement with the observation that vitamin D increase ALP activity and in osteoblasts derived from patients on chronic bisphosphonate therapy with osteonecrosis of the jaw (32) and provide a rationale for the therapeutic value of vitamin D supplementation in the treatment or prevention of this condition. It is interesting to note that we observed the maximum positive effect of zoledronate on osteoblast metabolic activity with concentrations of around 10^{-6}M, which should correspond to the theoretical concentrations reached *in vivo*.

However, with reference to cell proliferation, in this concentration range, zoledronate seems to exert a biphasic effect as with 10^{-7}M treatment proliferation is significantly increased compared with both basal conditions and lower zoledronate concentrations (10^{-10}M to 10^{-8}M) whereas when zoledronate is used at 10^{-6}M and 10^{-5}M, proliferation is reduced. Higher concentrations (10^{-4}M and 10^{-3}M) further decreased the proliferation activity in cultured osteoblasts. This can be due to an overall increase in osteoblastic activity (proliferation and metabolism) with 10^{-7}M concentration; the apparent discordance between the reduced cellular proliferation and the increased alkaline phosphatase production, observed with zoledronate 10^{-6}M and 10^{-5}M treatment, could be explained by the ability of these dosages in increasing osteoblast differentiation, favouring the transition from the proliferating stage of development to the non-proliferating matrix maturation stage (33). Contrary to findings reported in previous studies performed with other BPs analogues, zoledronate does not affect cell viability (34) as Caspase 3 activity remains unchanged with all zoledronate concentrations compared to untreated osteoblasts, both in basal conditions and with vitamin D_3, except after treatment with the higher zoledronate doses (10^{-4}M and 10^{-3}M) that induces a significant increase in Caspase 3 activity (Fig. 4).

In agreement with previous published data concerning other BPs analogues, we found that very high zoledronate concentrations exert an inhibitory effect on both metabolism and proliferation of osteoblasts, reducing cell viability by raising apoptosis. Some authors (35) have explained this negative effect as a consequence of a possible cytotoxic action of BPs at high concentrations (17), but probably this mechanism is only partially involved as metabolic activity is reduced but not entirely suppressed and vitamin D response also persisted with the highest zoledronate concentration we used. These results are partially consistent with a previous published study which showed that zoledronate inhibits osteoblast proliferation and enhances the expression of osteogenesis-related genes (16). However, in this previous study only the effect of a single zoledronate concentration was tested on osteocalcin and phosphatase alkaline production. Furthermore, osteoblast cell population was not homogenous as cell cultures derived from both healthy subjects and osteoarthritis patients (16).

The available data confirm that BPs can modify osteoblast behaviour at different levels, affecting the metabolic activity, proliferation and viability of these cells, but the exact mechanisms by which these compounds act are not completely understood. Igarashi *et al.* (36) showed that three different BPs inhibit endogenous prostaglandin E_2 production and enhance alkaline phosphatase production and mineralisation of MC3T3-E1 osteoblast-like cells. Alendronate and etidronate enhance IL-6 production in osteoblasts (20). Alendronate and risedronate enhance gene expression of BMP-2, type I collagen and osteocalcin (17, 37).

Some BPs (risedronate and etidronate) stimulate the formation of osteoblast precursors from bone marrow cells, stimulate osteoblastogenesis from osteoblast precursors, both in animal and in human experimental *in vitro* models (38, 39), and promote osteoblast differentiation (clodronate, etidronate), whereas pamidronate has no effects (17) and data on alendronate are discordant (37, 40).

Data on the effects of BPs on osteoblast proliferation are also very heterogeneous. Our results are in agreement with previous published data showing that both alendronate and risedronate increased primary human trabecular bone and MG-63 cell proliferation over a concentration range of 10^{-11}M to 10^{-5}M but inhibited cell proliferation at 10^{-4}M (17). On the other hand, some authors (41) have reported that concentrations of alendronate ranging between 10^{-12}M and 10^{-5}M exert no effect on the proliferation of normal human ostoblasts *in vitro*.

The reasons for these contradictory and extremely various data (17, 38) are not completely clear, but can depend on differences in cell types, the duration of treatment, the type of BP analogue tested, the concentration of drug used and on individual patient differences. Nevertheless, taken together these data provide strong evidence that the effects of BPs on bone tissue can be due BPs to their action on both osteoblasts and osteoclasts (18).

The results of this study confirm the hypothesis that BPs may have an anabolic effect on osteoblasts and could play an important role in enhancing bone formation, acting synergically with vitamin D on the metabolic activity of bone forming cells.

Further investigations are needed to determine how these *in vitro* BPs effects can contribute to the improvement of bone quality and BMD increase observed in clinical practice.

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