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ORIGINAL ARTICLE

Nitrogen-containing bisphosphonates modulate the antigenic profile and inhibit the maturation and biomineralization potential of osteoblast-like cells

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Abstract
Objectives The aim was to evaluate the effect of three nitrogen-containing bisphosphonates at different concentrations on osteoblast growth, differentiation, and antigenic profile, using the MG-63 cell line as osteoblast model, in order to determine the role of osteoblasts in bisphosphonate-related osteonecrosis of the jaw (BRONJ).
Materials and methods Osteoblasts were incubated in culture medium with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate. Proliferative capacity of the osteoblasts was determined by spectrophotometry (MTT) at 24 and 48 h of culture. Flow cytometry was used to study antigenic profile (CD54, CD80, CD86, HLA-DR) and phagocytic activity. Cell differentiation was evaluated at 7, 15, and 21 days by the study of nodule formation and alkaline phosphatase activity (ALP) at 24 h by spectrophotometric assay.
Results Pamidronate, alendronate, and ibandronate each exerted a significant stimulatory effect on MG63 proliferation that depended on the dose and treatment duration ($p<0.05$). In general, a significantly decreased expression of CD54, CD80, and HLA-DR membrane antigens was observed after 24 h of treatment with each nitrogen-containing bisphosphonate ($p<0.05$), but there was no significant difference in phagocytic activity versus controls. A decrease in ALP activity was observed after 24 h of treatment and a decrease in calcium deposition after 15 and 21 days ($p<0.05$).
Conclusion Nitrogen-containing bisphosphonates can increase the proliferation of MG-63 osteoblast-like cells, modulate their expression of co-stimulatory molecules associated with immune function, and decrease their differentiation capacity, generally at low doses.
Clinical relevance These findings suggest that low doses of nitrogen-containing bisphosphonates exert their effect on osteoblasts by altering their physiology, which would explain the disruption of their repair capacity and may be directly related to the development of BRONJ.

Keywords Nitrogen-containing bisphosphonates · Osteoblast · Proliferation · Phenotype · Differentiation · BRONJ

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Introduction

Bisphosphonates (BPs) are a family of pyrophosphate synthetic analogues in which the oxygen linking the phosphates has been replaced by carbon. They are commonly used as antiresorptive agents in the treatment of metabolic bone diseases with increased bone resorption (e.g., osteoporosis, Paget's disease, multiple myeloma, malignant hypercalcemia, etc.) and in bone metastases of breast and prostate cancer [1–4]. There are two major types of BPs with different

molecular modes of action, non-nitrogen-containing BPs and nitrogen-containing BPs [5].

BPs are widely prescribed, and their ability to inhibit osteoclast formation and activity in vitro and in vivo is well documented [6, 7]. However, the mechanisms underlying their action on bone and their effects on osteoblasts are not fully understood, although various proposals have been made, including a decrease in bone turnover and subsequent accumulation of microfractures; a toxic effect on osteoblasts; an antiangiogenic effect, producing avascular necrosis; and a reduction in the viability of fibroblasts and oral keratinocytes [8–10]. Although useful in the treatment of some bone diseases, BPs have also been associated with the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ) [11, 12].

Bone formation and bone remodeling processes go beyond osteoblast proliferation and differentiation and are regulated by a large number of paracrine, autocrine, and endocrine factors, including growth factors, hormones, and cytokines [13, 14]. Besides their essential role in bone formation and repair, osteoblasts possess immunological functions, including T lymphocyte stimulation, phagocytic activity, and cytokine synthesis [15–23]. Osteoblasts express CD54, CD80, CD86, and HLA-DR surface antigens according to their degree of differentiation and/or activation, and their antigenic profile is modulated by the presence of different cytokines and growth factors [17, 18, 22].

The objective of the present study was to evaluate the effect of three nitrogen-containing BPs (pamidronate, alendronate, and ibandronate) at different concentrations on osteoblast growth, differentiation, and antigenic profile using the MG63 cell line, in order to determine the role of osteoblasts in BRONJ. MG63 cells are widely used as osteoblast model because they share the same characteristics.

Materials and methods

The nitrogen-containing BPs pamidronate (Sigma-Aldrich, St. Louis, MO), alendronate (Sigma), and ibandronate (Sigma) were selected for the study. The doses in all assays were 10^{-5}, 10^{-7}, or 10^{-9} M, which are within the therapeutic dose range [24].

Cell culture

Human MG63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA). MG63 cell line was maintained as described by Diaz-Rodríguez et al. [22] in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5 μg/mL amphotericin B (Sigma), 1 % glutamine (Sigma), and 2 % HEPES (Sigma), supplemented with 10 % fetal bovine serum (FBS) (Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Cells were detached from the culture flask with a solution of 0.05 % trypsin (Sigma) and 0.02 % ethylenediaminetetraacetic acid (EDTA) (Sigma) and then washed and suspended in complete culture medium with 10 % FBS.

Cell proliferation assay

Osteoblasts were seeded at 1×10^4 cells/mL per well into a 24-well plate (Falcon, Becton Dickinson Labware, NJ) and cultured at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2 for 24 h. Next, the medium was replaced with DMEM containing pamidronate, alendronate, or ibandronate at a dose of 10^{-5}, 10^{-7}, or 10^{-9} M. After 24 or 48 h of culture, the cell proliferation was measured by MTT assay, as described by Manzano-Moreno et al. [25], replacing media with phenol red-free DMEM containing 0.5 mg/mL MTT (Sigma) and incubating for 4 h. Cellular reduction of the MTT tetrazolium ring resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated, and dimethyl sulfoxide (DMSO, Merck Biosciences, Darmstadt, Germany) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise™, Tecan, Männedorf, Switzerland). Results were reported as mean absorbance (570 nm)±standard deviation (SD). At least three experiments were conducted for each treatment, using the mean value in the analysis.

Antigenic phenotype by flow cytometry

Osteoblasts (MG-63 cell line) were previously treated with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate for 24 h at 37 °C. Cells were then detached from the cultured flask by treatment with 0.4 % (*w/v*) EDTA solution, washed, and suspended in phosphate-buffered saline (PBS) at 2×10^4 cells/mL. Cells were labeled by direct staining with anti-CD54, CD80, CD86, and HLA-DR monoclonal antibodies (MAbs) (CD54/IOL1b, CD80, CD86, and OKDR, respectively, all from Invitrogen Corp, Carlsbad, CA). Aliquots of 100 μL of cell suspension were incubated with 10 μL of the appropriate MAb for 30 min at 4 °C in the dark. Cells were washed, suspended in 1 mL of PBS, and immediately analyzed in a flow cytometer with diode laser (FACSCanton II, Becton Dickinson, Palo Alto, CAL) at a wavelength of 488 nm to determine the percentage of fluorescent cells. Untreated cells were used as controls. The percentage of antibody-positive cells was calculated from counts of 2,000–3,000 cells. At least three experiments were run for each antigen in all cultures.

Phagocytic activity

Phagocytic activity was studied by flow cytometry. Cultured human MG-63 cells were treated with10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate. Untreated cells were used as controls. Cells were detached from the culture flask by treatment with a solution of 0.05 % trypsin and 0.02 % EDTA, washed, and then suspended in complete culture medium with 10 % FBS at 2×10^4 cells/mL. Cells were labeled by direct staining with labeled latex beads. Then, 100 μL of cell suspension was incubated with 200 μL of carboxylated FICT-labeled latex beads with diameter of 2 μm (Aldrich, St Louis, MO) for 30 min at 37 °C in darkness. Cells were washed, suspended in 1 mL of PBS, and immediately analyzed in a flow cytometer (FACSCanton II). Results were expressed as percentage of cells positive for phagocytosis and mean channel fluorescence, which correlates with the number of phagocytosed particles.

Alkaline phosphatase activity

The effect on osteoblast-like cell differentiation was assessed by evaluating the alkaline phosphatase (ALP) activity of MG-63 cells cultured in non-osteogenic medium and treated with BPs. ALP activity was quantified by using a colorimetric assay (Diagnostic kit 104-LL, Sigma) to determine early osteoblastic differentiation. The assay measures the conversion of the colorless substrate *p*-nitrophenylphosphate by the enzyme ALP to the yellow product *p*-nitrophenol, with the rate of color change corresponding to the amount of enzyme present in solution. Standards of *p*-nitrophenol (0–250 μM) were prepared from dilutions of a 1,000 μM stock solution and assayed in parallel. The ALP assay was performed as described by Sandrini et al. [26]. The cells in non-osteogenic medium with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate or without drugs (control group) were seeded at 1×10^4 cells/mL per well into 24-well plates and cultured for 7 days under standard conditions. Then, cells were lysed with 0.1 % (*v*/*v*) Triton X-100 at 37 °C. The samples were centrifuged at 1,500 rpm and the supernatants stored at −70 °C until used. ALP activity was determined with *p*-nitrophenylphosphate as substrate. An aliquot (50 μL) of cell lysate solution was added to 50 μL of ALP substrate (Sigma) and then incubated at 37 °C for 45 min in darkness. The enzymatic reaction was stopped by adding 50 μL of 0.1 M NaOH, and the absorbance was measured at 405 nm with a spectrophotometer (Biotek ELx800). The total protein content was estimated by the Bradford method using a protein assay kit from Bio-Rad Laboratories (Nazareth-Eke, Belgium). All samples were run in triplicate, and the ALP activity was expressed as a percentage, considering enzymatic activity in the absence of BPs as 100 %.

Nodule formation and matrix mineralization

The presence of calcium deposits in the cell matrix was analyzed by the alizarin red S method. MG-63 cells were seeded (5×10^4 cells/mL/well) in a 6-well plate and cultured in osteogenic medium with different concentrations of the BPs at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. The medium was replaced after 4 days and then every 3 days. The matrix mineralization of each cell line was measured at 7,

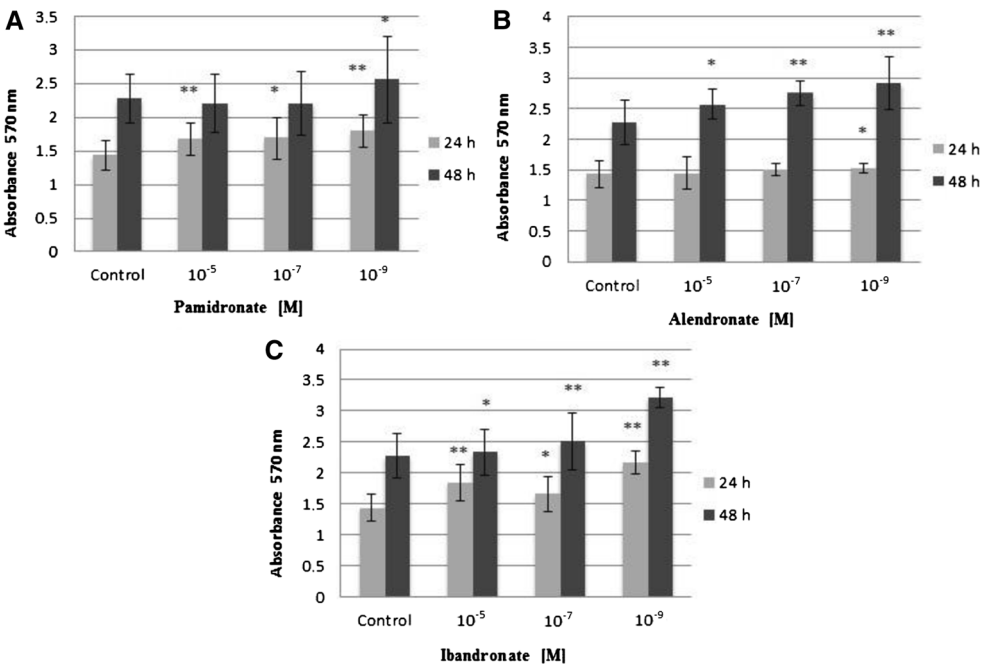

Fig. 1 Effect of **a** pamidronate, **b** alendronate, and **c** ibandronate at different doses (10^{-5}, 10^{-7}, 10^{-9} M) on osteoblast proliferation in MG-63 cell line after 24 and 48 h of incubation. Data are expressed as means+SEM. We compared data between each treatment and control culture by analysis of variance (ANOVA). **P<0.05*; ***P<0.001*

15, and 22 days of culture. Wells were washed with 150 mM sodium chloride, fixed in cold 70 % ethanol for 5 min, and rinsed three times with distilled water. Wells were then incubated for 10 min with 1 mL of 2 % (*u/v*) alizarin red S solution buffered at pH 4 with sodium hydroxide, then rinsed five times with distilled water, and finally washed with PBS to reduce non-specific staining. Calcium deposits present in the extracellular collagen matrix were colored red, revealing mineralization nodules, which were detained for 15 min with 10 % (*w/v*) cetylpyridiniumchloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured with a plate/reader spectrophotometer (Biotek ELx800).

Statistical analysis

SPSS 20.0 (IBM, Chicago, IL) was used for all data analyses. Mean values (±SD) were calculated for each variable. A two-way repeated-measures analysis of variance (ANOVA) was performed to examine the effects on proliferation, nodule formation, and ALP synthesis as a function of the BP type (pamidronate, alendronate, or ibandronate), treatment duration, and concentration. When a significant interaction was identified, the Bonferroni correction was applied for planned pair-wise comparisons. Antigenic profile and phagocytic activity were compared using the Student's *t* test; $p<0.05$ was considered significant. At least three experiments were performed for each assay.

Results

Cell proliferation assay

Each amino-BP studied stimulated osteoblast proliferation capacity as a function of dose and treatment duration (Fig. 1). In comparison to controls, a significant increase in proliferation ($p<0.05$) was observed with ibandronate at doses of 10^{-5}, 10^{-7}, and 10^{-9} M after 24 and 48 h of treatment; with alendronate at all doses after 48-h treatment but at only 10^{-9} M after 24-h treatment; and with pamidronate at all doses after 48-h treatment (10^{-5}, 10^{-7}, and 10^{-9} M), but at only 10^{-9} M after 24-h treatment.

Antigenic phenotype by flow cytometry

The flow cytometry results in Fig. 2 show that, in general, treatment with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate for 24 h significantly decreased the expression of CD54, CD80, and HLA-DR membrane antigens versus non-treated controls MG63 cells after 24 h of culture ($p<0.05$). The CD86 marker showed a variable expression depending on the BP and dose.

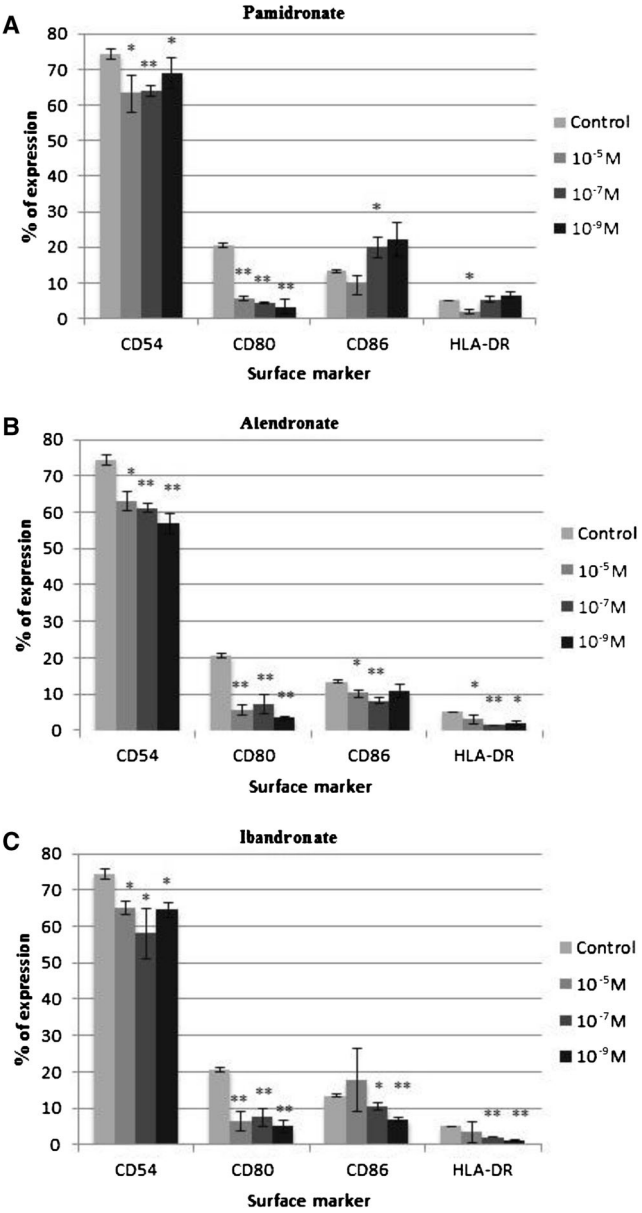

Fig. 2 Percentage expression of osteoblast MG63 cell surface markers after 24-h treatment with pamidronate (**a**), alendronate (**b**), or ibandronate (**c**) at doses of 10^{-5}, 10^{-7}, or 10^{-9} M.*$p<0.05$; **$p<0.001$

Phagocytic activity

Flow cytometry with fluorescent latex beads (Fig. 3) showed no significant differences in phagocytic activity between MG63 cells treated with different doses of BPs and non-treated controls cells after 24 h of culture.

Alkaline phosphatase activity

Results in Fig. 4 show a decrease in the expression of this differentiation marker versus control cells in all 3 drugs after 24 h of treatment; this difference only reached significance at doses of 10^{-7} and 10^{-9} M with pamidronate ($p=0.047$ and

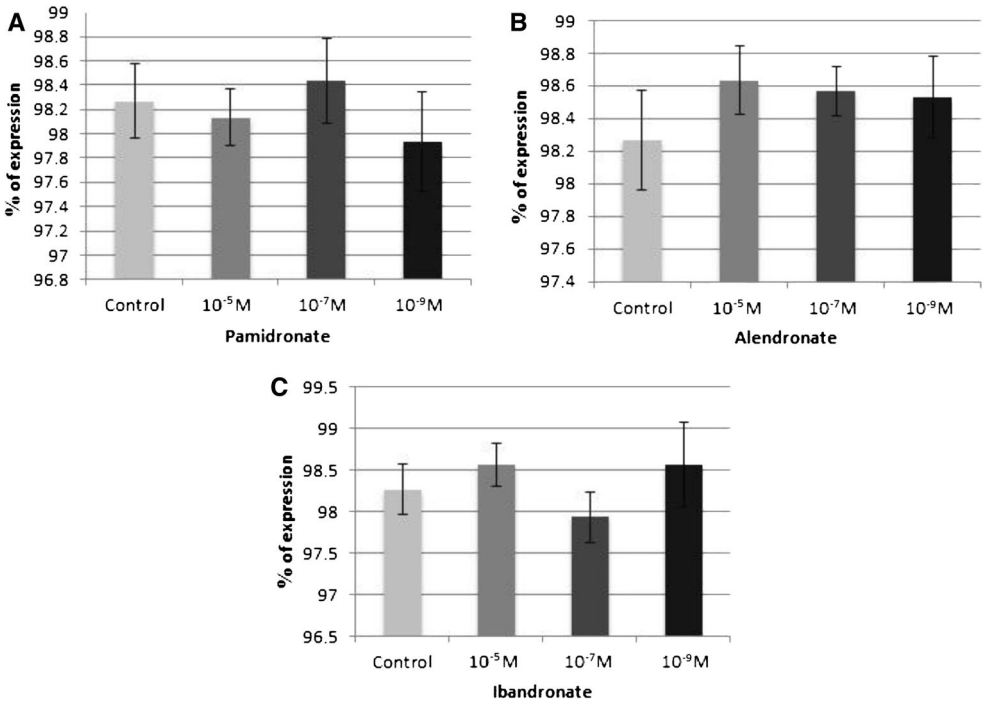

Fig. 3 Percentage of cells with flow cytometry-determined phagocyte capacity after 24-h treatment with pamidronate (**a**), alendronate (**b**), and ibandronate (**c**) at doses of 10^{-5}, 10^{-7}, or 10^{-9} M

p=0.002) or ibandronate (p=0.034 and p=0.028) and at a dose of 10^{-9} M with alendronate (p=0.032).

Nodule formation and matrix mineralization

The effect of the three BPs on nodule mineralization is depicted in Fig. 5. It shows the amount of Alizarin Red staining measured colorimetrically at 7, 15, and 21 days of culture in osteogenic medium with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate. There were no significant differences between treated and control (untreated) groups at 7 days of culture. However, in comparison to the control group, the calcium deposition was reduced (p<0.05) after treatment with each BP for 15 or 21 days at all concentrations tested.

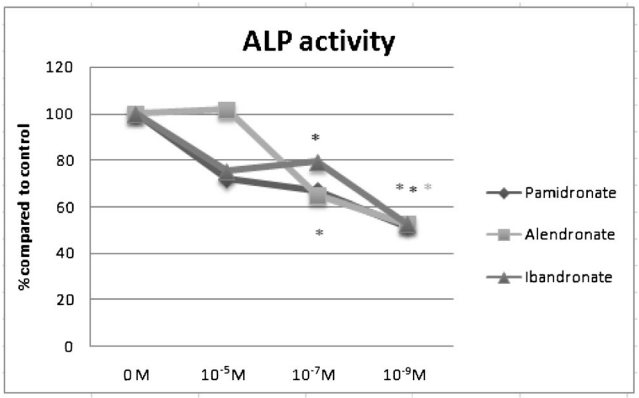

Fig. 4 ALP activity of MG63 line cell after 24-h treatment with pamidronate, alendronate, or ibandronate at doses of 10^{-5}, 10^{-7}, or 10^{-9} M, in non-osteogenic medium, expressed as a percentage, considering the enzymatic activity value in the absence of bisphosphonates as 100 %. *p<0.05

Discussion

The results of this in vitro study demonstrate that therapeutic doses of pamidronate, alendronate, or ibandronate exert an effect on osteoblasts, increasing their growth capacity, inhibiting their differentiation and mineralization, and modulating their antigenic profile. This implies major changes in the complex physiology of the osteoblast, which is regulated by multiple local and systemic factors that may regulate the activity of a specific transcription factor [16, 19, 27]. TGF-β1 is known to play an important role in regulating and stimulating the differentiation of osteoprogenitors during fracture repair [28] and has been described as responsible, among other growth factors, for the biostimulatory effect on osteoblast cells of various treatments [29, 30]. The MG63 human osteosarcoma line was selected as osteoblast model in this study because it is the most widely used cell line in studies on the effects of drug or other treatments on the osteoblast [31–33], although the limitations of studying a tumor line should be borne in mind.

The three BPs investigated in the present study stimulated osteoblast growth as a function of the dose and treatment duration, observing the highest proliferation rates at the lowest concentrations. These results are consistent with previous

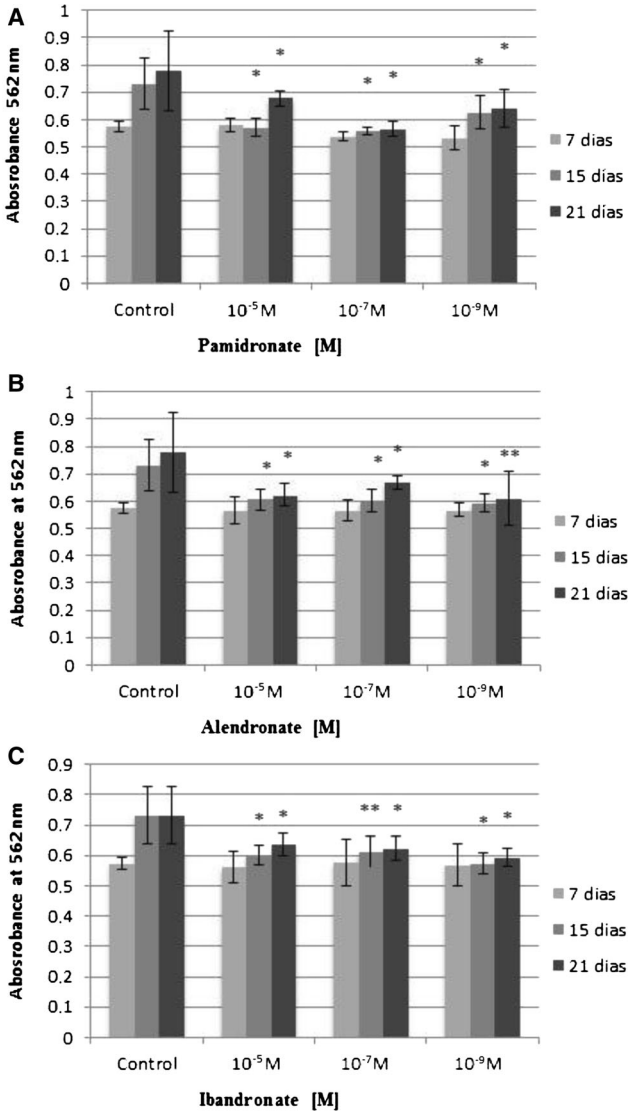

Fig. 5 Quantitative study of the mineralization of MG63 cells (nodule formation) as a function of treatment duration after culture in osteogenic medium supplemented with pamidronate (**a**), alendronate (**b**), or ibandronate (**c**) at doses of 10^{-5}, 10^{-7}, or 10^{-9} M.*$p<0.05$;**$p<0.001$

in vitro findings that BPs exert a pro-osteoblastogenic effect at lower concentrations (10^{-5} to 10^{-9} M) but an inhibitory effect at higher concentrations [34–37]. In common with previous reports, the present results suggest that BPs exert a beneficial effect on the osteoblast by enhancing its proliferative capacity, although account should be taken of the inverse dose-rate effect observed at the assayed doses.

The differentiation and maturation of osteoblasts involve the synthesis of specific bone proteins (e.g., ALP) that contribute to the synthesis of the extracellular matrix followed by its mineralization. The effects of BPs on this differentiation and maturation process are controversial, with some studies finding that BPs reduce ALP synthesis [38] and others suggesting that low doses of nitrogen-containing BPs increase ALP activity and nodule formation. In the present study, low doses of BPs reduced ALP activity and nodule formation [34, 35, 39], indicating an inhibitory effect on the differentiation and maturation of the osteoblast as bone-forming cell.

In addition, the BP treatment modulated the expression of antigens involved in the immunologic function of the osteoblast. Thus, 24 h of treatment produced a significant decrease in CD54, CD80, and HLA-DR antigens, whose expression is elevated in the MG63 cell line, although its effect on CD86 expression was variable and depended on the BP type and dose. No published data are available on the effects of BPs on the osteoblastic expression of these biomarkers.

CD54 expression on pre-osteoblasts and immature osteoblasts is elevated and depends on the degree of cell differentiation and maturation [20]. However, its expression can be modulated in the presence of various substances, notably cytokines, growth factors, platelet-rich plasma, bacterial lipopolysaccharide (LPS), and certain pharmaceuticals [19, 40–43]. In in vitro studies, human osteoblasts obtained by primary culture from bone samples showed a significantly reduced expression of CD54 and CD86, with no change in their expression of CD80 or HLA-DR after TGFβ1 treatment; and no change in these molecules after treatment with FGFb, PDGF-BB, or IL-2 but a significant increase in their expression after treatment with IL-1β, IFNγ, and LPS [19]. These data, alongside findings on the expression of cytokines (IL-4, IL-12, IL-15, IL-18, and IFNγ) in the osteoblast and their modulation by different factors (FGF, TGFβ1, and PDGF) and cytokines (IL-1 and IFNγ), suggest that the functional capacity of osteoblasts is modified during their differentiation and maturation, with a gain in their bone-forming function at the expense of their immunological function.

Naidu et al. [44] reported that TGF-β1 expression was increased by BPs, and others have found that this growth factor enhances proliferation and osteoblast differentiation in its initial phase but inhibits its differentiation and maturation and suppresses matrix mineralization in later phases [45, 46]. In the present study, the three BPs increased osteoblast proliferation and reduced ALP synthesis and mineralization nodule formation in inverse relation to their dose, which may be explained by the BP-induced increase in TGF-β1 synthesis, which would in turn be consistent with the reduced expression of co-stimulatory molecules in the presence of this growth factor. Nevertheless, under physiologic conditions, this inhibition of osteoblast differentiation would imply an increased expression of these markers (CD54, CD80, CD86, and HLA-DR), but this was not observed in the presence of these BPs. It should be borne in mind that TGF-β1 exerts its action via three receptors (types I, II, and III), and there is a need to investigate the effect of BPs on the expression of these receptors in order to improve our understanding of the response of osteoblasts in their presence.

On the other hand, numerous studies have associated mandibular osteonecrosis with BP treatment [11, 12, 47, 48]. The

underlying mechanism has not been elucidated, but the lack of vascular supply or bone remodeling and/or regeneration has been implicated as possible causes. Bone tissue loses its capacity to remove bone areas that are unhealthy or have completed their useful life, limiting or reducing its functional adaptation capacity [49].

The BPs most frequently related to BRONJ are those that contain nitrogen [11, 12, 50, 51], which were assayed in the present study. These drugs bind strongly to hydroxyapatite crystals. Although all BPs share the same phosphorus-carbon-phosphorus core, distinct BPs differ markedly in their affinity for hydroxyapatite binding, and this may play an important role in determining their duration of action [52]. The lesser or greater binding of the BP to hydroxyapatite as a function of the type of BP may explain the different degrees of adverse effects on bone tissue exerted by these drugs in long-term treatments.

In physiologic conditions, the osteoblast loses its immunologic capacity in favor of its bone-forming capacity. Thus, the differentiation and maturation of the osteoblast imply a reduced expression of markers of T cell activation, cytokine synthesis, and phagocyte capacity with an increase in the synthesis of bone morphogenetic proteins and in the mineralization of the extracellular matrix. However, the osteoblast loses its bone-forming capacity in the presence of BPs through their inhibition of its differentiation and maturation. In parallel, this inhibition of differentiation would lead to an increased expression of co-stimulating molecules, which is related to its functional capacity as an immune cell, although BPs inhibit the expression of these molecules, as evidenced in the present study.

In conclusion, the results of the present study suggest that low doses of amino-BPs exert their effect on osteoblasts by altering their physiology, which would explain the impaired repair capacity of the cell population; however, further studies are necessary to determine the mechanisms by which this effect takes place in order to understand the development of BRONJ. It would also be of interest to study the effects of these BPs on primary osteoblast culture lines or other typed osteoblast lines in order to confirm the effects of these drugs on the osteoblast.

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

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