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High doses of bisphosphonates reduce osteoblast-like cell proliferation by arresting the cell cycle and inducing apoptosis

Francisco Javier Manzano-Moreno [a, b, c], Javier Ramos-Torrecillas [b, c], Elvira De Luna-Bertos [b, c], Concepción Ruiz [b, c, d, *], Olga García-Martínez [b, c]

[a] Department of Stomatology, School of Dentistry, University of Granada, Spain
[b] Biomedical Group (BIO277), Department of Nursing, Faculty of Health Sciences, University of Granada, Spain
[c] Instituto Investigación Biosanitaria, ibs.Granada, Spain
[d] Institute of Neuroscience, Parque Tecnológico Ciencias de la Salud, University of Granada, Armilla, Granada, Spain

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ABSTRACT

Objectives: The study objective was to evaluate the effect on osteoblast growth of high concentrations of three nitrogen-containing bisphosphonates (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing bisphosphonate (clodronate), using the MG-63 cell line as an 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 different doses of pamidronate, alendronate, ibandronate or clodronate. The proliferative capacity of the osteoblasts was determined by spectrophotometry (MTT-based) at 24 h of culture. Flow cytometry was used to determine the percentage of cells in each cell cycle phase (G0/G1, G2/M, and S) and to discriminate apoptotic cell death from necrotic cell death in the cell cycle at 24 h of treatment.
Results: All the bisphosphonates assayed produced a significant and dose-dependent reduction in MG-63 proliferation at the high doses assayed (10^{-4} and 5×10^{-5} M) in comparison with controls ($p < 0.001$). Cell cycle study revealed that all assayed bisphosphonates significantly arrested the cell cycle in phase G0/G1 at doses of 10^{-4} and 5×10^{-5} M, increasing the percentage of cells in this phase ($p < 0.05$). Apoptosis/necrosis studies showed significant changes compared with control cells, with an increased percentage of cells in apoptosis after treatment with 10^{-4} or 5×10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate ($p < 0.05$).
Conclusions: High doses of nitrogen-containing or non-nitrogen-containing bisphosphonates can reduce the proliferation of MG-63 osteoblast-like cells by arresting the cell cycle and inducing apoptosis/necrosis.

1. Introduction

Bisphosphonates (BPs) are a family of pyrophosphate synthetic analogues in which the oxygen linking the phosphates has been replaced by carbon. They are widely used to treat bone disorders, including osteoporosis, Paget's disease, hypercalcemia of malignancy, fibrous dysplasia, and the bone metastases of breast and prostate cancer (Ralston et al., 1989; Eggelmeijer et al., 1994; Lala et al., 2000; Rodan and Martin, 2000; Lane et al., 2001). BPs can be divided between nitrogen-containing and non-nitrogen-containing forms. Nitrogen-containing BPs, such as pamidronate, alendronate, or ibandronate, interrupt the mevalonate pathway of cholesterol synthesis, inhibiting the enzyme farnesyl diphosphate synthase and blocking prenylation of small GTPases, leading to the impairment of osteoclast function (Lane et al., 2001; Reszka and Rodan, 2004). For their part, non-nitrogen-containing BPs such as clodronate suppress bone resorption by being metabolized into non-hydrolysable ATP analogues that have no releasable energy content, producing osteoclast death (Frith et al., 1997, 2001).

BPs bind to hydroxyapatite crystal but vary in the strength of their binding, which may play an important role in the duration of their action. Differences in the severity of adverse effects on bone

* Corresponding author. University of Granada, Avda. de la Ilustración s/n, 18016 Granada, Spain. Tel.: +34 958243497; fax: +34 958242894.
E-mail address: crr@ugr.es (C. Ruiz).

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tissue in long-term BP treatment have been attributed to the lesser or greater binding of the different BPs to hydroxyapatite (Nancollas et al., 2006; Idris et al., 2008). According to some authors, although therapeutic doses are not very high (10^{-5} to 10^{-9} M) (Chen et al., 2002), very high concentrations may reach the bone after prolonged treatment because of the strong binding of these drugs to hydroxyapatite (Marx, 2014).

BPs are widely prescribed, and their ability to inhibit osteoclast formation and activity in vitro and in vivo is well documented (Russell, 2007; Silverman and Maricic, 2007). 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; a decrease in collagen production of osteoblasts (Açil et al., 2012); an anti-angiogenic effect producing avascular necrosis; and a reduction in the viability of fibroblasts and oral keratinocytes (Santini et al., 2002; Mashiba et al., 2005; Landesberg et al., 2008). Although useful in the treatment of some bone diseases, BPs have also been associated with the development of BP-related osteonecrosis of the jaw (BRONJ) (Marx, 2003; Ruggiero et al., 2004). Probably, the infectious conditions that often precede the onset of BRONJ support recent pathogenesis theories stating that local inflammation and associated pH-changes may trigger the release and activation of nitrogen-containing bisphosphonates ultimately resulting in necrosis (Otto et al., 2012).

The objective of this study was to evaluate the effect on osteoblast growth of high concentrations of three nitrogen-containing BPs (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing BP (clodronate), using the MG-63 cell line, in order to determine the role of osteoblasts in BRONJ. The MG-63 cell line is commonly used as an osteoblast model because it shares the same characteristics.

2. Materials and methods

We studied three nitrogen-containing BPs, pamidronate (Sigma–Aldrich, St. Louis, MO, USA), alendronate (Sigma), and ibandronate (Sigma), and one non-nitrogen-containing BP, clodronate (Sigma).

2.1. Cell culture

Human MG-63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA, USA) and maintained as described by De Luna-Bertos et al., 2013, in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA) 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 (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 were then washed and suspended in complete culture medium with 10% fetal bovine serum.

2.2. Cell proliferation assay

Osteoblasts were seeded at 1×10^4 cells/mL per well into a 24-well plate (Falcon, Becton Dickinson Labware, NJ, USA) 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, ibandronate, or clodronate at a dose of 10^{-4}, 5×10^{-5}, or 10^{-5} M. After 24 h of culture, the cell proliferation was measured by MTT assay, as described by Manzano-Moreno et al. (2013), 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.) The results were reported as mean absorbance (570 nm) ± SD. At least three separate experiments were conducted for each treatment, using the mean value in the analysis.

2.3. Cell cycle

Cultured human MG-63 cells treated for 24 h with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate, or without BP (control), were detached from the culture flask by treatment with a solution of 0.05% trypsin (Sigma) and 0.02% EDTA (Sigma) and were then washed and suspended in PBS and prepared for study of the cell cycle as reported by García-Martínez et al. (2011). The suspension obtained was placed in 200 μL PBS with 2 mL ice-cold 70% ethanol and 30% distilled H_2O and vigorously mixed. Cells were left for at least 30 min in the cold and then harvested by centrifugation and resuspended in 800 μL PBS. Cells were microscopically examined and, if clumped, passed through a 25-gauge syringe needle. Cells were then incubated at 37 °C for 30 min with 100 μL ribonuclease (RNase) (1 mg/mL) and 100 μL propidium iodine (PI). Finally, samples were analyzed by using an argon-ion laser tuned to 488 nm (Facs Vantage, Becton Dickinson, Palo Alto, CA, USA), measuring forward and orthogonal light scatter and red fluorescence, determining both the area and peak of the fluorescent signal.

2.4. Apoptosis and necrosis analysis

The study of apoptosis and necrosis was performed as described by De Luna-Bertos et al. (2014). Osteoblasts were previously treated with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate for 24 h at 37 °C. Next, cells were detached from the culture flask, washed, suspended in 300 μL PBS, and then labeled with annexin V and PI (Immunostep S.L., Salamanca, Spain), incubating 100 μL aliquots of the cell suspension with 5 μL annexin V and 5 μL PI for 30 min at 4 °C in the dark. Cells were then washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer with argon laser (Facs Vantage, Becton Dickinson) at a wavelength of 488 nm to determine the percentage of fluorescent cells. We calculated the percentage of annexin-positive (apoptotic) cells and PI-positive (necrotic) cells from counts of 2000–3000 cells.

2.5. Statistical analysis

SPSS 22.0 (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, apoptosis/necrosis induction, and cell cycle as a function of the BP type (pamidronate, alendronate, ibandronate, or clodronate), treatment duration, and concentration. When a significant interaction was identified, the Bonferroni correction was applied for planned pair-wise comparisons. $P < 0.05$ was considered significant. At least three separate experiments were performed for each assay.