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The response of dental pulp-derived cells to zoledronate depends on the experimental model

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Abstract

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Aim To investigate whether zoledronate (ZOL) can cause a cytotoxic response in dental pulp-derived cells (DPCs) *in vitro*.

Methodology Cell activity was assessed utilizing MTT tests, ^{3}[H]thymidine, and ^{3}[H]leucine incorporation assays in human DPCs in response to ZOL. Cell activity assays were also preformed on calcium phosphate-coated plates. Cell death was analysed with annexin V/propidium iodide, trypan blue staining and Western blot analysis.

Results Micromolar concentrations of ZOL were required to decrease the activity of DPCs. The decreased activity of DPCs was associated with the occurrence of apoptosis and necrosis. No adverse effects were observed when DPCs were cultured on calcium phosphate-coated plates with ZOL.

Conclusion High concentrations of soluble ZOL were required to cause adverse effects *in vitro*. These adverse effects are abolished when the bisphosphonate was bound to a mineralized surface. However, the clinical relevance of these results remains to be determined.

Keywords: bisphosphonate-related osteonecrosis of the jaw, bisphosphonates, cell death, cytotoxicity, dental pulp-derived cells, zoledronic acid.

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Introduction

The intravenous application of zoledronate (ZOL) is an established therapeutic strategy for the treatment of tumour-associated bone metastasis and severe hypercalcemia (Ibrahim *et al.* 2003, Michaelson & Smith 2005). ZOL is also approved for the prevention and therapy of osteoporosis, although at an approximately 10-fold lower dosage (Black *et al.* 2007). ZOL is generally well tolerated, and the rate of adverse effects, such as acute phase reaction, nephrotoxicity and gastrointestinal erosions, is low (Dunstan *et al.* 2007). An adverse effect of this therapy is the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ), which presents as exposed necrotic alveolar bone (Khosla *et al.* 2007, Almazrooa & Woo 2009). The incidence of BRONJ is between 1% and 10% in patients with oncological diseases, while BRONJ is a rare event in patients with metabolic osteopathies (Badros *et al.* 2006, Pazianas *et al.* 2007).

The pharmacological mechanism that is responsible for the antiresorptive potential of this drug has been identified (Roelofs *et al.* 2006, Russell 2007). Bisphosphonates bind to the mineralized bone matrix and are exclusively released by osteoclasts. When internalized, bisphosphonates inhibit the mevalonate pathway, which is mandatory for osteoclast activity (Roelofs *et al.* 2006, Russell 2007). There are several hypothesis on the pathogenesis of BRONJ including suppression of bone remodelling, antiangiogenic effects, infection and cytotoxicity (Reid *et al.* 2007, Allen & Burr 2009). Despite these plausible hypotheses, very little data regarding the pathogenesis of BRONJ are available (Allen & Burr 2009).

Dental treatments, including tooth extraction, are associated with the occurrence of BRONJ and may

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therefore be considered a risk factor in pathogenesis of BRONJ (Woo *et al.* 2006, Dunstan *et al.* 2007). These observations, however, do not rule out the possibility that bisphosphonates might have a negative impact on the viability of the dental pulp. Bisphosphonates may thereby establish a vicious cycle that contributes to the development of BRONJ. However, whether bisphosphonates are toxic to the dental pulp cells is unclear.

Bisphosphonates have a high affinity to mineralized tissue such as dentine (Coxon *et al.* 2008); thus, ZOL may accumulate in the dentine adjacent to the dental pulp chamber. However, the chronic release of bisphosphonates from the dentine would require remodelling, which does not regularly occur in the adult. Dental pulp cells can, however, be exposed to bisphosphonates immediately after the infusion of ZOL; hence, acute adverse effects cannot be ruled out. Currently, data is limited to demonstrating the effects of bisphosphonates on mineralization during odontogenesis (Ogawa *et al.* 1989, Fuangtharnthip *et al.* 2000, Massa *et al.* 2006) and local treatment of tooth roots and canals before replantation (Mori *et al.* 2007, Komatsu *et al.* 2008). Thus, the clinical impact of bisphosphonates on pulp vitality remains to be determined.

The objective of this study was to determine the cytotoxic activity of ZOL in an *in vitro* situation with dental pulp-derived cells (DPCs). Based on *in vitro* models, ZOL and other bisphosphonates have been evaluated for toxicity including osteogenic cells (Almazrooa & Woo 2009, Simon *et al.* 2009, Walter *et al.* 2009), periodontal fibroblasts (Correia Vde *et al.* 2006, Simon *et al.* 2009, Walter *et al.* 2009, Agis *et al.* 2010) and endothelial cells (Hasmim *et al.* 2007, Walter *et al.* 2009). In this study, it was hypothesized that DPCs can also respond to ZOL by a decrease in cell activity and the occurrence of cell death. Advantage was taken of an *in vitro* model, where ZOL can bind to calcium phosphate (Schindeler & Little 2005, Patntirapong *et al.* 2009, Agis *et al.* 2010).

Material and methods

Experimental settings

Dental pulp-derived cells (DPCs) were isolated from the extracted third molars of three donors after informed consent was obtained (Ethics Committee of the Medical University Vienna, No. 631/2007). Standard access preparation was performed and pulp tissue removed (Papaccio *et al.* 2006). DPCs were obtained from explant cultures in minimal essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal calf serum (FCS; PAA Laboratories, Linz, Austria) and antibiotics (Invitrogen) in a humidified atmosphere at 37 °C.

For all experiments, 30 000 cells cm^{-2} up to passage five were plated on culture dishes. On the day following the initial plating, the growth medium was changed to serum-free medium with or without ZOL (30, 100 μmol L^{-1}; Novartis Pharma GmbH, Vienna, Austria) or staurosporine (STAU; 100 nmol L^{-1}; Sigma, St. Louis, MI, USA). Staurosporine is an ATP-competitive kinase inhibitor, which acted as a positive control for cell death (Bertrand *et al.* 1994, Karaman *et al.* 2008). Cells were cultured in serum-free condition to exclude interactions between ZOL and serum. To assess the impact of serum, cells were treated with ZOL in the presence of 10% FCS. DPCs were incubated for 24, 48 and 72 h before cells were subjected to analysis.

Where indicated, culture plates were coated with a layer of calcium phosphate. Calcium phosphate (CaP) coating was performed in two steps as previously described (Patntirapong *et al.* 2009). First, wells of 96-well tissue culture plates were incubated with a 2.5-fold Kokubo's simulated body fluid (2.5-fold SBF; 6.25 mmol L^{-1} $CaCl_2$, 0.34 mol L^{-1} NaCl, 3.75 mmol L^{-1} $MgCl_2$, 2.78 mmol L^{-1} Na_2HPO_4, 10.5 mmol L^{-1} $NaHCO_3$ in 50 mmol L^{-1} Tris pH 7.4) for 3 days. The 2.5-fold SBF solution was refreshed daily. Secondly, the wells were changed to a calcium phosphate solution (CPS; 2.25 mmol L^{-1} Na_2HPO_4, 4 mmol L^{-1} $CaCl_2.2H_2O$, 0.14 mol L^{-1} NaCl, 50 mmol L^{-1} Tris) for 3 days with daily refreshment. Then the wells were washed thoroughly with deionized water, dried at 50 °C overnight and sterilized. DPCs were seeded on calcium phosphate-coated culture dishes as described and treated with ZOL at 30 and 100 μmol L^{-1} for 48 h with and without the presence of serum. In another set of experiments calcium phosphate-coated culture wells were incubated with and without ZOL at 30 or 100 μmol L^{-1} for 2 h and washed three times with culture medium (Schindeler & Little 2005). DPCs were then plated on culture dishes and subjected to analysis after 48 h.

Cell activity: viability, proliferation and protein synthesis

For the analysis of cell viability, DPCs were exposed to MTT (1.25 mg mL^{-1}; Sigma) for 2 h at 37 °C, and the optical density of solubilized formazan crystals was

measured. For the analysis of proliferation and protein synthesis, DPCs were pulse labelled with ^{3}[H]thymidine or ^{3}[H]leucine (Amersham Pharmacia Biotech, Buckingham, UK). Labeled cells were washed with saline and allowed to dry before adding the scintillation cocktail. Then plates were subjected to liquid scintillation counting (Packard, Meriden, CT, USA). Data are expressed as relative percentage of untreated controls.

Cell death: apoptosis and necrosis

For Western blot analysis of apoptosis, DPCs exposed to ZOL for 48 h were applied. Untreated cells served as control. Both groups, ZOL-treated cells and untreated cells were lysed with Laemmli sample buffer containing phosphatase and protease inhibitors. Cell lysates were separated and transferred onto nitrocellulose membranes (Amersham). Membranes were washed with TBS-T and blocked in a 5% (w/v) milk powder solution. Membranes were exposed to antibodies for cleaved poly (ADP-ribose) polymerase (cleaved PARP; No. 9541; Cell Signalling Technology, Danvers, MA, USA) and actin (No. C-11, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 1000. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Rao *et al.* 1996). Western blots for actin were performed to test for equal loading of the sample. After washing in TBS-T, membranes were incubated with the appropriate secondary antibody diluted 1 : 2000. For cleaved PARP, an anti-rabbit (Dako, Glostrup, Denmark) and for actin, an anti-goat antibody (Dako) was used. To analyse the levels of apoptosis and necrosis, the Annexin V-FLUOS Kit (Roche, Mannheim, Germany) was used that contains Annexin-V-Fluorescein and propidium iodid. Analysis was performed by fluorescence and light microscope with a B-2A filter (excitation filter wavelengths: 450–490 nm). Furthermore, necrosis was analysed by trypan blue staining (0.4%). Staining was evaluated by light microscopy.

Statistical analysis

All experiments were performed at least twice with cells from three donors. Data were analysed with ANOVA and *post hoc* Student's paired *t*-test when data followed a normal distribution and standard deviations within the groups were approximately equal. If data have not been normal distributed, the Kruskal–Wallis and *post hoc* Mann–Whitney *U*-tests were used. Significant difference was assigned at the $P < 0.05$ level.

Results

Soluble ZOL reduces cell activity

Time-course and dose–response studies were performed to determine the sensitivity of isolated DPCs to ZOL. Viability was measured by the capacity of the cells to convert MTT into formazan crystals. Proliferation and protein synthesis were analysed by their ability to incorporate ^{3}[H]thymidine and ^{3}[H]leucine into DNA and protein, respectively. ZOL caused a reduction in viability, proliferation and protein synthesis at a concentration of 30 μmol L^{-1} after a 24-h incubation time. This cytotoxic effect became more severe when the concentration of ZOL was increased to 100 μmol $L^{-1,}$ and the incubation period was prolonged for up to 72 h (Fig. 1). Similar effects were observed in the presence of serum (Figure S1). Staurosporine, an inducer of cell death, revealed similar results (data not shown).

Soluble ZOL causes cell death by necrosis and apoptosis

To further elucidate the mechanisms that lead to the decrease in cell activity in ZOL-treated cells, the two pathways that lead to cell death including apoptosis and necrosis were considered. DPCs exposed to ZOL demonstrated significant changes in cell morphology, undergoing cell rounding and detachment as a result of necrosis and apoptosis. Based on these findings, necrosis was detected in cultures that were treated for 48 h with 30 μmol L^{-1} ZOL, as indicated by the permeability of the cell membrane for trypan blue and propidium iodide. Cells staining positive for annexin V but not for propidium iodide were also observed by fluorescence microscopy, indicating the presence of apoptosis (Fig. 2a). In line with these findings, the Western blot revealed a clear band for cleaved PARP, another characteristic marker for apoptosis (Fig. 2b). Staurosporine, an inducer of cell death, demonstrated similar results (data not shown). Together, these findings indicate that the decrease in cell activity induced by ZOL is the result of necrosis and apoptosis.

Calcium phosphate-bound ZOL has no effect on cell activity

Previous studies have shown that cell viability is maintained when cells are cultured on calcium phosphate-coated plates that have been pre-treated with

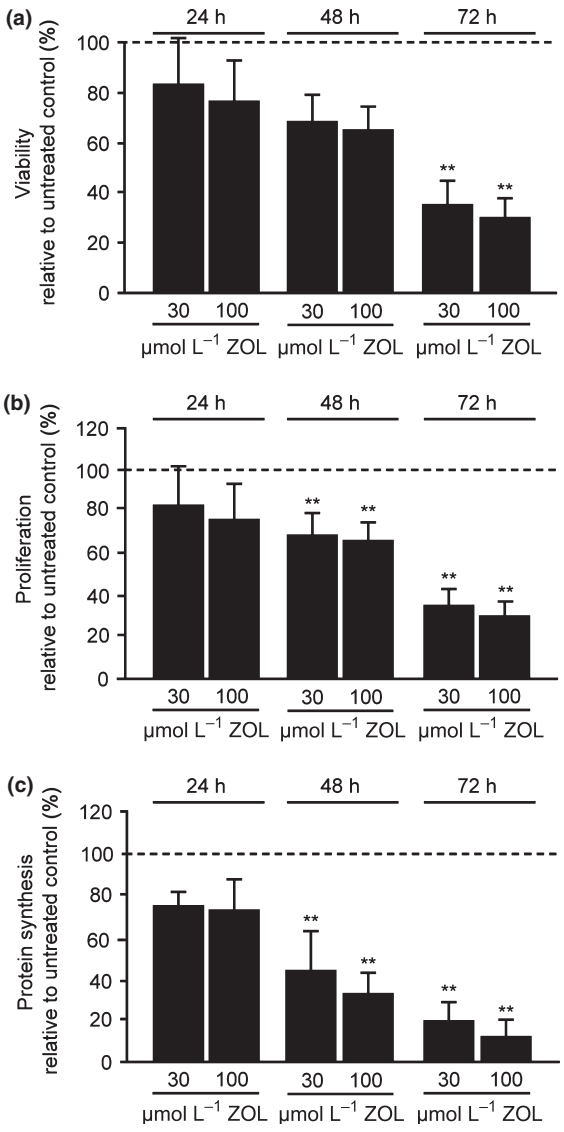

Figure 1 Soluble zoledronate reduces dental pulp-derived cells activity. Dental pulp-derived cells (DPCs) were stimulated with or without zoledronate (ZOL) at 30 and 100 μmol L^{-1} for the indicated time. All experiments were performed twice with cells from three donors. (a) Cell viability was measured via formation of formazan crystals. (b, c) Proliferation and protein synthesis were measured via incorporation of ^{3}[H]thymidine and ^{3}[H]leucine, respectively. Bars represent the mean + standard deviation relative to the untreated control (%). $^{**}P < 0.01$.

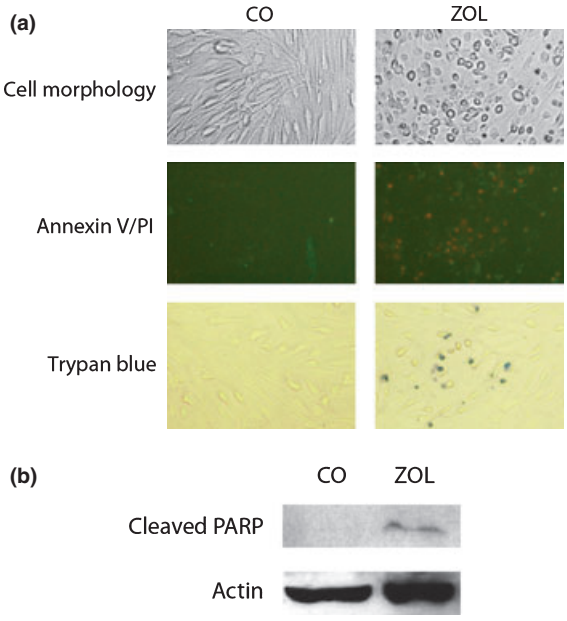

Figure 2 Soluble zoledronate causes necrosis and apoptosis in dental pulp-derived cells. Dental pulp-derived cells (DPCs) were exposed to zoledronate (ZOL) at 30 μmol L^{-1} for 48 h. As control untreated DPCs were used (CO). (a) The effect of ZOL on cell morphology, apoptosis and necrosis was evaluated with light microscopy, annexin V/propidium iodide staining (annexin V/PI) and trypan blue staining. (b) The effect of ZOL on the apoptosis marker–cleaved PARP was also examined by Western blotting. Western blots for actin were performed as a loading control.

ZOL (Agis *et al.* 2010). Therefore, the impact of ZOL on DPCs in similar *in vitro* models was determined. DPCs were cultured on calcium phosphate-coated plates and treated with ZOL under serum-free conditions and in the presence of serum (Fig. 3a–c; Figure S2a–c). In addition, calcium phosphate-coated plates were pre-treated with ZOL to exclude the effect of soluble ZOL (Fig. 4a–c). As expected, ZOL did not cause any detectable toxic effects in any of these *in vitro* models (Figs 3 and 4 and Figure S2). At these concentrations, ZOL effectively inhibited osteoclast activity (data not shown). These results suggest that once ZOL is bound to calcium phosphate, it becomes inert for the DPCs. These findings thus demonstrate that the selection of *in vitro* conditions has a significant impact on the cellular response to ZOL. *In vitro* models that more closely resemble the *in vivo* situation suggest that once ZOL is adsorbed to the mineralized surface, the cytotoxic effects are restricted to those cells that can release ZOL from the mineralized surface, i.e. osteoclasts.

Discussion

The results revealed that ZOL can reduce cell viability and associated parameters of proliferation and protein synthesis *in vitro*. This is in line with studies on

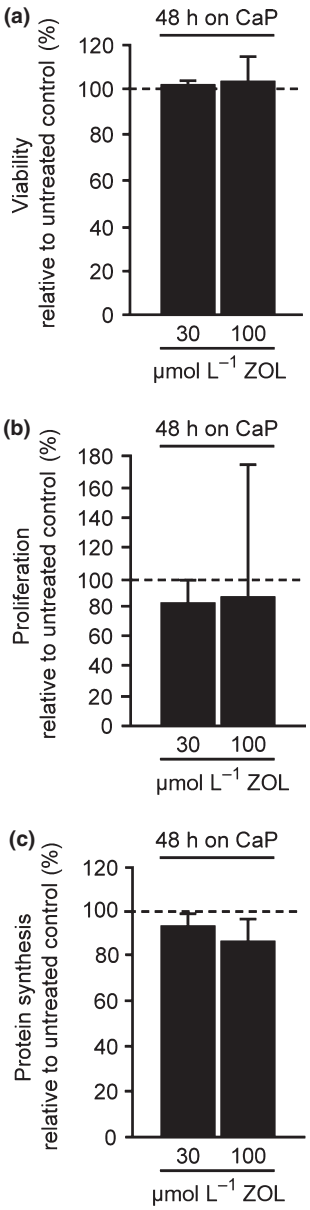

Figure 3 Zoledronate has no effect on cell activity of DPCs cultured on calcium phosphate-coated plates. Dental pulp-derived cells (DPCs) were seeded on calcium phosphate-coated plates. The next day DPCs were incubated with and without zoledronate (ZOL) at 30 and 100 μmol L^{-1} for 48 h. Experiments were performed at least twice with cells from three donors. (a) Cell viability in response to ZOL was measured as formation of formazan crystals. (b, c) Proliferation and protein synthesis were measured as incorporation of ^{3}[H]thymidine and ^{3}[H]leucine, respectively. Bars represent the mean + standard deviation relative to the untreated control (%).

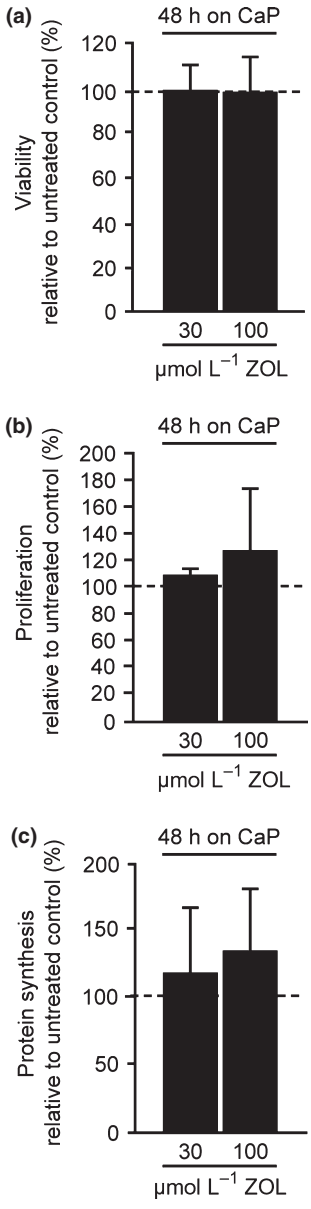

Figure 4 Calcium phosphate-bound zoledronate (ZOL) has no effect on cell activity. Dental pulp-derived cells (DPCs) were seeded on calcium phosphate-coated plates that were pre-incubated with and without ZOL at 30 and 100 μmol L^{-1}. Experiments were performed twice with cells from three donors. (a) Cell viability in response to ZOL was measured as formation of formazan crystals. (b, c) Proliferation and protein synthesis were measured as incorporation of ^{3}[H]thymidine and ^{3}[H]leucine, respectively. Bars represent the mean + standard deviation relative to the untreated control (%).

fibroblasts derived from the periodontium (Reinholz *et al.* 2000, Kellinsalmi *et al.* 2005, Scheper *et al.* 2009, Walter *et al.* 2009, Agis *et al.* 2010) and tumour cells (Senaratne *et al.* 2000, Corey *et al.* 2003, Dumon *et al.* 2004). Consistent with these observations, DPCs undergo necrosis and apoptosis, based on membrane leakage, annexin V staining and cleaved PARP. However, cells remained viable when ZOL was allowed to bind to calcium phosphate. Together, these results suggest that the selection of the *in vitro* culture conditions is critical to evaluate the cytotoxic activity of ZOL for DPCs.

It thus remains open whether the occurrence of BRONJ involves cell death in the dental pulp. Pharmacokinetic studies have shown that serum levels of ZOL transiently reach approximately 1 μmol L^{-1} (Chen *et al.* 2002). These concentrations are below the threshold level that caused short-term cytotoxic effects in the *in vitro* model used in this study. In addition, the pharmacokinetic of ZOL in the pulp chamber is unknown. The pharmacological mechanism of ZOL includes binding to bone (Roelofs *et al.* 2006, Russell 2007). The similarities of bone and dentine support the possibility that ZOL can accumulate in dentine. However, the results reveal that the cytotoxic effects are diminished when ZOL is bound to calcium phosphate. In bone tissue, a proportion of ZOL is released by osteoclasts and surrounding cells might be exposed to the drug (Coxon *et al.* 2008). In the dental pulp, it is likely that ZOL remains bound to the dentine matrix as the release of ZOL requires resorption by odontoclasts, which does not occur regularly in the adult (Silva *et al.* 2004, Harokopakis-Hajishengallis 2007, Coxon *et al.* 2008). The current state of knowledge does not rule out this possibility and preclinical studies are demanding. The *in vitro* study contributes towards evaluating these theories, as it can distinguish between soluble and calcium phosphate-bound ZOL and the consequences related to these modifications of the model. Although the *in vitro* model suggests that short-term effects of ZOL bound to calcium phosphate are unlikely, it cannot rule out long-term effects of the bisphosphonate *in vivo*. It is, however, not clear if ZOL binds to dentine of the dental pulp at all. Further studies are required that address this issue.

The data suggest the concept that high concentrations of soluble ZOL are required to cause toxic effects. The pharmacological mechanism of ZOL involves the inhibition of the mevalonate pathway in osteoclasts thereby inducing apoptosis. (Roelofs *et al.* 2006, Kimmel 2007, Russell 2007, Weinstein *et al.* 2009). Necrosis is usually a rare event when nontransformed cells are exposed to ZOL (Kellinsalmi *et al.* 2005). However, both apoptosis and necrosis were observed in other oral cells such as fibroblasts of the periodontal ligament (Agis *et al.* 2010). Whether the induction of apoptosis and necrosis in DPCs is mediated by the inhibition of the mevalonate pathway requires further studies. It can also not be ruled out that under inflammatory conditions or in the presence of chemotherapeutic agents, cells become more responsive to ZOL. This hypothesis will serve as the basis for subsequent studies aiming to understand the impact of the microenvironment on cellular response to ZOL.

Conclusion

In conclusion, micromolar concentrations of soluble ZOL, approximately an order of magnitude above the serum level after infusion, decreased the activity of DPCs and force cells to undergo apoptosis and necrosis *in vitro*. When ZOL is bound to calcium phosphate, DPCs do not exhibit these adverse response to the drug. The results suggest that it is unlikely that ZOL has a substantial negative effect on pulp viability. However, the clinical relevance of these results remains to be determined.

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References

Agis H, Blei J, Watzek G, Gruber R (2010) Is zoledronate toxic to human periodontal fibroblasts? *Journal of Dental Research* **89**, 40–5.

Allen MR, Burr DB (2009) The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. *Journal of Oral and Maxillofacial Surgery* **67**, 61–70.

Almazrooa SA, Woo SB (2009) Bisphosphonate and non-bisphosphonate-associated osteonecrosis of the jaw: a review. *Journal of the American Dental Association* **140**, 864–75.

Badros A, Weikel D, Salama A *et al.* (2006) Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors. *Journal of Clinical Oncology* **24**, 945–52.

Bertrand R, Solary E, O'Connor P, Kohn KW, Pommier Y (1994) Induction of a common pathway of apoptosis by staurosporine. *Experimental Cell Research* **211**, 314–21.

Black DM, Delmas PD, Eastell R *et al.* (2007) Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. *The New England Journal of Medicine* **356**, 1809–22.

Chen T, Berenson J, Vescio R *et al.* (2002) Pharmacokinetics and pharmacodynamics of zoledronic acid in cancer patients with bone metastases. *Journal of Clinical Pharmacology* **42**, 1228–36.

Corey E, Brown LG, Quinn JE *et al.* (2003) Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. *Clinical Cancer Research* **9**, 295–306.

Correia Vde F, Caldeira CL, Marques MM (2006) Cytotoxicity evaluation of sodium alendronate on cultured human periodontal ligament fibroblasts. *Dental Traumatology* **22**, 312–7.

Coxon FP, Thompson K, Roelofs AJ, Ebetino FH, Rogers MJ (2008) Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. *Bone* **42**, 848–60.

Dumon JC, Journe F, Kheddoumi N, Lagneaux L, Body JJ (2004) Cytostatic and apoptotic effects of bisphosphonates on prostate cancer cells. *European Urology* **45**, 521–9.

Dunstan CR, Felsenberg D, Seibel MJ (2007) Therapy insight: the risks and benefits of bisphosphonates for the treatment of tumor-induced bone disease. *Nature Reviews Clinical Oncology* **4**, 42–55.

Fuangtharnthip P, Yamada Y, Takagi Y, Ohya K (2000) Autoradiographic investigation of the effect of 1-hydroxyethylidene-1, 1-bisphosphonate on matrix protein synthesis and secretion by secretory ameloblasts in rat incisors. *Archives of Oral Biology* **45**, 495–506.

Harokopakis-Hajishengallis E (2007) Physiologic root resorption in primary teeth: molecular and histological events. *European Journal of Oral Sciences* **49**, 1–12.

Hasmim M, Bieler G, Ruegg C (2007) Zoledronate inhibits endothelial cell adhesion, migration and survival through the suppression of multiple, prenylation-dependent signaling pathways. *Journal of Thrombosis and Haemostasis* **5**, 166–73.

Ibrahim A, Scher N, Williams G *et al.* (2003) Approval summary for zoledronic acid for treatment of multiple myeloma and cancer bone metastases. *Clinical Cancer Research* **9**, 2394–9.

Karaman MW, Herrgard S, Treiber DK *et al.* (2008) A quantitative analysis of kinase inhibitor selectivity. *Nature Biotechnology* **26**, 127–32.

Kellinsalmi M, Monkkonen H, Monkkonen J *et al.* (2005) *In vitro* comparison of clodronate, pamidronate and zoledronic acid effects on rat osteoclasts and human stem cell-derived osteoblasts. *Basic & Clinical Pharmacology & Toxicology* **97**, 382–91.

Khosla S, Burr D, Cauley J *et al.* (2007) Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. *Journal of Bone and Mineral Research* **22**, 1479–91.

Kimmel DB (2007) Mechanism of action, pharmacokinetic and pharmacodynamic profile, and clinical applications of nitrogen-containing bisphosphonates. *Journal of Dental Research* **86**, 1022–33.

Komatsu K, Shimada A, Shibata T *et al.* (2008) Long-term effects of local pretreatment with alendronate on healing of replanted rat teeth. *Journal of Periodontal Research* **43**, 194–200.

Massa LF, Bradaschia-Correa V, Arana-Chavez VE (2006) Immunocytochemical study of amelogenin deposition during the early odontogenesis of molars in alendronate-treated newborn rats. *Journal of Histochemistry and Cytochemistry* **54**, 713–25.

Michaelson MD, Smith MR (2005) Bisphosphonates for treatment and prevention of bone metastases. *Journal of Clinical Oncology* **23**, 8219–24.

Mori GG, Garcia RB, Gomes de Moraes I, Bramante CM, Bernardineli N (2007) Morphometric and microscopic evaluation of the effect of a solution of alendronate as an intracanal therapeutic agent in rat teeth submitted to late reimplantation. *Dental Traumatology* **23**, 218–21.

Ogawa Y, Adachi Y, Hong SS, Yagi T (1989) 1-Hydroxyethylidene-1,1-bisphosphonate (HEBP) simultaneously induces two distinct types of hypomineralization in the rat incisor dentine. *Calcified Tissue International* **44**, 46–60.

Papaccio G, Graziano A, d'Aquino R *et al.* (2006) Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *Journal of Cellular Physiology* **208**, 319–25.

Patntirapong S, Habibovic P, Hauschka PV (2009) Effects of soluble cobalt and cobalt incorporated into calcium phosphate layers on osteoclast differentiation and activation. *Biomaterials* **30**, 548–55.

Pazianas M, Miller P, Blumentals WA, Bernal M, Kothawala P (2007) A review of the literature on osteonecrosis of the jaw in patients with osteoporosis treated with oral bisphosphonates: prevalence, risk factors, and clinical characteristics. *Clinical Therapeutics* **29**, 1548–58.

Rao L, Perez D, White E (1996) Lamin proteolysis facilitates nuclear events during apoptosis. *Journal of Cell Biology* **135**, 1441–55.

Reid IR, Bolland MJ, Grey AB (2007) Is bisphosphonate-associated osteonecrosis of the jaw caused by soft tissue toxicity? *Bone* **41**, 318–20.

Reinholz GG, Getz B, Pederson L *et al.* (2000) Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. *Cancer Research* **60**, 6001–7.

Roelofs AJ, Thompson K, Gordon S, Rogers MJ (2006) Molecular mechanisms of action of bisphosphonates: current status. *Clinical Cancer Research* **12**, 6222s–30s.

Russell RG (2007) Bisphosphonates: mode of action and pharmacology. *Pediatrics* **119**(Suppl. 2), S150–62.

Scheper MA, Badros A, Chaisuparat R, Cullen KJ, Meiller TF (2009) Effect of zoledronic acid on oral fibroblasts and

epithelial cells: a potential mechanism of bisphosphonate-associated osteonecrosis. *British Journal of Haematology* **144**, 667–76.

Schindeler A, Little DG (2005) Osteoclasts but not osteoblasts are affected by a calcified surface treated with zoledronic acid *in vitro*. *Biochemical and Biophysical Research Communications* **338**, 710–6.

Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW (2000) Bisphosphonates induce apoptosis in human breast cancer cell lines. *British Journal of Cancer* **82**, 1459–68.

Silva TA, Rosa AL, Lara VS (2004) Dentin matrix proteins and soluble factors: intrinsic regulatory signals for healing and resorption of dental and periodontal tissues? *Oral Diseases* **10**, 63–74.

Simon MJ, Niehoff P, Kimmig B, Wiltfang J, Acil Y (2009) Expression profile and synthesis of different collagen types I, II, III, and V of human gingival fibroblasts, osteoblasts, and SaOS-2 cells after bisphosphonate treatment. *Clinical Oral Investigations* **14**, 51–8.

Walter C, Klein MO, Pabst A, Al-Nawas B, Duschner H, Ziebart T (2009) Influence of bisphosphonates on endothelial cells, fibroblasts, and osteogenic cells. *Clinical Oral Investigations* **14**, 35–41.

Weinstein RS, Roberson PK, Manolagas SC (2009) Giant osteoclast formation and long-term oral bisphosphonate therapy. *The New England Journal of Medicine* **360**, 53–62.

Woo SB, Hellstein JW, Kalmar JR (2006) Narrative [corrected] review: bisphosphonates and osteonecrosis of the jaws. *Annals of Internal Medicine* **144**, 753–61.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Soluble zoledronate reduces cell activity of DPCs under the presence of serum.

Figure S2. Zoledronate has no effect on the cell activity of DPCs cultured on calcium phosphate-coated plates in the presence of serum.

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