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Zoledronic acid differently affects long bone and jaw bone turnover and induces molar root resorption in female mice

Jenny A.F. Vermeer
Greetje A.P. Renders
Marion A. van Duin
Ineke D.C. Jansen
Lieneke F. Bakker
Sophie A. Kroon
Teun J. de Vries
Vincent Everts

Submitted for publication
Abstract

Bisphosphonates (BPs) are widely used to treat bone diseases such as osteoporosis. However, BPs can negatively affect the jaw bone by causing osteonecrosis of the jaw. Previously, we showed that BPs differently affected long bone and jaw osteoclast precursors. Here, we investigated whether \textit{in vivo} exposure to BPs has a different effect on long bone and jaw osteoclasts and the turnover of these two types of bone. Zoledronic acid (ZA, 0.5 mg/kg weekly) was administered i.p. to 3-month-old female C57BL/6J mice for up to 6 months and its effect on osteoclasts and bone formation was studied. Long-term treatment with ZA reduced the number of jaw bone marrow cells, without affecting the number of long-bone marrow cells. ZA treatment did not affect the osteoclastogenic potential of long bone and jaw bone marrow cells \textit{in vitro} nor the number of osteoclasts \textit{in vivo}. Yet, ZA treatment increased bone volume and mineral density of both long bone and jaw. Interestingly, 6 months of treatment inhibited bone formation in the long bones without affecting the jaw. Finally, we showed that BPs can cause molar root resorption. Our results show that BPs differently affect long bone and jaw bone marrow cells and bone turnover in those bones. These findings provide more insight into bone-site-specific effects of bisphosphonates. Also, we showed that BPs can stimulate osteoclasts and their activity at the molar roots.
Introduction

Bisphosphonates (BPs) such as zoledronic acid (ZA) are commonly used to treat diseases that are characterized by excessive bone resorption. They inhibit osteoclastic bone resorption, thereby improving bone quality and reducing fracture risk. As BPs have a strong affinity for calcium, they rapidly bind to bone mineral after administration. During bone resorption they are released from the bone and taken up by osteoclasts. However, BPs have also been shown to be internalized by surrounding cells such as monocytes and macrophages [1-3]. Long-term BP treatment also reduced the number of osteoclast precursors in human peripheral blood [4,5]. These data show that next to osteoclasts, other cell types can also be affected by BPs, indicating that BPs may affect bone turnover in several ways.

A side effect of high-dose BP use in humans is osteonecrosis of the jaw (ONJ), which is defined as an exposure of bone that lasts for at least 8 weeks [6]. Risk factors for this condition are dental extractions and other oral trauma [7], however it is not clear why specifically the jaw is negatively affected by BPs. With the increasing life expectancy, it is estimated that these drugs will be subscribed more often, and the side effects will become an emerging problem. Although several in vivo models have shown that BP treatment induces necrotic jaw bone, these signs of ONJ were not solely the result of BP treatment. After tooth extraction, BPs delayed alveolar bone healing, especially in combination with an immunosuppressive agent [8,9]. Also, BP treatment induced signs of ONJ in rat models for periodontal disease [10,11]. Despite the development of these models, it remains unclear how and why specifically the jaw is affected by BP treatment. To explain bone-site-specific effects of BPs, it is essential to gain more insight into the bone remodeling activity of the different bones.

Previously, we hypothesized that different effects of BPs on osteoclasts in the jaw and in long bone may explain the etiology of BP-related ONJ [12]. We showed that BPs did not differently affect long bone and jaw osteoclastogenesis in vitro. However, osteoclast precursors from the jaw internalized more BPs than long-bone marrow precursors. It remains unclear whether long bone and jaw bone marrow cells are differently affected by BPs in vivo.

Here, we studied the effect of BP treatment on the jaw and on long bones of female mice in vivo. We also investigated the osteoclastogenic potential of bone marrow cells isolated from BP-treated mice. Finally, we studied the effect of BP treatment on markers of bone turnover in the different bones. This study will give us more insight into bone-site-specific effects of bisphosphonates and may provide a better understanding of the pathophysiology of osteonecrosis of the jaw.
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Materials and methods

Mice and bone marrow isolation

Animal experiments were approved by the Animal Welfare Committee of the VU University (Amsterdam, The Netherlands). Female, 3-month-old C57BL/6J mice were divided into 4 groups. Two groups were ovariectomized and two groups were sham operated. The ovariectomized mice were used for other experiments, not mentioned in this study. Sham operated mice were injected intraperitoneally with a high dose (0.5 mg/kg) zoledronic acid (ZA; Novartis, Basel, Switzerland) or saline once a week. Mice (6 per group) were sacrificed at baseline and 1, 3, and 6 months after the start of the experiment. Eight days before sacrifice, mice were injected intraperitoneally with fluorescently labeled calcein (10 mg/kg; Sigma, St. Louis, MO, USA), and 2 days before sacrifice with alizarin complexone (20 mg/kg; Sigma). Bone marrow cells were isolated from the right mandibles and long bones (tibia and femur) as described before [12]. Left mandibles, maxillas, and left humeri were fixed with 4% phosphate-buffered formaldehyde and stored at 4°C until microCT analysis and processing for histology.

Osteoclastogenesis and TRACP staining

Cells were stained with Türk’s solution (Merck, Darmstadt, Germany) and counted with a hemocytometer. Freshly isolated long bone and jaw bone marrow cells were seeded in 96-well plates (10^5 cells per well) and osteoclastogenesis was induced with 30 ng/mL recombinant mouse macrophage-colony stimulating factor (rmM-CSF; R&D Systems, Minneapolis, MN, USA) and 20 ng/mL recombinant mouse receptor activator of nuclear factor κB ligand (rmRANKL; R&D Systems). After 6 days, cells were fixed and a TRACP staining was performed with the leukocyte acid phosphatase kit (Sigma). Nuclei were visualized with 4′6-diamidino-2-phenylindole dihydrochloride (DAPI). The number of tartrate-resistant acid phosphatase (TRACP)-positive, multinucleated (≥3 nuclei) cells was assessed using an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Micro-computed tomography

Micro-computed tomography (microCT; Scanco Medical AG, Brüttisellen, Switzerland) was used to assess bone volume and the tissue mineral density (TMD) of humeri and hemi mandibles. Scanning was performed in fixation fluid with an 8 µm voxel size, a peak voltage of 55 kV, and an integration time of 250 ms. Imaging processing included Gaussian filtering and segmentation: sigma = 0.8, support = 1, threshold unit 170 (= 618 mg HA cm^-3) and 200 (= 727 mg HA cm^-3) for humeri and mandibles, respectively, for all analyzed time points and groups. Total humeri were analyzed by exactly outlining the bones, i.e. without
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including empty volume. The volume of interest for the jaw is indicated in Figure 2A. For both long bone and jaw, 20-30 slices per sample were analyzed.

Histology and histomorphometry

Following fixation, tissue samples were dehydrated with ethanol and subsequently embedded in methyl methacrylate (MMA). Transverse sections of the left maxillary bone and longitudinal sections of the proximal humeri were made using a Jung K microtome (Leica). Five µm sections were attached to gelatin-coated microscope slides and dried for at least 3 days at 37°C. Sections were mounted in depex and stored at room temperature in the dark for the analysis of dynamic bone formation in the diaphysis of the proximal humeri and the alveolar bone surrounding two or three roots of the second molar. Analyses were performed using Leica QWin (Leica Microsystems Image Solutions, Rijswijk, The Netherlands) and Image Pro-Plus Software (Media Cybernetics, Silver Spring, MD, USA).

Right maxillae and distal humeri were decalcified in 4.2% EDTA containing 0.8% formaldehyde for 5 weeks at room temperature, dehydrated, and embedded in paraffin. Transverse sections of the maxilla and longitudinal sections of the distal humeri were cut (7 µm thickness) and attached to silane coated microscope slides. Paraffin was removed with xylene substitute, sections were rehydrated and a TRACP staining was performed as described previously with minor changes [13]. A sodium-potassium tartrate concentration of 2 mM was used and sections were incubated for 3 h at 37°C. Counterstaining was performed with Mayer’s hematoxylin. TRACP-positive cells covering the bone in the endocortical, distal diaphysis and in the alveolar bone surrounding the roots of the second molar were analyzed and this was expressed as the percentage of bone covered by osteoclasts. Also, the osteoclasts attached to the cementum of the roots of the second molar were determined. Here, a distinction was made between osteoclasts that were attached to root-resorbed areas and those that attached to cementum that showed no signs of resorption. The periodontal ligament (PDL) area was also determined.

Statistical analyses

The Kruskal-Wallis test, followed by Dunn’s multiple comparison test were performed to test the effect of age on the number of bone marrow cells, osteoclast formation in vitro and in vivo, osteoclast size, and bone formation. To test the effect of age on microCT parameters we used a One-way ANOVA followed by Tukey’s multiple comparison tests. Unpaired, two-tailed t-tests were used to test the effect of ZA treatment on TMD and bone volume fraction. To test the effect of ZA treatment on the number of bone marrow cells, on the osteoclastogenic potential of bone marrow cells, bone formation parameters, and on the number of osteoclasts in vivo we used the Mann-Whitney U test. Graphs represent means
and standard deviations of six measurements, unless indicated otherwise. Differences were considered significant when $p<0.05$. 
Results

**Bisphosphonates affect jaw bone marrow cells without affecting long-bone marrow cells**

Bone marrow cells were isolated from mice treated with ZA to assess the osteoclastogenic potential of those cells. With age, fewer bone marrow cells were isolated from the long bone (Figure 1A). There was no effect of ZA treatment on the number of bone marrow cells isolated from long bones (Figure 1A). Interestingly, ZA gradually reduced the number of jaw bone marrow cells and after 6 months treatment, ZA significantly reduced the number of bone marrow cells that was isolated from the jaw (Figure 1B). These results indicate that long-term ZA treatment reduces the number of bone marrow cells present in the jaw. These results also show that BPs differently affect bone marrow cells in the jaw and in the long bones.

**Bisphosphonates do not affect the osteoclastogenic potential of bone marrow cells**

We investigated the osteoclastogenic potential of bone marrow cells from the long bones and jaws of mice treated with ZA and their controls at several time points. After 6 months, at the age of 9 months, significantly fewer long bone osteoclasts were formed from control bone marrow than after 1 and 3 months (Figure 1C). The age of the mice did not affect the number of osteoclasts that were formed from control bone marrow from the jaw (Figure 1D). Since 6 months of ZA treatment reduced the number of jaw bone marrow cells (see above), we were not able to isolate enough cells from the jaw at that time point to assess their osteoclastogenic potential. Therefore, the effect of ZA treatment on jaw osteoclastogenesis was only studied after 1 and 3 months of treatment (Figure 1D). There was no significant effect of ZA treatment on the number of osteoclasts that was formed from both long bone and jaw bone marrow precursors at any time point (Figure 1C,D). This indicates that BP treatment *in vivo* does not affect the *in vitro* osteoclastogenic potential of bone marrow cells at the included time points.
Figure 1. Bone marrow cell isolation and osteoclastogenic potential of bone marrow cells from long bones and jaws from mice treated with ZA. (A) The number of long bone marrow cells from tibia and femur was not affected by ZA treatment. (B) Six months of ZA treatment inhibited the number of jaw bone marrow cells in the hemi-mandible. (C,D) The osteoclastogenic potential of (C) long bone and (D) jaw bone marrow was not affected by ZA treatment. The x-axes show the time after the start of treatment at the age of 3 months. Means of 6 (A,B) or 4-6 (C,D) measurements and standard deviations are shown. **p=0.01 (Mann-Whitney U test) represents a significant difference between ZA treatment and control, #p<0.05, ##p<0.01 represent a significant difference of the controls at that time point versus baseline (0), $p<0.05$ represents a significant difference of the control group at 6 months versus 3 months.

Bisphosphonates increase bone volume and bone mineral density

ZA may reduce the number of bone marrow cells either directly by inducing bone marrow cell apoptosis, or indirectly by decreasing bone marrow space due to a higher bone volume or by a combination of both. To investigate whether a decrease in the number of jaw bone marrow cells (Figure 1B) was the result of such an indirect effect, we assessed bone volume by microCT (Figure 2A). With age, bone volume increased up to 6 months in the long bones and up to 9 months in the jaw (Figure 2B). ZA increased bone volume fraction (BV/TV) of
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the long bones and of the jaw after 1, 3, and 6 months of treatment (Figure 2B). This effect was similar in long bones (Figure 2B, left panel) and in the jaw (Figure 2B, right panel). However, the jaw bone volume fraction was higher than long bone BV/TV and increased to above 90% after ZA treatment. This might explain the decreased number of jaw bone marrow cells as indicated in Figure 1B. An increased bone volume by ZA decreased the periodontal ligament space of the lingual root after 6 months of treatment, whereas the PDL space surrounding the other roots were not affected (Supplementary Figure 1).

The TMD of both types of bone increased with the age of the mice and was higher in the jaw than in long bones (Figure 2C). After 3 and 6 months of ZA treatment, both long bone (Figure 2C, left panels) and jaw (right panels) TMD were significantly higher than the TMD of the control animals. This indicates that BPs similarly increase the mineral content in long bones and jaws.

**Figure 2.** MicroCT analysis of long bones and jaws from mice treated with BPs. (A) 3D reconstructions and the volume of interest (VOI) of the humeri (top) and the 1st and 2nd molar region of the mandible (bottom, arrows) after 6 months of treatment. M1=first molar, bar=1 mm. (B) Bone volume (% of total volume) of long bones (left) and jaws (right) was higher after 1, 3, and 6 months of ZA treatment. (C) Tissue mineral density (TMD) was increased by 3 and 6 months of ZA treatment. The x-axes show the time after the start of treatment at the age of 3 months. Graphs show the means and standard deviations of 5-6 measurements. *p<0.05, **p<0.01, ***p<0.001 represent significant differences between ZA treatment and control, determined by an unpaired, two-tailed t-test.
**Osteoclast numbers in the long bones increase with age**

The bone surface that was covered by osteoclasts and the number of osteoclasts *in vivo* was assessed (see Supplementary Figure 2 for an example). In the long bones the osteoclast surface and osteoclast numbers increased with the age of mice (#p<0.05, 6 months versus 0, and 6 months versus 1 month). In the jaw, the osteoclast numbers did not change with age. Interestingly, after 3 and 6 months of study, when the mice were 6 and 9 months old, respectively, osteoclasts covered a 2 to 3.6 times larger long bone surface than jaw bone surface (p<0.05, Figure 3A versus B). ZA treatment did not significantly affect the number of osteoclasts in the long bones (Figure 3A), nor in the jaw (Figure 3B). The effect on the number of osteoclasts per bone perimeter was similar (Figure 3C,D). Yet, the long bone and jaw osteoclasts were slightly larger after BP treatment (Figure 3E,F). This was only significant in the jaw after 3 months of treatment. Since the bone volume fraction and TMD were higher after ZA treatment, these data may indicate that the osteoclasts are less active after treatment.

**ZA differently affects mineralization in long bone and jaw**

The higher BV/TV and TMD after ZA treatment could not be attributed to a lower number of osteoclasts. Therefore, we investigated the effect on the bone formation markers mineralizing surface and mineral apposition rate. The mineralizing surface in the control mice decreased with age, only in long bones (Figure 4A). Interestingly, the long bone mineralizing surface was 1.4 to 2.4 times higher than the mineralizing surface in the jaw (Figure 4A versus 4B). The mineral apposition rate was even 2.2 to 3 times higher in the long bones than in the jaw (Figure 4C versus 4D).

ZA treatment significantly reduced the bone formation parameters in the long bones after 3 and 6 months of treatment, whereas the jaw was only affected after 3 months (Figure 4). This might indicate that 6 months of ZA treatment reduces bone turnover in the long bones, whereas it is unaffected in the jaw. These results also indicate that the higher bone volume fraction and TMD were probably induced at an earlier stage of treatment, or that they were the result of reduced osteoclast activity.
Figure 3. Osteoclast counts on decalcified sections. (A) Long bone and (B) jaw osteoclasts were not significantly affected by ZA treatment. (C-D) The number of osteoclasts per bone perimeter in (C) long bone and (D) jaw, showing similar results as the osteoclast surface covering the bone. (E-F) Osteoclasts are generally larger after ZA treatment, however this was only significant in the jaw (F) after 3 months of treatment. The x-axes show the time after the start of treatment at the age of 3 months. Graphs show the means and standard deviations of 4-6 measurements or 3 measurements for the jaw at baseline. # indicates p<0.05, between 6 months and baseline, and between 6 months and 1 month, as determined by The Kruskal-Wallis test, followed by Dunn’s multiple comparison. **p<0.01 using the Mann-Whitney U test comparing ZA and control at that time point.
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Figure 4. Mineralization parameters. (A) Long bone mineralizing surface and (C) mineral apposition rate were inhibited by 3 and 6 months of ZA. (B) Jaw mineralizing surface and (D) mineral apposition rate were only affected after 3 months of ZA. Graphs show the means and standard deviations of n=4-6 for the long bone and n=3-6 for the jaw. The x-axes show the time after the start of treatment at the age of 3 months. *p<0.05, **p<0.01 using the Mann-Whitney U test between ZA and control at that time point. #p<0.05 as determined by The Kruskal-Wallis test, followed by Dunn’s multiple comparison.

ZA induces molar root resorption

Analysis of the root surfaces of the molars revealed the presence of osteoclasts attached to the cementum layer (Figure 5A). Osteoclasts were commonly found in the animals treated with ZA and very rare in the controls (Figure 5C). These molar-root-associated osteoclasts proved to be associated with resorption pits (Figure 5B). After 3 months, 9.1% of the osteoclasts were active, and after 6 months 15.4% of the osteoclasts was associated with a resorption pit (Figure 5B,D), thus indicating resorptive activity despite the presence of the bisphosphonate. These results show, for the first time to our knowledge, that BPs can induce osteoclast activity at a certain location.
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Figure 5. Osteoclast attachment to root cementum. (A) Active (arrow) and non-active (dashed arrow) osteoclast attached to the root cementum. d: dentin, ab: alveolar bone, pdl: periodontal ligament, * cementum. (B) Higher magnification of the resorbing osteoclast, clearly showing disrupted cementum. Bars: 30 µm. (C) Resorption is exclusive to ZA treatment; in controls, cementum associated osteoclasts are very rare. (D) The percentage of osteoclasts after 3 and 6 months that was associated with a resorption pit. The x-axes show the time after the start of treatment at the age of 3 months. The graphs show the means and standard deviations of 5-6 measurements or 3 measurements for the jaw at baseline. ##p<0.01 represents a significant differences between ZA treatment and control using the Mann-Whitney U test.
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Discussion

In this study we showed that osteoclast numbers and bone turnover rate are bone-site specific. We showed that the osteoclast numbers increased with age in the long bones, whereas their number remained the same in the jaw (Figure 3). Also, the diaphyses of the long bones contained more osteoclasts per bone perimeter than the alveolar bone after 3 and 6 months of study, when the mice were 6 and 9 months old, respectively. This was accompanied by higher mineral apposition rates in the long bones than in the alveolar bone, as measured by dynamic bone parameters (Figure 4). These results strongly suggest that the bone turnover rate in old mice is higher in the long bones than in the jaw. This is in contrast with the paradigm, stating that bone turnover in the jaw is higher than in long bones [14]. However, only few studies directly compared turnover at both sites. Also, variation in turnover rate exists between locations within both bone sites [15]. Huja and co-workers showed that the bone formation rate in young, growing dogs is higher in the femur than in the jaw [16], whereas the opposite was found in mature dogs [17]. In line with our studies, Kubek et al. found similar mineral apposition rates in 6-months-old C3H female mice, which were also higher in the femur than at the periodontal ligament surface [15]. It has to be noted that they studied intracortical turnover in the femur, whereas we investigated endocortical turnover in the humerus. Together these results show that bone turnover is not necessarily higher in the jaw than in the long bones; on the contrary, in mice it seems to be higher in the long bones.

Besides the difference in long bone and jaw osteoclast numbers found in the current study, we previously showed that osteoclast precursors from these sites can be differently affected by bisphosphonates. Jaw osteoclast precursors internalized more BPs and accumulated more unprenylated Rap1a than long bone precursors, however, this was not accompanied by differences in osteoclastogenesis [12]. In the present study, ZA did not significantly affect the number of osteoclasts in the long bones and in the jaw, thereby confirming that ZA has a similar effect on long bone and jaw osteoclasts. Yet, more and larger osteoclasts seemed present in the jaw after 6 months of treatment, whereas the number was inhibited in the long bones (Figure 3). This is in line with a previous study by Kuroshima et al., who showed fewer long bone osteoclasts and slightly more jaw osteoclasts after subcutaneous injections of 0.1 mg/kg ZA twice a week for 13 months [18]. Therefore, it requires further investigation whether jaw osteoclasts are less susceptible to BPs than long bone osteoclasts after a longer treatment.

Although we previously showed that jaw bone marrow cells might be less sensitive to BPs than long-bone marrow cells in vitro [12], the current study showed that the bone marrow cells in the jaw were more susceptible to BP treatment than those in the long bones. Six months of ZA treatment reduced the number of bone marrow cells in the jaw and
not in long bones (Figure 1B). This might be attributed to the very limited bone marrow cavity in the jaw (Figure 2) or by higher BP uptake by the bone marrow cells at that site [12]. Perhaps, jaw bone marrow cells are exposed to higher BP concentrations in vivo. Jaw and long bones from rats were shown to contain a similar amount of BPs per unit of dry weight or calcium [19,20]. Since the jaw contains more mineral per bone volume [20] (Figure 2), it likely adsorbed more BPs than long bones. Together with the finding that jaw osteoclasts resorb at least as much bone or dentin as long bone osteoclasts in vitro [12,21], more BP is likely released from the jaw than from long bone, making it available for the surrounding cells in the bone marrow [1,2]. The resulting high local BP concentration might cause apoptosis of bone marrow cells, leading to a reduced number of jaw bone marrow cells. Also, Hokugo et al. recently showed that newly administered BPs can replace previously adsorbed BP [22]. Since we applied ZA weekly, it is likely that the BP replaces the previously administered ZA, releasing it into the bone microenvironment and making it available for internalization by other cell types than osteoclasts.

The reduced number of jaw bone marrow cells after 6 months of ZA treatment did not result in fewer osteoclasts at the alveolar bone (Figure 1,3). This may indicate that the osteoclasts are not formed from precursors in the bone marrow or that the osteoclast precursors still present in the bone marrow were not affected by BPs. This was supported by the finding that ZA treatment did not affect the osteoclastogenic potential of bone marrow cells, although it should be noted that the osteoclastogenic potential after 6 months could not be measured (Figure 1D). Yet, since ZA reduced the number of jaw bone marrow cells, our findings seem to provide support for the hypothesis that BPs may affect other cell types in the bone marrow, such as macrophages [23]. Also, the jaw is more exposed to microbes and pathogens and a negative effect of BPs on macrophages and the inflammatory response may explain why specifically the jaw is vulnerable for osteonecrosis. Inhibition of inflammation was also shown to be involved in the induction of ONJ [8,9]. Therefore, it would be of interest to study the BP release from the bone and uptake by bone marrow cells in the jaw in a mouse model of inflammation and bisphosphonates, such as the previously designed models for ONJ [10,11]. This could also provide more insight into whether the different response of long bone and jaw osteoclasts to BPs we showed here are related to the development of ONJ. This requires further investigation, since with our study design, the mice did not develop any signs of osteonecrosis of the jaw.

Another hypothesis regarding the etiology of ONJ is the oversuppression of bone turnover. A reduced bone remodeling capacity and reduced ability to repair bone damage can lead to the accumulation of microdamage [24,25]. Our results however, do not support a reducing effect on bone turnover markers in the jaw, whereas bone turnover was inhibited by ZA in the long bones on the long term (Figure 4). Also, the bone surface covered with
osteoclasts and osteoclast numbers increased with age in mice only in long bone, to levels that were 2 to almost 4 times higher in the long bones than in the jaw (Figure 3A-D). Therefore, our results suggest that oversuppression of bone turnover and the alleged microdamage would more likely cause side effects in the long bones than in the jaw. Indeed, others have shown that BPs were associated with atypical femur fractures, which are probably caused by the inability to heal microcracks [26].

Interestingly, under the influence of BPs, active osteoclasts accumulated at the molar root cementum (Figure 5). Previous studies on the effect of bisphosphonates during orthodontic treatment reported an inhibiting effect of BPs on root resorption during orthodontic tooth movement [27,28]. However, under our regime with ZA, but without an external force, approximately 10-15% of the root osteoclasts proved to be active. Therefore, we demonstrate that under the influence of BPs, more osteoclasts are associated with tooth cementum and moreover, that these cells actively resorb this mineralized tissue. A stimulating effect on osteoclast activity has, as far as we know, never been reported. This was unexpected and surprising, since BPs are used as a treatment to inhibit osteoclast activity. Possibly, those molar-root-associated osteoclasts were resistant to BPs as little BP accumulates at the root cementum [29].

Another explanation for the induction of osteoclast activity may be similar to the mechanism of osteoclast activity that is induced during orthodontic tooth movement. Mechanical loading induces bone resorption in the direction of the load. We hypothesize that through a similar mechanism, osteoclasts accumulate at the root after BP treatment. The increased bone volume after BP treatment (Figure 2) may induce mechanical loading that is transmitted through the periodontal ligament, and induces root resorption in order to make space for the newly formed bone. The periodontal ligament fibroblasts themselves could play a critical role in this process as they can stimulate osteoclast formation [30,31]. However, further studies are required to investigate the role of the periodontal ligament fibroblasts in osteoclast formation after BP treatment. Also, it would be highly interesting to investigate whether root resorption and a possible loss of teeth could be related to the positive correlation between a tooth extraction and the occurrence of BP-related ONJ.

In conclusion, our results support previous findings that osteoclasts are bone-site specific. Also, we show that BPs can differently affect long bone and jaw osteoclasts and bone turnover in vivo. Furthermore, we found resorption of tooth root cementum, in spite of the presence of the bisphosphonate ZA, indicating that the drug is able to stimulate osteoclast-mediated resorption. This stimulated resorption was site-specific, again indicating the diverse effects the drug has on osteoclasts at different bone sites. Finally, the evidence that BPs affect specifically jaw bone marrow cells may improve our understanding of the pathogenesis for BP-related ONJ.
Acknowledgments

The authors would like to thank Dr. Geerling E.J. Langenbach for his input in the design of the study and Dr. Clara M. Korstjens for the development of analysis protocols for the measurement of dynamic bone parameters. We are grateful to Carla Prins and Rika van der Laan for conducting the animal experiments. We thank Dr. Behrouz Zandieh Doulabi for development of the TRACP staining protocol on decalcified sections. Finally, we thank The Dutch Organization for Scientific Research for funding (NWO grant number: 021.001.050 to GAPR).
Supplementary data

**Supplementary Figure 1.** Periodontal ligament area. ZA reduced the periodontal ligament area surrounding the root on the lingual side, only after 6 months of treatment as tested with a Mann-Whitney U test (**p<0.01). The x-axes indicate the locations of the molar roots.

**Supplementary Figure 2.** TRACP staining on decalcified sections. (A) transverse section of the maxilla, showing roots of the second molar. ab: alveolar bone, pdl: periodontal ligament, d: dentin. (B) Longitudinal section of the distal humerus. Squares (A-B) indicate the area that is magnified in C (maxilla) and D (humerus). Arrows indicate osteoclasts. Bars: 100 µm (A-B), 30 µm (C-D)
References


