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Equilibrium-dependent bisphosphonate interaction with crystalline bone mineral explains the pharmacokinetics and osteonecrosis of the jaw in rats

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Abstract

Bisphosphonates (BPs) are chemically stable analogs of pyrophosphate exhibiting strong affinity to bone and have been used for the treatment of diseases characterized by excessive bone resorption. Contrary to the widely accepted BP accumulation model in bone after repeated applications, we report here that an equilibrium-dependent BP-crystalline bone mineral interaction may better explain BP bio-distribution and anti-catabolic bone remodeling and may be relevant to the appearance of osteonecrosis of the jaw (ONJ) in rats. Fluorescent-labeled BP analogs were synthesized and used to evaluate the mode of bone adsorption. After fluorescent-labeled BP adsorbed on crystalline calcium phosphates *in vitro*, subsequent BP application replaced the previously absorbed BP depending on the dose and the relative binding affinity to hydroxyapatite. The *in vivo* intravenous zoledronate (ZOL) injection of repeated fractional doses resulted in lower serum CTX and TRAP5b measurements than a single bolus injection in spite of the equivalent cumulative dose. Repeated injections resulted in the distribution of fluorescent-labeled BP on the large area of bone surfaces; whereas the single bolus injection gave rise to the intense BP bio-distribution at selected bone sites such as the alveolar process of jawbones. Necrotic maxillary alveolar bone was predominantly observed in vitamin D deficiency rats treated with bolus ZOL injection. The palatal necrotic bone was characteristically sequestered by the fistulation of hyperplastic oral epithelium, suggesting the initial development of ONJ-like lesions in rats. Our results suggest that equilibrium-dependent BP-bone interaction may, in part, determine the effectiveness and influence side effects of long-term and repeated applications of BPs.

Keywords

Bisphosphonate; Adsorption; Fluorescence; Equilibrium; Osteonecrosis of the Jaw

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Author contributions A.H. performed *in vitro* experiments and animal experiments. S.S. synthesized and characterized fluorescent-labeled bisphosphonates. S.S. also performed the fluorescence measurement of liquid samples. S.P. and I.N. performed animal experiments. C.E.M. and I.N. jointly conceived the study, analyzed the data and wrote the paper.

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1. Introduction

Methylenebisphosphonic acids ((HO)₂P(O)CR¹R²P(O)(OH)₂), commonly referred to as “bisphosphonates” (BPs) are analogs of pyrophosphoric acids in which a carbon atom replaces the bridging oxygen atom between the two phosphate groups. The current generation of BP drugs have in common a hydroxy-R¹ side chain and a nitrogen-containing-R² substituent, and exhibit strong affinity to inorganic mineral in bone [1, 2]. As highly stable chemical compounds, BPs are rapidly adsorbed to bone crystalline mineral after entering the biological system and may remain in skeletal tissues without biological metabolism [3–6]. Once internalized in osteoclasts during bone resorption, nitrogen-containing BPs act as a de-coy substrate of the mevalonate metabolism pathway [7–9], leading to insufficient post-translational prenylation of cell membrane small GTPases [10]. As a result, the osteoclasts undergo premature detachment from bone surface and apoptosis. With this unique pharmacological effect targeting osteoclasts, BPs are among the most widely prescribed drugs for prevention and treatment of hypercalcemia caused by bone resorbing tumors such as multiple myeloma and bone metastases of breast or prostate cancers, as well as for the treatment of osteoporosis.

Since 2003, a phenomenon collectively referred to as osteonecrosis of the jaw (ONJ) has been reported as a rare but potentially severe adverse event in patients receiving BP [11–13]. ONJ is clinically characterized as unresolved exposure of partially necrotic jawbone to the oral cavity and is frequently associated with dentoalveolar procedures in cancer patients receiving high doses of intravenously administered BPs [12, 14–17]. Repeated BP administrations are believed to result in the accumulation of bioavailable doses in bone and thus have been postulated to reach the accumulated “toxic” BP level causing ONJ [18]. This hypothesis currently provides the underlining rationale for assessing the risk of developing ONJ particularly for osteoporosis patients with long-term BP use [19], significantly affecting the dental treatment decisions.

The objective of this study is to establish the bio-distribution of BPs with different administration protocols *in vitro* and *in vivo* using fluorescently labeled BP analogs. We report here that unlike currently accepted accumulation kinetics, BP and bone crystalline mineral appear to establish an equilibrium-based molecular interaction and the initially adsorbed BP can be removed and further replaced by the later applied BP in the dose and affinity dependent manner. This “competitive” equilibrium appeared to better explain BP bio-distribution and pharmacological outcomes, and influence the development of ONJ-like lesions in rats.

2. Materials and Methods

2.1. Synthesis and purification of fluorescent BP analogs: 5-FAM-ZOL, 5(6)-FAM-RIS, 6-ROX-RIS

Zoledronate (ZOL) was purchased from Molekula Limited, UK. Risedronate (RIS) was a kind gift from Procter & Gamble Pharmaceuticals. Fluorescent BP analogs: ZOL conjugated with 5-carboxyfluorescein (FAM-ZOL), RIS conjugated with 5(6)-carboxyfluorescein (FAM-RIS) and RIS conjugated with 6-Carboxyl-X-Rhodamine (ROX-RIS); were synthesized via a linker strategy (Fig. 1A) [20]. The conjugates were purified by TLC and HPLC (purity > 95 %), and were characterized by ¹H, ³¹P NMR, HRMS, UV-VIS and fluorescent spectroscopy as described elsewhere [20].

2.2. *In vivo* effect of FAM-ZOL on femur of vitamin D-deficient rats

All animal experiments were approved by the UCLA Animal Research Committee. Eight week-old Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were fed a diet lacking vitamin D (TD.89123: 0.47% calcium, 0.3% phosphorus; Harlan Teklad, Madison, WI) for two weeks. All rats had free access to water and were kept in regular housing with a 12-hr light/dark cycle in a UV-restricted environment; UVB emission (290–305 nm) overlapping the action spectrum. Rats (n=3 in each group) received intravenous injection of the equal molar dose of ZOL or FAM-ZOL. Rats in the control group (n=3) received an intravenous injection of vehicle solution (0.9% NaCl). Six weeks later, rats were euthanized in a carbon dioxide chamber and femur was harvested. The femur specimens were fixed in 10% buffered formalin. Micro-CT scanning (μ CT 40, Scanco Medical, Bassersdorf, Switzerland) was performed distally from the growth plate at an x-ray energy level of 55 kVp with a current of 72 μ A. Out of this measurement volume, a region located 0.5 mm below the lower end of the growth plate and extending 1.6 mm distally was consistently selected for evaluation. Nominal isotropic resolution was 16 μ m, and the analysis region was represented by 100 slices. Three-dimensional image of femur was constructed using a customized computational program by digitally extracting the bone image using the predetermined threshold [220]. The trabecular and cortical regions were separated by semi-automatically drawn contours [21], and the trabecular bone structure of the proximal femur was evaluated to assess bone volume ratio (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N) [22]. The data were analyzed by one-way analysis of variance (ANOVA) and statistical significance was accepted for $p < 0.05$.

2.3. Dose-dependent adsorption of FAM-ZOL to CaP discs *in vitro*

Submicron synthetic mineralized calcium phosphate (CaP) thin films coated discs (BioCoat Osteologic Discs, BD Bioscience, San Jose, CA) were used to investigate the adsorption pattern of FAM-ZOL. The CaP disc has been used as an alternative material for direct assessment of osteoclast and osteoblast activity *in vitro* [23]. Incrementally increased doses (1, 5, 10, 50, 100, 200 and 500 μ M) of FAM-ZOL were applied on the discs placed in 24-well plate (n=3 in each group) and incubated for 1 hr at room temperature. After vigorous and repeated washes with phosphate buffered saline (PBS), the standardized fluorescent biophotonics image of the CaP disc was obtained using an excitation wavelength of 460 nm and the 515 nm filter (LAS3000, FUJIFILM Corp, Tokyo, Japan). An image evaluation program (Image J, NIH, Bethesda, MD) was used to determine the fluorescent intensity of each CaP disc.

2.4. FAM-ZOL adsorption to CaP discs after repeated applications

A group of CaP discs (n=3) were incubated with FAM-ZOL (12.5 μ M) for 1 hr followed by vigorous washes with PBS. These CaP discs were repeatedly incubated with FAM-ZOL (12.5 μ M) for a total of 4 applications (the cumulative dose of 50 μ M). In a separate group (n=3), 50 μ M FAM-ZOL was applied on the CaP disc followed by vigorous washes with PBS. For a negative control (n=3), the CaP disc was treated with PBS followed by vigorous washes with PBS. The standardized fluorescent images of the CaP disc were assessed as described above. The data were analyzed by Fisher's protected least significance difference (PLSD) test, and statistical significance was accepted for $p < 0.05$.

2.5. Alternate application of BP analogs to CaP discs

A group of CaP discs (n=3) was first incubated with 50 μ M ZOL. After vigorous washes with PBS, 50 μ M FAM-ZOL was then applied followed by vigorous washes. In another group, the CaP discs were first treated with 50 μ M FAM-ZOL and then 50 μ M ZOL. CaP

discs treated only with PBS or 50 μ M FAM-ZOL were used as references. The standardized fluorescent images of the CaP disc were assessed. The data were analyzed by *Tukey-Kramer* test, and statistical significance was accepted for $p < 0.05$. In this study, the wash solutions were collected and fluorescent emission spectra of each wash solution were recorded on Jobin Yvon Nanolog spectrometer, using an excitation wavelength of 490 nm; the excitation and emission slits were set at 5 nm; scan ranges from 495 nm to 700 nm. Spectra were calibrated and the emission peak was found at 522 nm. Fluorescent intensity of each sample at 522 nm was collected and plotted.

In a separate experiment, synthetic CaP-coated wells (Bone Resorption Assay Plate 24, Cosmo Bio Co., LTD, Tokyo, Japan) wells were first incubated with 50 μ M ROX-RIS (n=18) or 50 μ M FAM-RIS (n=18) for 1 hr at 37°C followed by vigorous and repeated washes in PBS. Then 0, 0.1, 1, 5, 10 or 50 μ M FAM-RIS or ROX-RIS, respectively, was applied to CaP-coated wells (n=3 in each condition) and incubated for 1 hr at 37°C. After vigorous and repeated washes in PBS, the fluorescent intensity of CaP plate was examined using an excitation wavelength of 460 nm and the 510 nm filter for FAM fluorescence and an excitation wavelength of 520 nm and the 670 nm filter for ROX fluorescence (LAS3000, FUJIFILM Corp, Tokyo, Japan). An image evaluation program (Image J, NIH, Bethesda, MD) was used to determine the fluorescent intensity of both FAM and ROX in each CaP-coated well.

2.6. FAM-ZOL bio-distribution in rat femur and mandible

Rats fed with vitamin D deficient diet for 2 weeks received either a single bolus FAM-ZOL (180 μ g/Kg) intravenous injection or 4 weekly-injections of fractional dose of FAM-ZOL (45 μ g/kg per injection; a cumulative dose of 180 μ g/Kg). As controls, rats received an intravenous injection of vehicle solution (0.9% NaCl). Six weeks after the single FAM-ZOL injection or 2 weeks after the last weekly injection, rats were euthanized. The femur and mandible specimens were immediately harvested and fixed in 10% buffered formalin. In the standardized fluorescent biophotonics images, fluorescent intensity at proximal and distal areas of femur primary spongiosa; anterior and posterior areas of mandibular ramus; and alveolar bone at the 3rd mandibular molar were quantitatively evaluated. The data were analyzed by Fisher's PLSD test, and statistical significance was accepted for $p < 0.05$.

2.7. Effect of different ZOL administration protocols on femur bone morphometry and serum chemistry as well as on the development of osteonecrosis at the maxillary tooth extraction site

Rats fed with vitamin D deficient diet for 2 weeks received 2 monthly intravenous injections of 70 μ g/Kg ZOL (the cumulative dose of 140 μ g/Kg) or 8 weekly injections of 17.5 μ g/kg ZOL (the cumulative dose of 140 μ g/Kg). After the cumulative ZOL dose reached 70 μ g/Kg, maxillary left molar teeth were extracted using the previously established procedure [24] under general anesthesia by inhalation of 2% isoflurane. Subcutaneous injections of 0.05 mg/kg buprenorphine were given every 12 hours for 48 hours after tooth extraction, but no antibiotics were administered. Four weeks after tooth extraction, rats were euthanized and approximately 8 ml of periphery blood was extracted from each animal after 6 hours of fasting. Femur and maxillary bones were harvested and fixed in 10% buffered formalin. Serum alkaline phosphatase was measured by a conventional clinical chemistry system (VetACE™, Alfa Wassermann, West Caldwell, USA). C-terminal cross-linking telopeptide of type I collagen (CTX) and serum band 5 of tartrate-resistant acid phosphatase (TRACP-5b) were measured by enzyme-linked immunosorbent assays (RatLaps ELISA, Nordic Bioscience Diagnostics, Herlev, Denmark; RatTRAP Assay, IDS, Inc., Fountain Hills, AZ, USA, respectively). The data were analyzed by Dunnett test, and statistical significance was accepted for $p < 0.05$. The femur specimens were subjected to micro CT

evaluation as described previously. The micro CT bone morphometric data of femurs were analyzed by Fisher's PLSD test, and statistical significance was accepted for $p < 0.05$.

The maxillary bones harvested 4 weeks after tooth extraction were scanned by micro CT and then decalcified with 10% ethylenediaminetetraacetic acid (EDTA) followed by paraffin embedding. A series of 4-mm-thick sections in the frontal plane were prepared through the second to third molars of the non-extraction side as the reference. The palatal and buccal alveolar processes of the tooth extraction side were subjected for further investigations. Osteonecrosis was histologically defined by the presence of empty or pyknotic osteocyte lacunae. The bone sequestration was histologically determined by a piece of separated necrotic bone surrounded by the abnormal elongation of hyperplastic oral epithelium [25]. The effect of different ZOL administration protocols was compared by the percentage of animals positive for histologic necrotic bone sequestration lesion(s) in the group. Fisher's exact test was used for statistical evaluation and statistical significance was accepted for $p < 0.05$.

3. Results

3.1. FAM-ZOL retained the pharmacological effect of ZOL *in vivo*

In this study, ZOL was conjugated with 5-carboxyfluorescein (FAM-ZOL) through 'magic linker' chemistry as previously described [20]. The approach is based on the use of a functionalized epoxide to attach a universal linker group to the heterocyclic nitrogen of BP under mild reaction conditions (aqueous, near neutral pH, 21–40 °C). The drug-linker is facilely conjugated to succinimidyl ester of FAM, yielding FAM-ZOL (Fig. 1A). Previously, FAM-RIS has been shown to prevent prenylation of Rap1A of J774.2 macrophages *in vitro* [20, 26, 27]. In the present study, to evaluate the effect of FAM conjugation on the overall pharmacological efficacy of ZOL, we carried out a direct comparison of FAM-ZOL vs. ZOL for relative anti-resorptive activity *in vivo*. Nutritionally induced vitamin D deficiency increased osteoporosis-like catabolic bone remodeling in rats [25, 28, 29]. Compared to the control VitD(–) group, the FAM-ZOL group shows a significant ($p < 0.05$) increase in the trabecular bone volume and improvements of trabecular structural indices, while the anti-resorptive effect of FAM-ZOL *in vivo* was approximately 50% of that of ZOL (Fig. 1B). This study provided direct evidence that the dye-drug conjugate can maintain anti-resorptive drug activity *in vivo* albeit at reduced rates.

3.2. BP absorption kinetics to CaP disc *in vitro*

When increasing concentrations of FAM-ZOL in PBS were incubated, commercially available synthetic calcium phosphate (CaP)-coated quartz substrate discs adsorbed FAM-ZOL in a dose-dependent linear increase manner (Fig. 2A) and did not show any sign of adsorption saturation up to 500 μM of applied drug. Our data suggested that the binding characteristics of BP to hydroxyapatite crystals follow the classical Langmuir adsorption isotherm [30] and that CaP discs are an appropriate model for BP adsorption studies.

One-time application of 50 μM FAM-ZOL consistently gave rise to approximately 70 pixel-intensity fluorescence in CaP discs. Four repeated applications of 12.5 μM FAM-ZOL resulted in significantly less FAM fluorescence intensity in the CaP discs than the single bolus application of 50 μM FAM-ZOL (Fig. 2B). Cumulative doses of FAM-ZOL were equivalent in these groups; however, repeated applications of a fractional FAM-ZOL dose did not reach the adsorption level of a high dose-single time application.

Because the Langmuir isotherm is influenced by the adsorption affinity, we postulated that the first application of BP to the CaP disc might condition the adsorption affinity and thus interfere with the subsequent BP adsorption. To test this hypothesis, CaP discs were

alternately treated with ZOL, then FAM-ZOL or FAM-ZOL, then ZOL. In the group of CaP discs pretreated with 50 μM ZOL followed by a second application of 50 μM FAM-ZOL, the CaP disc fluorescent intensity reached nearly at the level of FAM-ZOL application alone (Fig. 2C). In the reverse addition order group, the FAM-derived fluorescence intensity of the CaP discs from pre-adsorbed FAM-ZOL decreased to an undetectable level after the second application of unlabeled ZOL (Fig. 2C).

The applied solutions were saved from the above experiment and the FAM fluorescence intensity of these solutions was determined. Non-adsorbed FAM-ZOL appeared to be effectively removed by sequential PBS washes to a negligible level (Fig. 2D). After unlabeled ZOL solution was applied, a small but distinct peak of FAM fluorescence intensity was detected in the ZOL solution (arrow in Fig. 2D), suggesting that the loss of FAM fluorescence intensity in the CaP discs was likely to be due to displacement of previously adsorbed FAM-ZOL from the discs.

While the so-called tridentate structure of BP is thought to play the primary role in the binding to bone mineral, it is well established that the chemical moiety of R^2 chain can also influence the binding affinity. The addition of chemical structures such as FAM and ROX to the R^2 chain is anticipated to affect the binding affinity. Roelofs et al. (2012) demonstrated that ROX-RIS has significantly higher affinity than FAM-RIS in mouse tibiae [31]. Taking advantage of the relative difference in affinities of ROX-RIS (higher affinity) and FAM-RIS (lower affinity), we further investigated whether the BP affinity influenced the adsorption to CaP discs. CaP-coated culture wells were pre-treated by 50 μM ROX-RIS or FAM-RIS, giving rise to uniform adsorption as detected by the RIS or FAM fluorescence intensity. When the pre-adsorbed 50 μM ROX-RIS was challenged by FAM-RIS, ROX-RIS was not completely replaced even at the same molar concentration (Fig. 3A). On the contrary, when the pre-adsorbed 50 μM FAM-RIS was challenged by ROX-RIS, FAM-RIS was completely removed (Fig. 3A). ROX-RIS showed initial increase in CaP adsorption and reached the plateau between 10 μM and 50 μM (Fig. 3B). It was noted that the adsorption concentration of ROX-RIS did not seem to increase beyond the previously established adsorption concentration between FAM-RIS and CaP.

3.3. Bio-distribution pattern of FAM-ZOL with different administration protocols *in vivo*

The *in vivo* bio-distribution of FAM-ZOL was examined in rats with nutritionally induced vitamin D deficiency. A single injection of 180 $\mu\text{g}/\text{Kg}$ FAM-ZOL resulted in localized intense fluorescence in the femur trabecular bone proximal to the poorly labeled primary spongiosa located at the distal end of epiphysis (Fig. 4A). The fluorescence intensity varied significantly in the femur of this group. Contrarily, 4-weekly injections of 45 $\mu\text{g}/\text{Kg}$ FAM-ZOL showed more diffused and uniform fluorescent labeling in femurs (Fig. 4A). In the mandibular ramus, repeated injections of 45 $\mu\text{g}/\text{Kg}$ FAM-ZOL resulted in uniform but less intense fluorescent labeling, whereas a single 180 $\mu\text{g}/\text{Kg}$ FAM-ZOL injection generated a line of intense fluorescent labeling (Fig. 4B). In the mandibular alveolar bone, the fluorescence intensity was found to be much stronger in the single injection group than in the repeated injection group, despite the fact that both groups received the equivalent cumulative FAM-ZOL dose (Fig. 4C).

3.4. Effect of different ZOL administration protocols on anti-catabolic bone remodeling

The biological effect of different administration protocols with the equivalent cumulative dose was examined in vitamin D deficient rats. All rats were treated by the extraction of left maxillary molars. One group received 2 monthly-injections (70 $\mu\text{g}/\text{Kg}$ ZOL per injection) and the other group received 8 weekly-injections (17.5 $\mu\text{g}/\text{Kg}$ ZOL per injection). At the

end of the experiment, whole blood, femur and maxillary bone samples were harvested (Fig. 5A).

The serum samples were examined for bone remodeling markers: alkaline phosphatase [32], carboxy-terminal collagen crosslinks (CTX) [32, 33], and secreted tartrate-resistant acid phosphatase 5b (TRACP-5b) [34]. The weekly injection protocol significantly decreased all serum bone remodeling markers compared to the monthly injection protocol (Table 1). On the contrary, the micro CT bone morphometry data of the femur trabecular bone clearly indicated the more robust increase in bone mass in the monthly injection group (Fig. 5B).

3.5. Effect of different ZOL administration protocols on the development of ONJ-like lesions in the rat maxilla

At the time of maxillary molar extraction and the end of the experimental period, the cumulative ZOL doses in both groups were equivalent (Fig 5A). Four weeks after maxillary molar extraction, the maxillary tissues were harvested. The micro CT scanning indicated that all of 6 rats received 2 injections of high dose ZOL (70 $\mu\text{g/Kg}$ per injection) developed the abnormal bone sequestration, which appeared to be separated from the palatal alveolar process at the maxillary molar extraction wound (Fig. 6A). The histological cross section of the maxilla revealed the bone sequestrum was indeed necrotic and associated with oral epithelial fistulation and inflammatory cell infiltration (Fig. 6B and 6C). There was 1 out of 8 rats (or 12.5%) received the 8 repeated weekly injections of low dose ZOL (17.5 $\mu\text{g/Kg}$ per injection) developed the bone sequestration. Fisher's exact test showed the significant difference on the presence of necrotic bone sequestration between 2 different ZOL administration groups ($p < 0.01$) (Fig. 6E). The buccal alveolar process of the tooth extraction side was covered by the buccinator muscle and separated from the oral epithelium (Fig. 6B). There were clusters of empty osteocyte lacunae in the buccal alveolar process (Fig. 6D). The percent of viable osteocytes were significantly lower in rats received the bolus injection of high dose ZOL (Fig. 6F).

4. Discussion

The results from this study collectively suggest that the establishment of a dynamic equilibrium between BP and the CaP substrate may better explain the BP adsorption pattern to bone tissue and its pharmacological effects than a simple dose accumulation model. Repeated applications of low-dose FAM-ZOL did not produce the adsorption level observed for a single high-dose FAM-ZOL application *in vitro* (Fig. 2) as well as *in vivo* (Fig. 4) albeit at equivalent cumulative doses. Repeated vigorous washes *in vitro* or creatinine clearance in rats [35] should not be the primary cause of the removal of the adsorbed BP. Instead, our *in vitro* data suggest the possibility that the initially adsorbed BP can be removed by freshly applied BP (Fig. 2C and 2D) and further replaced by the later applied BP in the dose and affinity dependent manner (Fig. 3).

Russell et al (2008) reported that near saturation occurred at bisphosphonate concentrations greater than 10 μM [36]; however, in the present study, FAM-ZOL did not demonstrate adsorption saturation within our study conditions (Fig. 2A). The addition of a bulky chemical moiety such as fluorescent molecules by conjugation to ZOL will influence the skeletal distribution [31, 37]. As previously reported [20], the FAM conjugate of risedronate (FAM-RIS) showed a moderate decrease (30 %) in retention time ($R_t = 7$ min) on a hydroxyapatite column compared to risedronate ($R_t = 10$ min). Duan estimated bone mineral binding of several clinically used bisphosphonates and related fluorescent conjugates using a HAP powder adsorption assay and subsequently reported a smaller decrease (10 %) [38]. The derived K_d (equilibrium dissociation constant) value for 5(6)-FAM-RIS (i.e., as a mixture, thus the K_d is apparent) was $K_d = 14.56 \pm 3.65 \mu\text{M}$, while risedronate had a $K_d =$

6.63±0.43 μ M. Despite differences in methodologies, the results are consistent with the FAM conjugates having comparable but slightly reduced HAP binding affinity, relative to the parent BP drug. Roelofs *et al.* (2012) tested the bone mineral affinity of a wider range of BP conjugates with several fluorescent dyes including FAM, ROX, and Alexa Fluor 647 (AF647) using a dentine disc adsorption assay [31]. The addition of FAM to various BP derivatives gave rise to lowered affinity, although the exact mechanism is unknown [31]. In the present study, the FAM conjugation apparently attenuated the anti-resorptive activity of ZOL (Fig. 1B). This may also explain the discrepancy of the FAM-ZOL binding saturation pattern with the published data of unmodified FAM. We further found that ROX-RIS with higher affinity was not completely replaced by FAM-RIS with lower affinity (Fig. 3). These observations are consistent with the establishment of a dynamic equilibrium during the initial adsorption of BP to bone mineral.

Cremers *et al.* (2005) estimated the uptake of BP to skeletal tissue from 24-hr urine excretion data in humans [39]. Repeated intravenous administration of pamidronate appeared to be accumulated in skeletal tissue, which mirrored progressive decrease in bone resorption assessed by urine N-terminal telopeptide of type I collagen and serum alkaline phosphatase. In the present study, rat femurs in the group that received repeated injections of low dose ZOL exhibited more reduction in catabolic bone remodeling assessed by serum CTX and TRAP5b assays (Table 1) than those treated with less frequent injections of high dose ZOL. Fluorescent imaging of the rat bones revealed that the repeated injections resulted in wide distribution of FAM-ZOL on bone surfaces, suggesting a net increase in the amount of BP adsorbed in the skeletal tissues (Fig. 4), which might have contributed to the significant reduction of serum bone remodeling markers as reported by Cremers *et al.* (2005) [39]. Also, in the clinical context, some of the adsorbed bisphosphonate may be buried by new bone formation, and virgin surfaces are continually being laid down on which subsequent bisphosphonate doses can be adsorbed. This would, of course, decrease the total fluorescence signal intensity. On the contrary, a high local BP concentration established by the bolus injection appears to contribute to the retention of bone mass in femurs as measured by micro CT (Fig. 5B).

A single bolus injection resulted in highly localized FAM-ZOL adsorption in the alveolar process of jawbone, where bone remodeling was thought to be active [40, 41] (Fig. 4). Therefore, we hypothesized that the probability of developing ONJ, if associated with a BP, might be influenced by different administration protocols. The extended exposure of necrotic bone in patients treated with BP but not with radiation therapy has been proposed as the clinical definition of ONJ [12]. ONJ biopsy specimens frequently demonstrated the presence of hyperplastic epithelium surrounding the bone sequestrum [25, 42]. In our rat model study, the bolus injections of high dose ZOL resulted in this abnormal extension of oral epithelium that sequestered the necrotic alveolar bone (Fig. 6B and 6C). The sign of osteonecrosis was also observed in the buccal alveolar process in this group (Fig. 6D). Furthermore, our studies may suggest that bolus injections of high dose ZOL can establish a BP-bone equilibrium during the initial exposure and the resulting high concentration of ZOL bio-distribution at the alveolar bone may contribute to the development of ONJ-like lesions (Fig. 6).

Palaska *et al.* (2009) summarized the published literatures on the clinical incidences of ONJ and reported that the mean duration of ZOL treatment was 1.8 years but the range varied significantly [43]. Van der Wyngaert *et al.* (2006) reported that the duration of bisphosphonate therapy at the time of diagnosis of ONJ ranged from 1 to 94 months with the average 2.5 years [44]. Hoff *et al.* (2008) reported that the duration of bisphosphonate treatment in ONJ patients was 1 to 50 months (median 1.67 years) for pamidronate; and 2 to

71 months (median 2.02 years) for ZOL [15]. These studies suggest that ONJ can occur without a clear time-dependency.

Saad et al (2011) reported a three-blinded active-controlled phase III trial of humanized anti-RANKL antibody (denosumab) compared to ZOL in cancer patients [45]. The development of ONJ-like lesions was reported in both denosumab- and ZOL-treated patients and the cumulative prevalence at the end of trial or 36 months was 1.8% and 1.3%, respectively. Among ZOL-treated patients, ONJ cases were steadily reported during 0–12 months, 12–24 months and 24–36 months of treatment. The time-independent pattern of ONJ occurrences appeared to be consistent with previous reports. The development of ONJ may associate with other drugs such as denosumab and its etiology is not clearly established. It must be noted that among denosumab-treated patients, all of the ONJ cases were reported during the first 24-month period and none was found in the 24–36-month period [45]. Although preliminary, the possible difference in the timing of ONJ development in denosumab- and ZOL-treated patients may provide a clue to elucidate the similarity and differences in the pathological mechanism contributed by these anti-resorptive agents.

Currently, there are no definitive causal relationship between low dose BP treatment and the development of ONJ in osteoporosis patients. Within the limited experimental period of our study, repeated injections of low dose BPs did not result in the accumulation of BP at the alveolar process of rat jawbone and the infrequent development of ONJ-like lesions.

In conclusion, this study suggests evidence for an equilibrium process of BP adsorption to bone tissue. The postulated equilibrium model may better explain BP bio-distribution, anti-catabolic bone remodeling and the prevalence of ONJ. Furthermore, the possibility to remove adsorbed BP molecules may suggest a novel opportunity for widening the therapeutic options.

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Highlights

- Repeated applications of fluorescent-labeled BPs to CaP substrate demonstrated the removal of pre-adsorbed BP in an equilibrium-dependent manner.
- Single bolus and repeated fractional doses of ZOL demonstrated different pharmacological effects in vivo despite the equivalent cumulative dose.
- The prevalence of ONJ-like lesion in a vitamin D deficient rat model was dependent on the initial ZOL dose.
- The equilibrium-dependent BP-bone interaction model suggests the possibility of removing pre-adsorbed BP from skeletal tissues by displacement.

Figure 1.

A: Chemical structures of fluorescent-labeled BP analogs: ZOL conjugated with 5-carboxyfluorescein (FAM-ZOL), RIS conjugated with 5(6)-carboxyfluorescein (FAM-RIS) and RIS conjugated with 6-Carboxyl-X-Rhodamine (ROX-RIS) via a linker strategy. **B:** The pharmacological function of FAM-ZOL was compared with ZOL in vitamin D deficient (VitD(-)) rats. ZOL, FAM-ZOL or vehicle solution (0.9% NaCl) was injected via tail vein into VitD(-) rats (n=3 per group). Six weeks after the injection, the trabecular bone morphology of femurs was evaluated by micro-CT. FAM-ZOL significantly blocked catabolic bone remodeling resulting in increased trabecular bone structure; however, the effectiveness of FAM-ZOL was reduced approximately 50% of ZOL *: p<0.05 by one-way ANOVA.

Figure 2.

A: FAM-ZOL showed a dose-dependent linear absorption to CaP discs and did not saturate below 500 μM in PBS. **B:** Although the 4 repeated applications of 12.5 μM FAM-ZOL should deliver the equivalent cumulative dose of the single application of 50 μM FAM-ZOL, the CaP discs showed less fluorescent intensity level in the former protocol than those of single high-dose application. *: $p < 0.05$ by Fisher's PLSD test. **C:** Unlabeled ZOL (50 μM) and FAM-ZOL (50 μM) were alternately applied to CaP discs. The fluorescence intensity of CaP discs was largely influenced by the lastly applied compound. *: $p < 0.05$ by Tukey-Kramer test. **D:** The PBS wash solutions collected from the group of FAM-ZOL followed by ZOL application in **Fig. 2C** revealed a distinct peak of FAM fluorescence (arrow) during the second ZOL application, suggesting the displacement of FAM-ZOL from CaP discs.

Figure 3.

CaP-coated wells were pre-treated with either ROX-RIS or FAM-RIS (50 μM). After challenged by serially diluted doses of FAM-RIS or ROX-RIS (from 0 to 50 μM), respectively, ROX and FAM fluorescence intensities of the CaP-coated wells were measured. **A:** ROX-RIS in CaP (red bars) remained near the pre-adsorbed 50 μM level until 5 μM or greater concentrations of FAM-RIS challenged. On the contrary, FAM-RIS (green bars) was rapidly displaced by as low as 1 μM ROX-RIS. **B:** The pre-adsorbed FAM-RIS was completely replaced by the challenged ROX-RIS between 10 μM to 50 μM range.

Figure 4.

In vivo absorption pattern of FAM-ZOL in rat bone tissue. Through the tail vein, a group of vitamin D-deficient rats ($n=3$) received 4 repeated-weekly injections of $45\mu\text{g/Kg}$ FAM-ZOL, another group ($n=3$) received a single-injection of $180\mu\text{g/Kg}$ FAM-ZOL and the control rats ($n=3$) received a single-injection of vehicle solution (0.9% NaCl). Rat femurs and mandibles were harvested 8 weeks after the initial injection and subjected to the fluorescent CCD detector imaging analysis. **A:** Fluorescence distribution in femurs showed more diffused and less intense adsorption of FAM-ZOL in the repeated injection group. In the single application group, an intense fluorescence signal was found localized at the proximal (P) area of femur primary spongiosa (arrowheads) contrasted to lesser labeling in the distal (D) primary sponsiosa of the more recent growth area. **B:** Mandibular ramus specimens similarly indicated bone growth-dependent discrepancy in FAM-ZOL bio-distributions at the anterior (A) and distal (D) areas (arrowheads). **C:** Mandibular molar alveolar bone (arrowheads; M3: the 3rd molar) completed the growth by 6 to 8 weeks of age and showed the intense FAM-ZOL labeling in the single-injection group. *: $p<0.05$ by Fisher's PLSD test.

Figure 5.

Different biological consequences to the modulated administration of ZOL. **A:** Experimental protocols involving vitamin D deficient treatment and ZOL injections in rats. The equal cumulative ZOL dose of 140 $\mu\text{g/Kg}$ was given in 2 rat groups; however, one group received 8 weekly-injections of 17.5 $\mu\text{g/Kg}$ ZOL (n=8), whereas the other group received 2 monthly-injections of 70 $\mu\text{g/Kg}$ ZOL (n=6). Control rats (n=4) received injections of vehicle solution (0.9% NaCl). **B:** Micro-CT bone morphometry of femur distal primary spongiosa indicated significantly greater anti-catabolic effect by 70 $\mu\text{g/Kg}$ monthly-injections than by 17.5 $\mu\text{g/Kg}$ weekly-injections. *: $p < 0.05$ by Fisher's PLSD test.

Figure 6.

A. C: Micro-CT evaluations of maxillary alveolar bone after molar extraction revealed the partially necrotic bone sequestra (arrow) in rats received 70.0 µg/Kg monthly injections. (Bar = 500 µm) **B.** Histological cross section of maxilla of rats received 70.0 µg/Kg monthly injections showed the necrotic bone sequestrum (arrow) that appeared to be derived from palatal alveolar bone (P). The bony socket housed molar (M) was filled with new bone (Nu) between palatal (P) and buccal (B) alveolar bone. (H & E staining; Bar = 500 µm) **C.** Necrotic bone sequestrum was associated with abnormal hyperplasia of oral epithelium (arrows). (H & E staining; Bar = 100 µm) **D.** Buccal alveolar process of rat maxilla received weekly injections of 17.5 µg/Kg ZOL or monthly injections of 70.0 µg/Kg ZOL showed the different distributions of empty osteocyte lacunae (arrowhead). (H & E staining; Bar = 100 µm) **E.** The rate of rats developed necrotic bone sequestrum. ** $p < 0.01$ by Fisher's Exact test. **F.** The rate of viable osteocytes in the buccal alveolar process. ** $p < 0.01$ by Student's t test.

Table 1

Serum bone remodeling markers of vitamin D deficient rats with different ZOL injection protocols.

ZOL Treatment	Parameters		
	ALP (U/L)	CTX (ng/mL)	TRACP-5b (U/L)
8 × 17.5μg/Kg	89.50±10.75	16.61±4.82	2.95±0.69
		1 *	1 *
2 × 70.0μg/Kg	145.17±70.17	39.44±14.35	4.63±1.22
Reference (VD- rats)	270.20±46.82	63.85±13.26	14.37±3.81

*
p<0.05 Student t-test