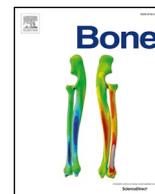




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Rescue bisphosphonate treatment of alveolar bone improves extraction socket healing and reduces osteonecrosis in zoledronate-treated mice



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ABSTRACT

Bisphosphonate (BP)-related osteonecrosis of the jaw, previously known as BRONJ, now referred to more broadly as medication-related osteonecrosis of the jaw (MRONJ), is a morbid condition that represents a significant risk for oncology patients who have received high dose intravenous (IV) infusion of a potent nitrogen containing BP (N-BP) drug. At present, no clinical procedure is available to prevent or effectively treat MRONJ. Although the pathophysiological basis is not yet fully understood, legacy adsorbed N-BP in jawbone has been proposed to be associated with BRONJ by one or more mechanisms. We hypothesized that removal of the pre-adsorbed N-BP drug common to these pathological mechanisms from alveolar bone could be an effective preventative/therapeutic strategy. This study demonstrates that fluorescently labeled BP pre-adsorbed on the surface of murine maxillo-cranial bone *in vivo* can be displaced by subsequent application of other BPs. We previously described rodent BRONJ models involving the combination of N-BP treatment such as zoledronate (ZOL) and dental initiating factors such as tooth extraction. We further refined our mouse model by using gel food during the first 7 days of the tooth extraction wound healing period, which decreased confounding food pellet impaction problems in the open bony socket. This refined mouse model does not manifest BRONJ-like severe jawbone exposure, but development of osteonecrosis around the extraction socket and chronic gingival inflammation are clearly exhibited. In this study, we examined the effect of benign BP displacement of legacy N-BP on tooth extraction wound healing in the *in vivo* model. Systemic IV administration of a low potency BP (lpBP: defined as inactive at 100 μM in a standard protein anti-prenylation assay) did not significantly attenuate jawbone osteonecrosis. We then developed an intra-oral formulation of lpBP, which when injected into the gingiva adjacent to the tooth prior to extraction, dramatically reduced the osteocyte necrosis area. Furthermore, the tooth extraction wound healing pattern was normalized, as evidenced by timely closure of oral soft tissue without epithelial hyperplasia, significantly reduced gingival inflammation and increased new bone filling in the extraction socket. Our results are consistent with the hypothesis that local application of a rescue BP prior to dental surgery can decrease the amount of a legacy N-BP drug in proximate jawbone surfaces below the threshold that promotes osteocyte necrosis. This observation should provide a conceptual basis for a novel

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strategy to improve socket healing in patients treated with BPs while preserving therapeutic benefit from anti-resorptive N-BP drug in vertebral and appendicular bones.

1. Introduction

An oral complication involving unhealed jawbone lesions has been reported in cancer patients and, more rarely, in osteoporosis patients treated with potent antiresorptive nitrogen-containing bisphosphonates (N-BPs) such as zoledronate (ZOL), risedronate (RIS) and alendronate (ALN), termed BP-related osteonecrosis of the jaw (BRONJ) [1]. The updated position paper released by the American Association of Oral and Maxillofacial Surgeons (AAOMS) redefines BRONJ as medication-related ONJ (MRONJ) [2], expanding the scope of a previous position paper, reflecting the inclusion of antiresorptive medications other than BPs, such as receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitors (e.g. denosumab) and antiangiogenic therapies. MRONJ symptoms primarily consist of painful bone exposure, which does not resolve over several months to years [3]. Clinical reports have indicated that dental procedures such as routine tooth extraction markedly increase the risk of developing MRONJ [4], thus causing uncertainty and apprehension among dental healthcare professionals and patients in recent years. This has led to dramatically decreased acceptance of BP and other antiresorptive agents in patient populations, despite the demonstrated clinical benefits of these drugs for the serious primary diseases [5].

Among MRONJ diseases, BRONJ has been extensively reported to date. Using the AAOMS definition, the incidence of BRONJ among the cancer patient cohorts in the US was 5.3/1000 person-years, which was significantly higher than BP unexposed sex- and age-standardized cancer groups of 0.29/1000 person-years [6]. By contrast, the BRONJ incidence rate in the osteoporosis cohort was almost negligible as 0.15/1000 person-years in the US [6], 0.69–0.82/1000 person-years in Taiwan [7] and 1.05–1.31/1000 person-years in Japan [8]. It has been reported that a non-exposed variant of BRONJ, which was not included in these epidemiological studies, presents clinical symptoms including jaw pain, sinus tract, radiological abnormalities and gingival swelling [9,10]. The non-exposed variant was recognized in the updated AAOMS definition as Stage 0 [2]. Taguchi et al. (2019) reported that the Stage 0 and Stage 1 BRONJ in a Japanese osteoporosis cohort treated with minodronate was 4.44 and 1.71/1000 person-years, respectively, while the exposed variant of BRONJ was not identified [11]. Bone biopsy specimens from Stage 0 BRONJ [12,13] and Stage 0 denosumab-related ONJ patients [14] demonstrated an extensive osteonecrosis with neutrophil infiltration, identical to the histopathological observation of typical BRONJ bone biopsy observations. Twenty (20) to fifty (50) percent of non-exposed BRONJ patients were reported to develop subsequent bone exposure [9,12]. Therefore, non-exposed variant, Stage 0 BRONJ may be considered a sign of disease development or a latent form [15]. However, without a bone biopsy report, it would be difficult to differentially diagnose BRONJ in the non-exposed variant cases. The International Task Force on Osteonecrosis of the Jaw therefore expressed concern about overdiagnosis led by the use of such Stage 0 terminology [3].

The pathophysiology of BRONJ has not been fully established. Current hypotheses accept that BRONJ primarily occurs in the oral cavity [16], a unique environment comprising a thin layer of periosteum and epithelium with an attenuated layer of connective tissue, covering the alveolar bone, that is subject to a variety of stresses [17]. Altered bone remodeling caused by antiresorptive agents localized in the jaw area has been postulated to increase bone necrosis when osseous repair is needed [18]. The presence of *Actinomyces* [19,20] and its role in the pathogenesis of BRONJ has also been suggested [21] based on bone biopsies. Periodontitis is a common inflammatory and

infectious disorder in the oral cavity and has been shown to increase the risk of developing BRONJ in humans [22] and animal models [23,24]. Histopathological reports on biopsy samples of BP-related [25] and denosumab-related [14] ONJ lesions reveal a chronic neutrophil infiltration on the surface of necrotic bone. Thus, aberrant oral barrier immunity may also be associated with the development of MRONJ [26].

Despite the different pathological mechanisms that have been linked to BRONJ, a common attribution is exposure of alveolar bone to a BP drug. Antiresorptive BP such as ZOL are avidly bound by the hydroxyapatite (HAP) component of bone [27]. We have therefore hypothesized that if BP adsorbed on the jawbone could be removed or dissociated, BRONJ might be prevented or reduced in severity. Because BPs are highly stable chemical compounds, repeated administrations are believed to accumulate the BP bioactive dose in bone, creating a depot of adsorbed drug [28]. In an animal study, it was reported that BP concentration in bone was linear with the dose and independent of the administration schedule [29]. We demonstrated using fluorescent imaging BP probes that, over a range of concentrations, BP in solution reached an apparent concentration-dependent equilibrium with BP adsorbed on the surface of HAP and importantly, could be removed by displacement using a subsequently applied different BP in aqueous solution [30]. We report here a demonstration of bisphosphonate-bisphosphonate (BP-BP) replacement on a bone surface using an *in vivo* mouse model. We further present evidence from the model that local administration of a “rescue” BP after systemic exposure to ZOL dramatically reduces development of osteonecrosis symptoms triggered by proximate molar extraction, suggesting a conceptual basis to improve socket healing in patients treated with BPs.

2. Materials and methods

2.1. Ethics statement

All of the experimental protocols using animals were reviewed and approved by the UCLA Animal Research Committee (ARC# 1997–136) and followed the PHS Policy for the Humane Care and Use of Laboratory Animals and the UCLA Animal Care and Use Training Manual guidelines.

2.2. Chemistry and reagents

ZOL (Reclast®, Novartis, East Hanover, NJ) and ETI (Didronel®, Norwich Pharmaceuticals, Inc., North Norwich, NY) were purchased from the UCLA Pharmacy. Commercially available formulations of Reclast® and Didronel® were diluted to the specified concentration in medical grade saline (0.9%NaCl) solution. MHDP (1-hydroxyethylidenebisphosphonic acid) was purchased from Aroz Technologies (Cincinnati, OH). AF647-ZOL and FAM-ZOL provided by BioVinc LLC (Pasadena, CA), were characterized by ¹H and ³¹P NMR, MS and where appropriate, by HPLC according to published procedures [31]. FAM (5-Carboxyfluorescein) was purchased from Sigma-Aldrich (St. Louis, MO). MHDP, AF647-ZOL and FAM-ZOL were diluted to the specified concentration in medical grade saline (0.9%NaCl) solution. In a separate experiment, medical grade phosphate-buffered saline (PBS) was used as the vehicle solution for AF647-ZOL, FAM-ZOL and FAM. For *in vivo* experiments, carprofen (Rimadyl, Zoetis, Parsippany, NJ) and gel diet (DietGel 76A, ClearH2O, Portland, ME) were obtained from UCLA DLAM Pharmacy. For histological experiments, tartrate-resistant acid phosphatase (TRAP) staining agent was obtained from Sigma-

Aldrich (St. Louis, MO). Anti-cytokeratin 14 antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). For cell culture experiments, J774.2 macrophages, minimum essential medium- α medium (MEM- α), fetal bovine serum (FBS), penicillin/streptomycin (Pen/Strep) and mouse receptor activator of nuclear kappa-B ligand (RANK Ligand) were obtained from Sigma-Aldrich (St. Louis, MO). A modification of Basal Medium Eagle medium (DMEM) was obtained from Life Technologies (Carlsbad, CA). RAW 264.7 cells were obtained from ATCC (Manassas, VA). For Western blot assay, antibodies against Rap 1 (E-6, sc-398755), the unphosphorylated form of Rap1A (uRap1A, sc-373968) and the house-keeping protein β -actin, as well as Horseradish Peroxidase (HRP) conjugated secondary antibodies (sc-516102) were obtained from Santa Cruz Biotechnology (Dallas, TX).

2.3. Refinement of mouse model involving a combination of ZOL treatment and tooth extraction

We have previously documented a BRONJ mouse model: 7 to 10-week-old female C57Bl6/J (B6) mice (Jackson Laboratory, Bar Harbor, ME) were treated by a bolus IV injection through the retro-orbital venous plexus of 500 μ g/Kg ZOL followed by maxillary left first molar extraction [26,32,33]. We noted that the BRONJ-like extended exposure of mouse alveolar bone was often associated with food impaction. In this study, we refined our mouse model by introducing a gel diet for 7 d after tooth extraction and compared the disease development with our original pellet diet protocol. One w after 500 μ g/Kg ZOL IV injection, maxillary left first molar was extracted using a dental explorer following the previously published method [26,32]. Immediately prior to tooth extraction, 5.0 mg/kg Carprofen was subcutaneously injected, and this injection was repeated every 24 h for 48 h. At 2 and 4 w after tooth extraction, mice were euthanized by 100% CO₂ inhalation and the maxillary tissue containing the tooth extraction site was harvested and digitally photographed. The area of oral mucosa swelling around the tooth extraction site was measured (ImageJ, NIH, Bethesda, MD) and normalized to the circumferential area of the remaining right maxillary first molar.

The maxillary tissue was fixed in 10% buffered formalin. EDTA-decalcified histological frontal cross-sections were then prepared containing the tooth extraction wound healing site. Some histological sections were stained with tartrate-resistant acid phosphatase. The osteonecrosis area, as measured by necrotic osteocytes, within the palatal bone was measured in histological frontal sections containing tooth extraction socket and the maxillary right first molar. From each animal, two histological sections 50 μ m–100 μ m apart were selected representing the middle and distal area of maxillary first molar. This osteonecrosis area was defined by a cluster of four or more non-vital osteocytes (*i.e.* empty osteocyte lacunae or pyknotic osteocytes), which was normalized by the maxillary/palatal bone area of the tooth extraction side.

2.4. Determination of ZOL dose required to induce maxillary osteonecrosis in mice

Seven to ten-week-old female B6 mice received a bolus IV injection of increasing doses of ZOL (0, 100, 300, 500, 700 or 900 μ g/Kg). One week later, maxillary left first molar tooth was extracted as described above. After tooth extraction, mice were fed gel diet. Two w later, the mouse maxilla was harvested. The osteonecrosis area within the palatal bone was measured in histological frontal sections as described above. Femur bones were also harvested from each mouse and fixed in 10% buffered formalin and subjected to microCT scanning (Scanco Medical, Brüttisellen, Switzerland). Twenty-five slices (0.5 mm) were evaluated in the distal femur metaphysis at a threshold of 200. The volume of interest only included secondary spongiosa [32]. The trabecular bone morphometry was evaluated by the proprietary algorithm (Scanco Medical, Brüttisellen, Switzerland).

2.5. Competitive equilibrium-based BP replacement *in vivo*

B6 mice received a bolus systemic injection of 50 μ M FAM-ZOL through the retro-orbital venous plexus. Six d later, mice received an intra-oral injection of 2- μ l of 5 μ M AF647-ZOL or 0.9% NaCl solution. Under a surgical microscope, intraoral injection to the palatal gingiva adjacent to the left first molar was carried out using a Hamilton syringe with a 33-gauge needle. Separately, mice received a bolus systemic injection of 500 μ g/kg ZOL through the retro-orbital venous plexus. After 6 d, a group of mice received 2- μ l of 250 μ M AF647-ZOL intra-oral injection to the palatal gingiva of left first molar. The other group received the IV injection of AF647-ZOL through the retro-orbital venous plexus. Mouse tissues were harvested one day after the second injection and maxillary and femur bones were subjected to standardized fluorescent biophotonics image obtained using an excitation wavelength of 460 nm and a 515 nm filter (LAS3000, FUJIFILM Corp, Tokyo, Japan).

For a quantitative examination of the postulated competitive equilibrium-based BP displacement *in vivo*, mouse cranial bone was selected as the model due to its simple anatomical structure. Skeletally matured 24-week-old female B6 mice received a bolus systemic injection of 50 μ M AF647-ZOL in 0.9% NaCl vehicle solution through the retro-orbital venous plexus. After 24 h, mice received a sub-periosteal injection to cranial bone with 0, 1, 10 or 50 μ M FAM-ZOL in 0.9% NaCl vehicle solution. The reverse order of injections (systemic injection of FAM-ZOL, then sub-periosteal injection of AF647-ZOL) was also performed. In a separate experiment, 50 μ MAF647-ZOL in PBS vehicle solution were IV injected into B6 mice followed by sub-periosteal cranial *in situ* injection of 50 μ M FAM-ZOL or 50 μ M FAM in PBS vehicle solution. The PBS vehicle solution *in situ* injection was performed in the control group.

Two h later, the cranial bone was harvested and soft tissues were removed. The fluorescent signals of AF647 and FAM were measured by standardized fluorescent biophotonics (LAS3000, FUJIFILM Corp, Tokyo, Japan) using an excitation wavelength of 460 nm and a 515 nm filter for FAM, or 520 nm and the 670 nm filter for AF647. Quantitative analysis for fluorescent intensity of each cranial bone was performed with an image evaluation program (ImageJ, NIH, Bethesda, MD). The region of interest was set within the injection site on cranial bone. The cranial bones were then prepared for non-decalcified cryosection using Kawamoto's tape method [34]. Cross-sections were observed by a confocal laser scanning microscopy (Confocal SP1 MP-Inverted, Leica Microsystems Inc., Buffalo Grove, IL) using oil magnification, and images were recorded.

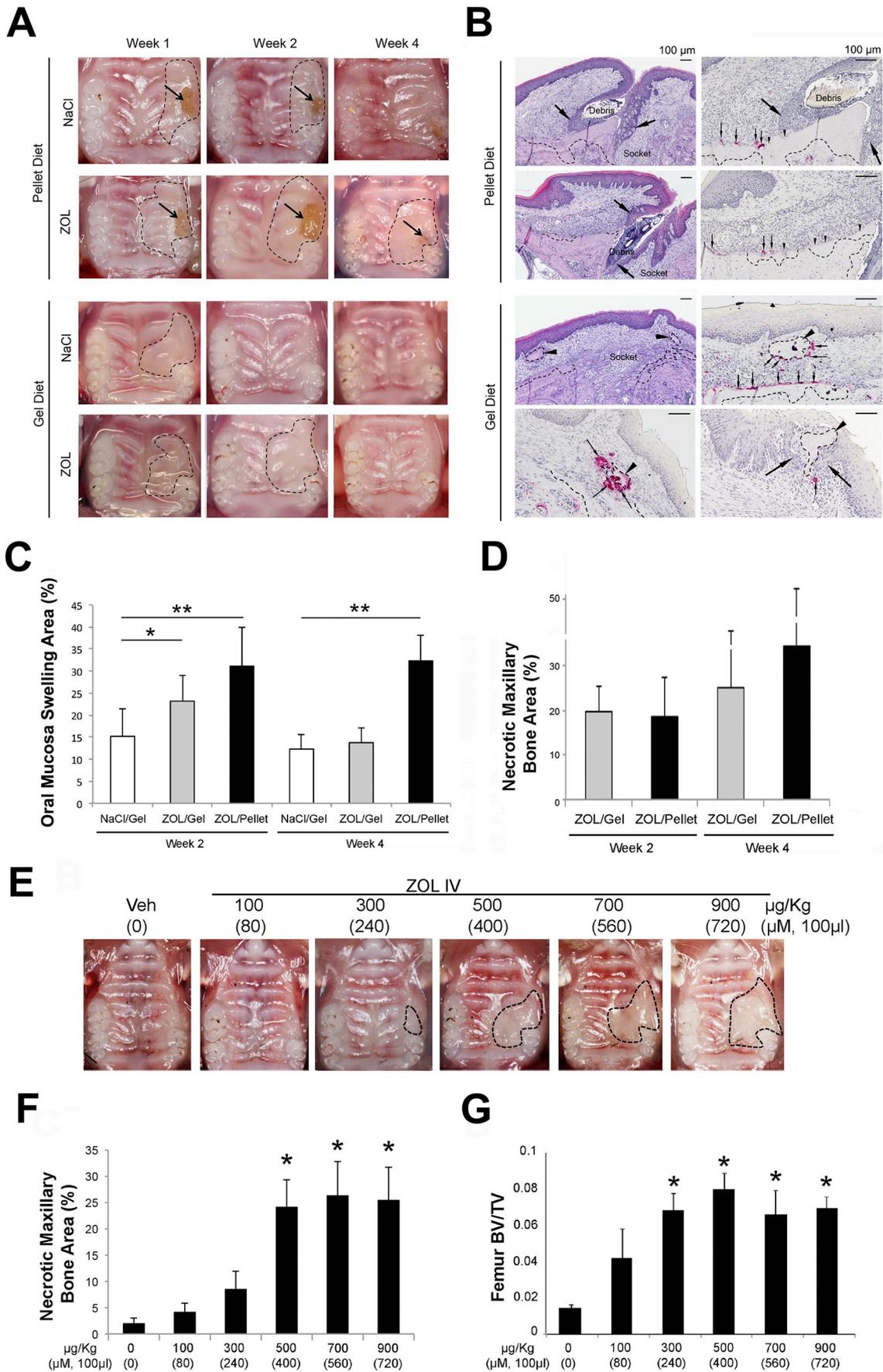
2.6. Western blot assay for inhibition of Rap1 prenylation

J774.2 cells were maintained in DMEM containing 10% FBS plus 1% Pen/Strep. Cells were seeded into 6-well plates at a density of 2×10^5 cells/well and left to adhere overnight. Cells were then treated with 10 or 100 μ M of ZOL, MHDP, ETI, or AF647-ZOL for 24 h. Cells were lysed in RIPA lysis buffer. Then Rap1 prenylation were evaluated by SDS-PAGE and Western blotting using antibodies to Rap 1, the unphosphorylated form of Rap1A and the house-keeping protein β -actin [35]. A low potency BP (lpBP) was defined as a BP showing no detectable anti-prenylation activity in this assay at 100 μ M compound.

2.7. Effect of lpBP on femur trabecular bone morphometry *in vivo* and resorption pit formation *in vitro*

Seven to ten-week-old female B6 mice received a bolus IV injection of ZOL, AF647-ZOL and MHDP (400 μ M in 100 μ l 0.9% NaCl solution). Control mice received a 0.9% NaCl vehicle injection. Two w later, mice were euthanized by 100% CO₂ inhalation and right femur from each mouse was harvested and evaluated by microCT as described above.

The RAW 264.7 cells were plated on a Corning Osteo Assay Surface Plate (Corning Inc., Corning, NY, USA) and stimulated with osteoclast



(caption on next page)

Fig. 1. ZOL dose threshold causing alveolar bone necrosis induced by tooth extraction in the refined mice model with minimal confounding food impaction. A. C57Bl6J mice received a bolus IV injection of 500 µg/Kg ZOL followed by maxillary left first molar extraction. In a conventional pellet diet protocol, control mice received a vehicle 0.9% NaCl vehicle solution IV injection showed a progressive wound healing over 4 w. The ZOL-treated mice sustained the open wound throughout the 4 w period. By contrast, the refined protocol with gel diet for 7 d after tooth extraction generated faster wound healing. However, the ZOL-treated group sustained gingival swelling at 2 w. B. Histological examination revealed the extensive oral epithelial hyperplasia (large arrows) facilitating the alveolar bone exposure in the conventional pellet diet group. The open wound contained debris and food impaction. By contrast, the refined gel diet protocol eliminated the debris and food impaction. ZOL-treated mice sustained the development of gingival inflammation and osteonecrosis (dotted line) including bone sequestration associated with osteoclasts highlighted by TRAP staining (red, small arrows). C. The area of oral mucosa swelling was sustained in the ZOL-treated mice with the gel diet protocol for 2w. D. The development of osteonecrosis associated with tooth extraction was not affected by the refined gel diet protocol. E. C57Bl6J mice treated with 0, 100, 300, 500, 700 or 900 µg/Kg ZOL IV injection followed by maxillary left first molar extraction. After 2 w, the maxillary tissue including the tooth extraction wound was examined. The swelling area of oral mucosal tissue (dotted line) was visible at the tooth extraction site of mice received 500 µg/Kg or more ZOL treatment. F. The histological cross-sections through the extraction socket were used to measure the necrotic maxillary bone area. It was indicated that there was a ZOL dose threshold between 300 and 500 µg/Kg for the development of significant osteonecrosis in mice. The data show mean ± SEM. **p* < .05 (*n* = 6, 5, 6, 5, 6 and 6 for ZOL doses 0, 100, 300, 500, 700 and 900 µg/Kg). G. Femur bones of mice (*n* = 6 in each group) were scanned by microCT and bone volume/total volume (BV/TV) was calculated. The effect of ZOL on femur BV/TV linearly increased up to 500 µg/Kg ZOL treatment and reached a plateau. The data show mean ± SD. **p* < .05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentiation medium (MEM- α , 10% FBS, 1% Pen/Strep and 50 ng/mL mouse RANK Ligand) for 7 d. Then, medium was replaced with 100 µM of ZOL, MHDP, ETI, or AF647-ZOL diluted with the differentiation medium. The control group was continuously cultured with only differentiation medium alone. After 48 h, the culture medium was removed and the cells were treated with 5% sodium hypochlorite for 5 min at room temperature. After washing the plate with Milli-Q water and allowing it to dry, regions of each well were photographed using a microscope. The pit area was measured using ImageJ software.

2.8. Systemic IV injection of lpBP (AF647-ZOL or MHDP) to mice treated with ZOL IV injection prior to tooth extraction

Seven to ten-week old female B6 mice received a bolus IV injection of ZOL (400 µM; 100 µl) through the retro-orbital venous plexus. After one day, mice received IV injection of lpBP (AF647-ZOL:400 µM; 100 µl, MHDP: 400 or 4,000 µM; 100 µl) through the retro-orbital venous plexus. Six d after lpBP injection, mice were subjected to extraction of the left maxillary first molar and the oral wound healing was evaluated after 2 w by measuring the oral mucosa swelling area in the standardized photographs as described above. Then the maxillary bones were harvested for microCT imaging and EDTA-decalcified histological specimen preparation. The histological osteonecrosis area was measured as described above.

2.9. Local intra-oral injection of lpBP to mice treated with ZOL IV injection prior to tooth extraction

Seven to ten-week-old female B6 mice received a bolus IV injection of ZOL (400 µM; 100 µl). After eleven d, mice received the intra-oral injection of AF647-ZOL (250 µM; 2 µl), ETI (250 µM; 2 µl), or MHDP (250 µM; 2 µl). One day after intra-oral injection of lpBP, mice were subjected to extraction of the left maxillary first molar and the oral wound healing was evaluated 2 w after tooth extraction. The oral mucosa swelling area and the histological osteonecrosis area were measured as described above. Furthermore, extraction socket wound healing was determined in reconstructed 3D microCT images. The mouse maxillary first molar has three roots (mesial, distal and palatal roots). The bone formation in each bony socket was rated as 1 = poor, 2 = immature bone filling and 3 = mature bone filling, and the bone wound healing index was calculated as the sum of these ratings in each sample.

To examine the effect on tooth extraction-induced wound healing, histological sections were subjected to immunohistochemistry including antigen retrieval heating process and incubated with anti-cytokeratin 14 antibody [26]. Briefly, histological sections were deparaffinized and treated by an antigen retrieval procedure using citrate buffer (pH 6.0) and a microwave for 2 min followed by a conventional blocking process. The antibodies were diluted in blocking buffer and

applied to the sections. Following secondary antibody incubation, diaminobenzidine (DAB) chromogenic reaction assays were performed.

2.10. Statistics

In vitro and *in vivo* data in groups were compared by one-way analysis of variance (ANOVA) using a parametric method. When more than two groups were compared, ANOVA was adjusted by *post hoc* Dunnett's multiple comparisons for *in vitro* data and by Bonferroni/Holm test for *in vivo* data. The multiple experimental groups were compared with the ZOL-treated positive control group in each experiment.

3. Results

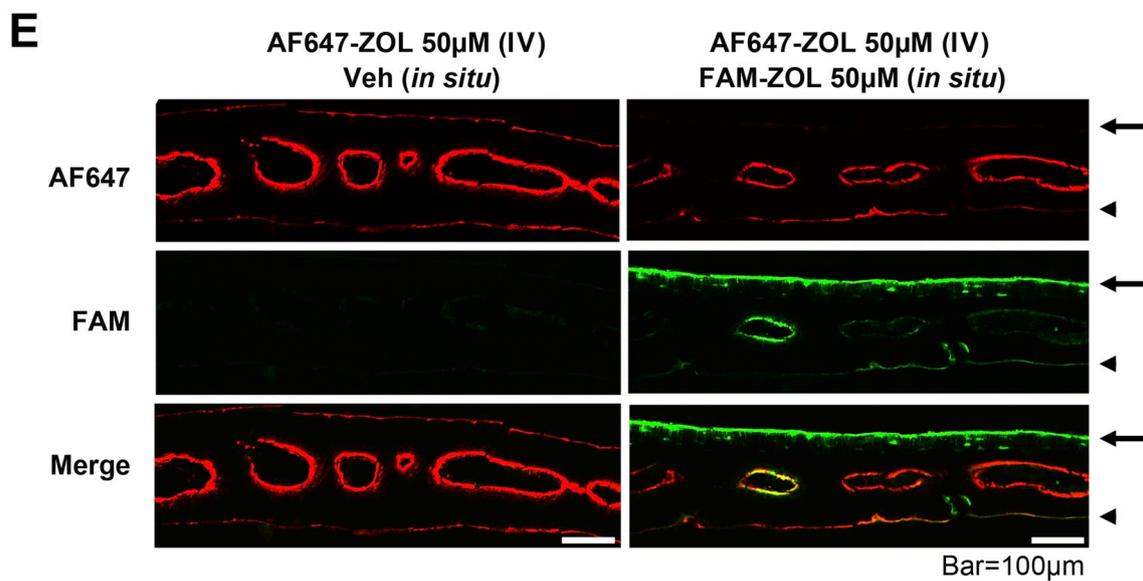
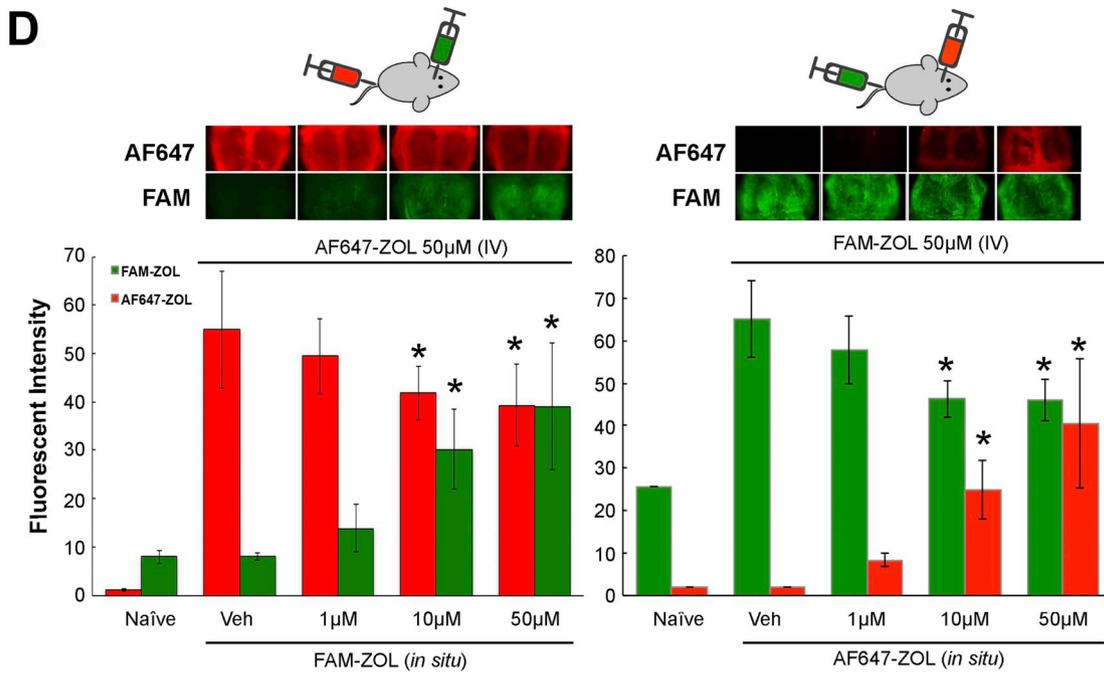
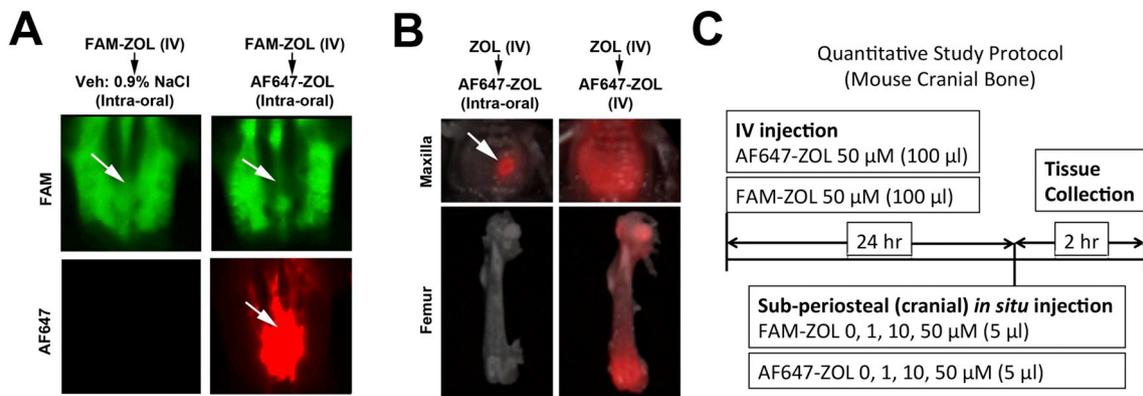
3.1. Refined mouse BRONJ lesion resembled the non-exposure variant

The maxillary tooth extraction wound of mice administered with ZOL developed BRONJ-like lesion with exposed bone and gingival swelling for 4 w (Fig. 1A). This disease development was consistent with our previous reports [26,32]. Histological examination revealed the oral epithelial hyperplasia contacting the surface of necrotic maxillary bone, facilitating the bone exposure. The refined protocol with gel diet effectively eliminated food impaction in both control and ZOL-treated mice (Fig. 1A and B). This model exhibited the sustained gingival swelling for 2 w in the ZOL-treated group (Fig. 1A and C) and a corresponding amount of osteonecrosis including bone sequestrations (Fig. 1B and D). In some histological specimens, the localized oral epithelial hyperplasia at the tooth extraction site was observed, which formed a fistula-like structure reaching the necrotic alveolar bone.

It must be noted that the refined mouse model lacked bone exposure and thus was no longer considered a typical BRONJ model. However, it was free from food impaction, which had confounded the significant variation of disease presentation in the previous BRONJ model. Therefore, the refined mouse model should isolate the effect of legacy ZOL adsorbed on the jawbone and was selected for this study addressing the effect of postulated treatment targeting the BP dissociation.

3.2. Threshold of ZOL IV injection dose leading to the development of jawbone osteonecrosis in mice

This study was conducted to determine threshold BP doses for the development of jawbone necrosis using ZOL. Single IV injection with various ZOL doses was administered to mice and maxillary left first molar extraction wounds were compared. We found that abnormal oral mucosa swelling (Fig. 1E) and the increased osteonecrosis area appeared when mice received 500 µg/Kg or higher doses of ZOL (Fig. 1F). By contrast, the mouse femurs exhibited a dose-dependent increase of bone volume/tissue volume (measured by micro CT imaging) up to 500 µg/Kg ZOL dose, beyond which the effect leveled off (Fig. 1G).



(caption on next page)

Fig. 2. *In vivo* assessment of competitive equilibrium-based BP displacement. A: Mice were treated with FAM-ZOL IV injection. One week later, AF647-ZOL or 0.9% NaCl vehicle solution was administered locally to maxilla by intra-oral injection. Adsorbed AF647-ZOL was observed at and around the injection site (white arrow) of maxilla, where the fluorescent signal of pre-injected FAM-ZOL was reduced. B: Mice pretreated with ZOL IV injection were subsequently administered AF647-ZOL by intra-oral injection. AF647-ZOL signal was observed only at and around the injection site in the maxilla (white arrow) whereas femur did not show any AF647-ZOL signal (left panel). By contrast, systemic IV administration of AF647-ZOL in the ZOL-pretreated mice resulted in a clear maxilla and femoral AF647-ZOL signal (right panel). C: To quantitatively examine the postulated competitive equilibrium-based BP displacement, mouse cranial bone was selected due to its simple anatomical structure. Mice were pretreated by IV injection of FAM-ZOL, followed by cranial bone sub-periosteal injection of sequential doses of AF647-ZOL. In a separate experiment, AF647-ZOL and FAM-ZOL were switched. D: Sub-periosteally injected FAM- or AF647-ZOL signal increased dose dependently, while pre-adsorbed AF647- or FAM-ZOL signal decreased. * $p < .005$ vs the sub-periosteal vehicle injection group (Veh) ($n = 6$ in each group). E: Cranial bone cryo-cross sections revealed localized displacement of AF647-ZOL on the external bone surface and replacement by FAM-ZOL. Black arrows indicate external (skin) side of cranial bone and black arrowheads indicate internal (dura) side.

These data indicate that a certain threshold concentration of bioavailable BP dose in the jawbone is necessary to trigger osteonecrosis pathogenesis. In this mouse model, the presence of threshold dose was determined to be 500 $\mu\text{g}/\text{kg}$ or 400 μM ZOL in a 100 μl IV injection.

We previously demonstrated that application of a BP in solution to a hydroxyapatite surface containing a previously adsorbed BP compound could replace the latter [30]. Thus, the mouse model in this study received a single bolus IV injection of the above threshold dose of ZOL instead of repeated administrations. Tooth extractions were performed within 3 w of drug administration to ensure the presence of “legacy” adsorbed drug.

3.3. Competitive equilibrium-based BP displacement *in vivo*

AF647-ZOL intra-oral injection gave rise to an AF647 signal at the injection site on the palatal bone and decreased the fluorescent signal from pre-adsorbed FAM-ZOL (Fig. 2A). Moreover, ZOL-treated mice receiving the AF647-ZOL intra-oral injection did not show a detectable AF647 signal in femurs, whereas systemic administration of AF647-ZOL resulted in a clear femoral AF647-ZOL signal (Fig. 2B). While we demonstrated successful intra-oral injection using a Hamilton syringe (Fig. S1A and S1B), the complex palatal/maxillary bone anatomy precluded quantitative measurement of BP displacement.

We then selected mouse cranial bone as an *in vivo* model to quantitatively evaluate the postulated competitive equilibrium-based BP displacement. Mice received a bolus systemic injection of 50 μM AF647-ZOL, then received a sub-periosteal injection to cranial bone with 0, 1, 10 or 50 μM FAM-ZOL (Fig. 2C). The reverse order of injections was also performed. The FAM signal of sub-periosteally injected FAM-ZOL showed a dose-dependent increase in loading on the cranial bone at the injection site. The signal of IV pre-injected AF647-ZOL at the corresponding site showed a dose dependent decrease of FAM-ZOL with statistical significance ($p < .05$ by ANOVA) reached at 10 μM and 50 μM (Fig. 2D). This effect was also observed when the FAM-ZOL and AF647-ZOL injection sequence was reversed, with similar cut point. The cranial bone cryo-cross sections revealed AF647-ZOL labeling on the external (skin) and internal (dura) sides of the bone surfaces, as well as on the trabecular surface. In a cranial bone specimen that received a FAM-ZOL sub-periosteal injection, a FAM-ZOL signal was observed primarily on the external bone surface and, occasionally, in the bone marrow space. The AF647-ZOL signal on the external bone surface was disproportionately reduced relative to the internal bone surface (Fig. 2E).

We additionally performed an experiment to elucidate whether FAM alone was adsorbed on the bone (Fig. S2). The results showed that treatment with FAM did not produce the intense fluorescent signal on the cranial bone that was seen with the FAM-BP conjugate. Furthermore, cranial *in situ* injection of FAM did not observably displace legacy AF647-ZOL (Fig. S2). This confirmed that the BP moiety was essential for bone mineral adsorption of the fluorescent dye conjugate and also for displacement of legacy BP in the bone. This additional experiment used PBS as the vehicle solution, which did not have any noticeable effect on the results, demonstrating that the presence of phosphate in this solution did not affect specific or non-specific binding

of either FAM or FAM-ZOL. As such, 0.9% NaCl or saline solution was used as the vehicle solution for all *in vivo* studies.

Taken together, these data provide compelling evidence in support of competitive equilibrium-based BP displacement from bone *in vivo*.

3.4. Candidate lpBPs: AF647-ZOL, Methylhydroxyl diphosphonate (MHDP), and Etidronate (ETI)

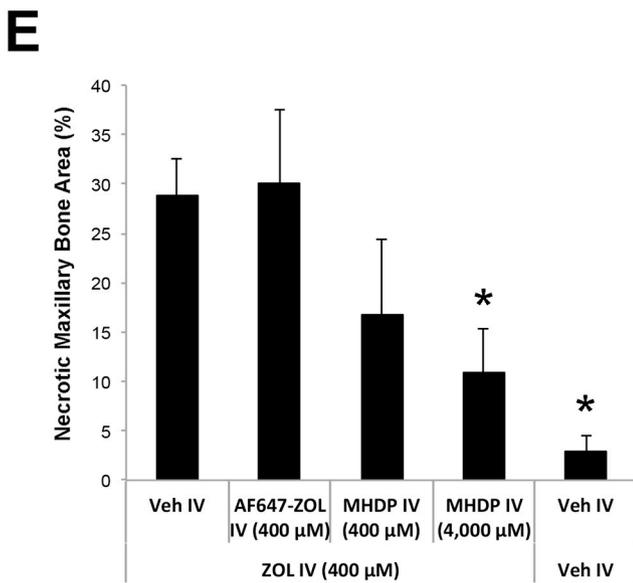
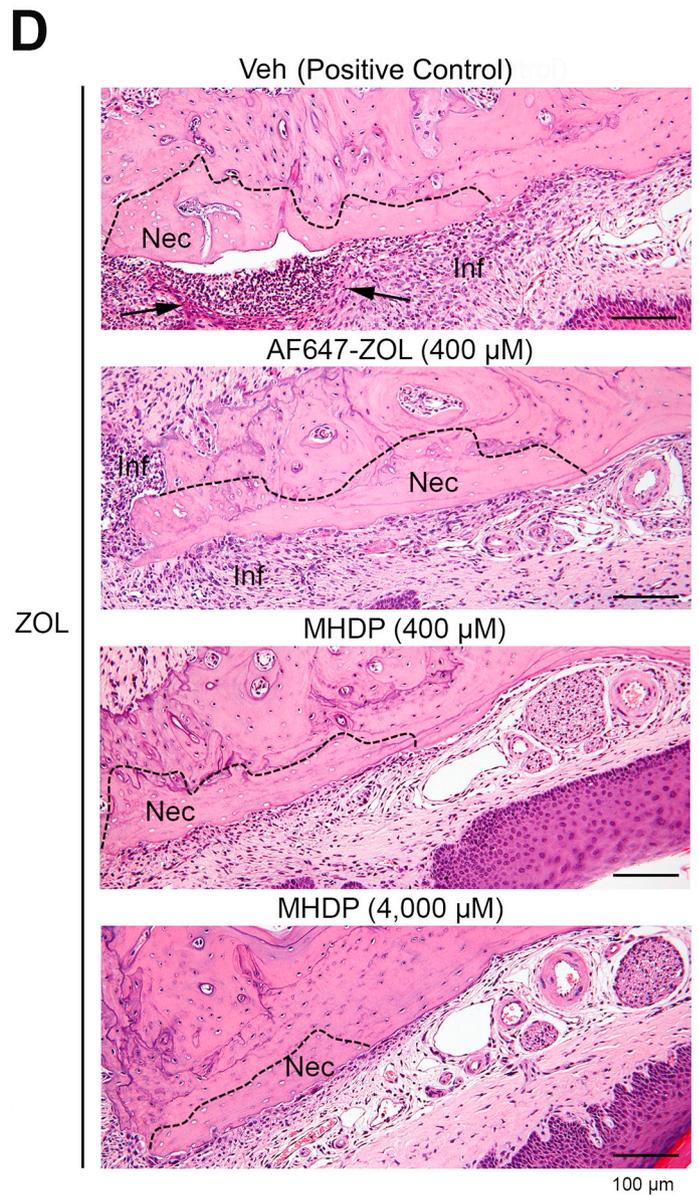
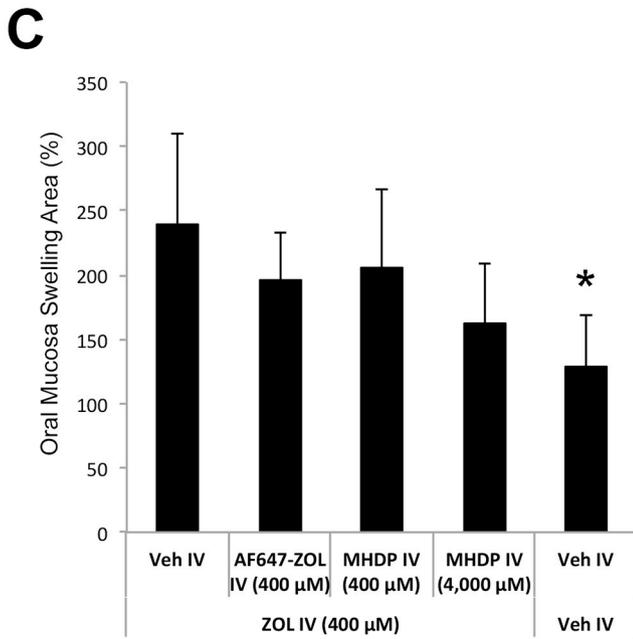
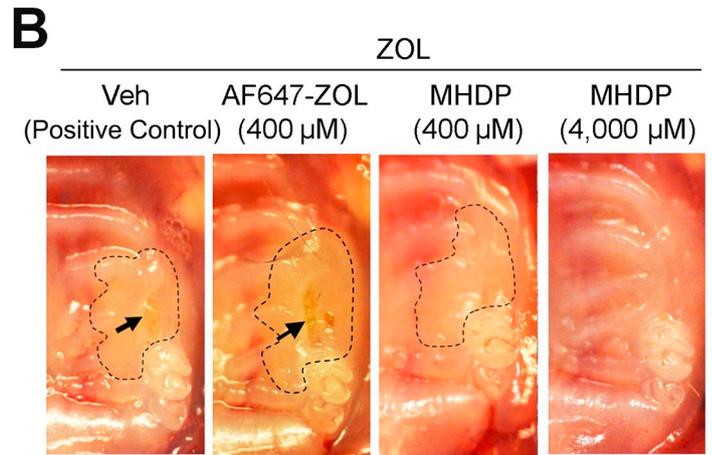
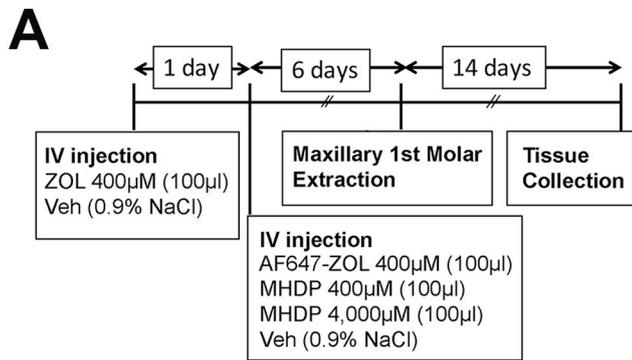
We selected AF647-ZOL, MHDP and ETI as the candidate lpBPs for this study (Fig. 3A). When J774.2 macrophage cells were treated with two different concentrations (10 and 100 μM) of BP compounds, un-prenylated Rap1A was detected by Western blotting after 24 h of ZOL treatment, whereas un-prenylated Rap1A was undetectable in cells treated with AF647-ZOL [31], MHDP or ETI (Fig. 3B). The reconstructed microCT images and quantitative microCT analysis of the distal epiphyseal trabecular bone indicated that the BV/TV ratio was significantly small for mice treated with AF647-ZOL and MHDP as compared to that of mice treated with ZOL (injection of 100 μl of 400 μM of each compound) (Fig. 3C). The formation of resorption pits *in vitro* by osteoclasts treated with ZOL was significantly less than the PBS-treatment control ($P < .001$). Resorption pit generated by osteoclasts treated with AF647-ZOL, MHDP and ETI did not significantly differ from the control (Fig. 3D).

3.5. Partial attenuation of jawbone osteonecrosis symptoms by systemic injection of lpBP

In this study, the effect of systemic lpBP injection on the development of jawbone osteonecrosis was evaluated using AF647-ZOL (400 μM , 100 μl) and MHDP (400 or 4000 μM , 100 μl) (Fig. 4A). In the standardized oral image, the ZOL-treated control and the AF647-ZOL systemic administration groups showed oral mucosa swelling and signs of fistula formation (Fig. 4B). The area of oral mucosal swelling appeared to be decreased in the MHDP (4000 μM) injection group as compared with the ZOL-treated control group; however statistical significance was not reached (Fig. 4C). Histological evaluation revealed inflammation in the oral mucosa connective tissue in both the ZOL-treated control and AF647-ZOL injection groups, whereas oral mucosa inflammation appeared to be less significant in the MHDP injection groups (Fig. 4D). Evidence of osteonecrosis adjacent to the tooth extraction socket was observed in all groups (Fig. 4D). The area of osteonecrosis appeared decreased in the MHDP injection groups, although statistical significance was only achieved at a relatively high dose of MHDP (4000 μM or 1000% of the pre-injected ZOL dose), which reduced the osteonecrosis area by approximately 50% (Fig. 4E).

3.6. Effective prevention of jawbone osteonecrosis by a single intra-oral injection of lpBP in mice

Mice pre-treated with an IV injection of ZOL (500 $\mu\text{g}/\text{kg}$ or 100 μl of 400 μM ZOL) received an intra-oral injection of 2 μl of lpBP: AF647-ZOL (250 μM), MHDP (250 μM) or ETI (250 μM) one day prior to the maxillary left first molar extraction (Fig. 5A). Two w after tooth extraction, oral wound swelling was observed in the ZOL-treated control mice;



(caption on next page)

Fig. 4. Systemic administration of lpBPs (AF647-ZOL and MHDP) and jawbone necrosis in ZOL-treated mice. A. Time course of mouse experiments. B. Gross observation of maxillary tooth extraction site: swelling (dotted line) and an open wound (arrow). ZOL-treated mice received MHDP (400 or 4000 μM) IV injection appeared to improve tooth extraction wound healing. C. The oral mucosa swelling area was normalized by the circumferential area of remaining maxillary first molar. Although MHDP IV injection reduced the size of swelling area, no statistical significance was reached. D. Histological evaluation revealed areas of osteonecrosis in all groups (dotted line, Nec). The ZOL-treated control and AF647-ZOL IV injection groups developed the alveolar bone necrosis associated with focal inflammation (*). The ZOL-treated control mice showed the epithelial hyperplasia (arrows) reaching to the bone surface. E. The standardized osteonecrosis area was not significantly improved by systemic injection of AF647-ZOL (400 μM) or MHDP (400 μM). However, MHDP (4000 μM) systemic injection demonstrated significant reduction. The data show mean \pm SEM. * $p < .05$ ($n = 6, 4, 3, 6$ and 3 for ZOL-pretreated mice with vehicle solution, AF647-ZOL (400 μM), MHDP (400 μM) or MHDP (4000 μM) systemic injection and the negative control group).

Histological evaluation further revealed extensive osteonecrosis, gingival swelling and focal inflammation on the alveolar bone in the ZOL-treated controls (Fig. 5E). Intra-oral injection of lpBP such as ETI and MHDP (1.25% of pre-injected ZOL dose) prevented the development of osteonecrosis lesion (Fig. 5E). However, mice receiving an intra-oral injection of AF647-ZOL appeared to retain some disease phenotypes including a focal inflammatory cell infiltration on the surface of necrotic alveolar bone (Fig. S3). The area of osteonecrosis was smaller in all lpBP intra-oral injection groups than that of the ZOL-treated control group, although statistical significance was reached only in the MHDP and ETI intra-oral injection groups, with a reduction in the osteonecrosis area of approximately 80%. (Fig. 5F).

In order to assess the effect of lpBP intra-oral injection on tooth extraction wound healing, 3D reconstructed microCT images were obtained and rated for the extent of bone filling in each socket (Fig. 5G). The bone wound healing index calculated as the sum of bone filling rate for all sockets, showed a significant increase in all lpBP intra-oral injection groups relative to the ZOL-treated control (Fig. 5H).

4. Discussion

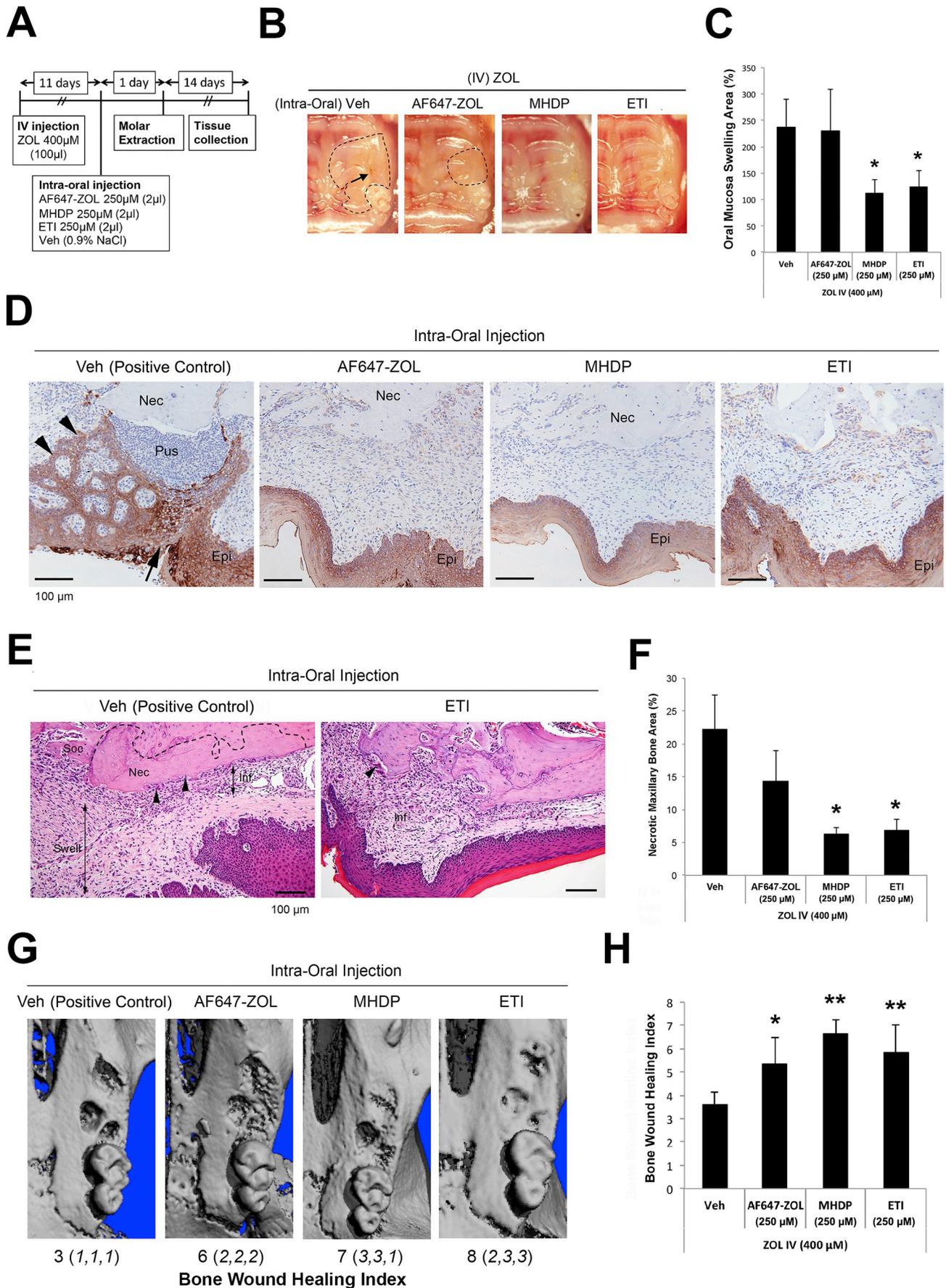
BRONJ is highly localized to the oral cavity, which is not the intended treatment site for BP antiresorptive agents. To investigate the pathogenesis of BRONJ, a number of rodent models have been reported, in which, in addition to BP treatment, systemic manipulations: *i.e.*, vitamin D deficiency [19,30]; and oral manipulations: *i.e.* tooth extraction [36,37], periodontitis [24,38–40] and periapical disease [41]; increased the prevalence and severity of BRONJ. Induced oral inflammation appeared to be critical for the disease onset. In fact, we have previously reported that the lack of BRONJ in B/T cell deficient *rag2*^{-/-} mice [32], supporting the pathological role of oral inflammation. The present study demonstrated that the pellet food aggravated tooth extraction-induced inflammation, which appeared to contribute to the severity of BRONJ (Fig. 1). Use of the alternative gel food during the first week after tooth extraction eliminated the food impaction and prevented the bone exposure; however, the development of osteonecrosis was still observed. This newly refined mouse model suggests that jawbone necrosis could occur without bone exposure. Furthermore, our previously reported BRONJ mouse model [26,32,33] and the current refined model (Fig. 5D and S3) similarly exhibited oral epithelial hyperplasia albeit at different frequencies and severities. Pseudoepitheliomatous hyperplasia (PEH) has been reported in a high percentage of BRONJ biopsy specimens [19,42]. Both epithelial hyperplasia in our mouse model and PEH of human BRONJ pathologically establish the contact between epithelial cells and necrotic bone surface. While our refined mouse model may not be considered BRONJ by definition, this model retains the pathological process causing gingival inflammation and osteonecrosis, which is consistent with the previously reported mouse BRONJ model. In this study, the consistent development of osteonecrosis without confounding food impaction was thought to be suitable for quantitative evaluation of the isolated effect of oral BP on the disease onset and the postulated preventive hypothesis.

Using the refined mouse model, we experimentally determined a ZOL dose threshold, above which osteonecrosis was likely to develop (Fig. 1), suggesting that reduction of legacy BP drug bioavailable in the jawbone might be an effective strategy to prevent BP-associated oral

pathology. Indeed, decreasing legacy BP by naturally occurring desorption and elimination is implicit in discontinuing drug administration in a ‘drug holiday’. A two-month BP drug holiday prior to and after dentoalveolar surgery has been recommended by AAOMS for BP-treated patients [2]; however, the effectiveness of this strategy is unclear as evidence to support it has not been forthcoming [43]. Systemic metabolism of skeletally adsorbed BPs is very slow due to their prolonged half-lives on bone, which may be as long as 10 years depending on the particular drug [44]. In fact, the FDA has determined that there are “no substantial data available to guide decisions regarding the initiation or duration of a drug holiday” and the effects of a BP drug holiday remain inconclusive after meta-analysis [45]. In a mouse model, BP discontinuation did not reverse radiological and histological findings of MRONJ, although discontinuation of a RANKL inhibitor, as a surrogate for denosumab, altered features of MRONJ 10 w after discontinuation [46].

To address this important clinical challenge, we have explored the potential of an approach based on displacement of the active legacy drug by a locally applied inactive BP [47]. In the present study, we demonstrate that displacement of one BP by another one can be realized *in vivo* (Fig. 2). Systemic administration of ETI has been recently proposed as a potential etiotropic procedure to treat BP-related ONJ [48,49]. In an informal clinical study, 25 patients with a previous BP therapy history who were subsequently diagnosed with stage 2 or 3 BRONJ underwent an experimental treatment of orally administered ETI (200 mg/d, two w’ ingestion and three w’ rest) and were reported to show some improvement in outcomes (statistical analysis was not possible in this uncontrolled study), including separation and removal of necrotic bone sequestra and improved recovery of affected soft tissue. However, our present study did not provide support for systemic administration of an lpBP as an effective prophylactic approach to BRONJ. Two BP compounds lacking significant anti-phenylation activity, AF647-ZOL and MHDP (Fig. 3B) were both found to attenuate only moderately the severity of gingival swelling and osteonecrosis when systemically administered in our mouse model (Fig. 4). After systemic administration, the distribution of BP within the skeleton is not homogeneous and systemic administration of an lpBP may not achieve a local concentration sufficient to displace pre-adsorbed BP in the jawbone to a level below the BRONJ threshold. In our study, only the systemic administration of the higher dose MHDP (4000 μM or $10\times$ of the pre-injected legacy ZOL dose) showed the potential feasibility for this approach (Fig. 4); however, it is unclear whether this dose level is practical for human application.

Systemic administration of an lpBP and drug holidays are contraindicated for patients with severe primary bone disease (*i.e.*, malignancy metastatic to bone or multiple myeloma) who may need to maintain BP drug levels at affected sites in the skeleton. At the same time, the significant morbidity of BRONJ argues for privileging prevention over treatment as a general strategy. Therefore, an ideal therapy must isolate the oral cavity and should exhibit little or no effect on the skeletal system as a whole. To address the unique concerns associated with this patient population, which is the one most at risk for BRONJ, we have envisioned an innovative approach, which involves the local administration of an lpBP directly to the oral cavity. Our results demonstrate that at an appropriate dose, a fluorescently labeled lpBPs bound avidly to local injection site in the jawbone without being



(caption on next page)

Fig. 5. Intra-oral *in situ* injection of lpBPs prevented the development of jawbone necrosis in ZOL-treated mice. A: Time course of mouse experiments of lpBP intra-oral administration prior to tooth extraction. B: Gross examination revealed the excellent tooth extraction wound healing in mice received MHDP or ETI intra-oral injection. C: The normalized oral mucosa swelling area was significantly smaller in the MHDP and ETI intra-oral injection group than that of the ZOL-treated control group. D: Anti-cytokeratin 14 immunohistology indicated the extensive epithelial hyperplasia (arrowheads), reaching the surface of necrotic alveolar bone (Nec) and forming a fistula (arrow) connecting to the pustule (Pus) of ZOL-treated control specimen. The epithelial abnormality was not observed in the lpBP intra-oral injection groups. E: A typical lesion in the ZOL-treated control specimen exhibited gingival swelling (double head arrow and Swell) and focal inflammation (Inf and double head arrow) on the surface of necrotic bone area (dotted line and Nec) with shallow osteoclastic lacunae (arrowheads), which contained numerous neutrophils (small arrows). ZOL-pretreated mice with ETI intra-oral injection demonstrated nearly normalized tooth extraction wound healing characterized by diffused inflammatory cell infiltration (Inf) and osteoclasts (arrowhead). The inflammatory cells were mostly lymphocytes. F: The significant reduction of osteonecrosis area was observed in MHDP and ETI intra-oral injection groups. G: Tooth extraction wound healing was assessed in 3D reconstructed microCT images. Three bony sockets of mouse maxillary first molar were separately rated for 1 to 3, 3 being the excellent healing. The bone wound healing index was calculated as the sum of these rates. H: The bone wound healing index showed significant increase in all lpBP intra-oral injection groups. The data show mean \pm SEM (n = 6, 4, 4, and 5 for ZOL-pretreated mice with vehicle, AF647-ZOL, MHDP or ETI intra-oral injection). *, p < .05; **, p < .01.

significantly distributed systemically to other skeletal bones (Fig. 2B).

In the present study, intra-oral injection of lpBP attenuated or completely prevented the development of oral abnormalities in ZOL-pretreated mice. In particular, the intra-oral injection of MHDP and ETI prior to the tooth extraction demonstrated the significant effect in preventing oral mucosa swelling and osteonecrosis areas, as well as improved tooth extraction wound healing (Fig. 5). However, AF647-ZOL administration led to mixed results (Fig. 5 and S3). BP species share a backbone chemical structure composed of a methylene carbon bridging two phosphonate groups (P-C-P moiety). The strong affinity of BPs for inorganic calcium phosphate mineral in bone is ascribed to bidentate chelation of Ca^{2+} by the two phosphonate groups. A hydroxy substituent on the carbon is known to augment bone mineral affinity by adding a tridentate interaction with the metal ion [27,50]. In our study, AF647-ZOL was the least effective among lpBPs tested (Fig. 5 and S4). AF647-ZOL was previously shown to have a smaller binding affinity to HAP than ZOL in an FPLC column elution assay [31,51]. By contrast, ETI exhibits strong HAP binding affinity in both the HAP FPLC column assay and in a competitive binding assay based on fluorescence detection [52]. In 1980, Francis et al. investigated the *in vitro* adsorption of C^{14} -labeled MHDP and ETI and found that MHDP was more strongly adsorbed on both amorphous calcium phosphate and HAP [53]. Therefore, we postulate that MHDP and ETI, but not AF647-ZOL, contribute significantly to its effectiveness in replacing the pre-adsorbed labeled BP, and will an important feature for any translational candidate.

Recently, an application of 17% EDTA with Q-tips to the exposed alveolar bone immediately after tooth extraction in ZOL-pretreated rats was reported to attenuate jawbone osteonecrosis development [54]. Chelation therapy had been applied for myocardial infarction with the postulated effect of EDTA for chelating the calcium in atherosclerotic plaques, although a recent clinical trial did not show a proven detectable clinical benefit [55]. Thus the mechanism of EDTA chelation therapy is still unclear. Nonetheless, the approach of reducing the jawbone BP bioavailable dose below the disease threshold level appears to have a merit as a therapeutic option.

The pathophysiological mechanism of BRONJ has not been fully established. Patients with multiple myeloma and bone metastatic diseases often present immunodeficiency or are treated with immunosuppressive agents [56], which might predispose a susceptibility to develop BRONJ [57]. It must be noted that osteonecrosis occurs nearly exclusively in the oral cavity. The spatial association between BRONJ lesions and a preceding oral wound or infection may further indicate the involvement of local pathological factors. Oral mucosa houses active barrier immunity. In our mouse model, oral mucosa immune function was shown to be significantly deficient, as characterized by decreased T cell cytokine expression [33] and persistent presence of neutrophils [26]. The current study demonstrated that the prevention of disease phenotypes by prophylactic intra-oral injection of lpBP (Fig. 5) was associated with the attenuation of abnormal oral inflammation.

Based on clinical reports, other potential preventative therapies proposed, namely oral hygiene care [58], prophylactic antibiotic treatments before and after oral surgical procedures [59] and antimicrobial mouth rinses [60] have been symptomatic procedures. The present study provides support for an effective mechanism-based approach, in which intra-oral administration of an lpBP, prior to dentoalveolar treatment, displaces pre-adsorbed BP from the local bone surface. This results in the lowering of the BP level accumulated by subsequent resorption activity, triggered by oral surgery, below an apparent threshold that can induce at least the initial pathogenesis characterized by the development of osteonecrosis without oral bone exposure. We therefore postulate that the prevention of osteonecrosis may further result in the uneventful healing of oral surgical manipulations. This novel concept would benefit patients who must maintain the therapeutic benefit of pre-adsorbed BP for their primary diseases in axial and appendicular skeletal sites, while significantly decreasing the prevalence and severity of BRONJ in the oral cavity triggered by dentoalveolar manipulation.

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Author contributions

The BP displacement approach presented herein was conceived by CEM and IN. AH, SS, FHE, MWL, CEM, and IN designed the experimental studies. The experiments were performed by AH, KeK, SS, KM, YS, QW, HS, KS, HO, CE and IN. AH, KeK, SS, YS, QW, HS, KS FHE, CEM and IN analyzed and interpreted the data. AH, SS, MWL, FHE, KS, CEM, and IN drafted the manuscript, and CEM and IN wrote the final version. All authors agreed on the content of the manuscript.

Competing interests

IN is a consultant for and CEM, SS, and FHE are founding members and shareholders of BioVinc, LLC. SS, FHE, KS, and MWL are or have been paid employees or consultants of BioVinc, LLC. Other authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2019.03.027>.

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