Title

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

Authors

Hokugo, Akishige
Kanayama, Keiichi
Sun, Shuting
et al.

Publication Date

2019-06-01

DOI

10.1016/j.bone.2019.03.027

Peer reviewed

HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2020 June 09.

Published in final edited form as:

Bone. 2019 June ; 123: 115–128. doi:10.1016/j.bone.2019.03.027.

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

Akishige Hokugo[a,b], **Keiichi Kanayama**[a,c,1], **Shuting Sun**[a,d,*,1], **Kenzo Morinaga**[a,e], **Yujie Sun**[a,f], **QingQing Wu**[a,g], **Hodaka Sasaki**[a,h], **Hiroko Okawa**[a,i], **Courtney Evans**[a], **Frank H. Ebetino**[d], **Mark W. Lundy**[d], **Keivan Sadrerafi**[d], **Charles E. McKenna**[j,**], **Ichiro Nishimura**[k,**]

[a]Weintraub Center for Reconstructive Biotechnology, UCLA School of Dentistry, Los Angeles, CA 90095, USA

[b]Regenerative Bioengineering and Repair Laboratory, Division of Plastic and Reconstructive Surgery, Department of Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA

[c]Department of Periodontology, Asahi University School of Dentistry, Gifu 501-0296, Japan

[d]BioVinc, LLC, Pasadena, CA 91107, USA

[e]Department of Oral Rehabilitation, Section of Implantology, Fukuoka Dental College, Fukuoka 814-0175, Japan

[f]Department of Dental Implant Centre, Beijing Stomatological Hospital, School of Stomatology, Capital Medical University, Beijing 10050, China

[g]Department of Oral Implantology, Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences, Stomatological Hospital of Chongqing Medical University, Chongqing Medical University, Chongqing 400015, China

[h]Department of Oral and Maxillofacial Implantology, Tokyo Dental College, Tokyo 101-0061, Japan

[i]Division of Molecular and Regenerative Prosthodontics, Tohoku University Graduate School of Dentistry, Sendai 980-8575, Japan

[*] Correspondence to: S. Sun, BioVinc LLC, Pasadena, CA 91107, USA . [**] Corresponding authors. shuting.sun@biovinc.com (S. Sun), mckenna@usc.edu (C.E. McKenna), inishimura@dentistry.ucla.edu (I. Nishimura). .

[1]These authors contributed equally.

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.

[j]Department of Chemistry, Dana and David Dornsife College of Letters, Arts and Sciences, University of Southern California, Los Angeles, CA 90089, USA

[k]Weintraub Center for Reconstructive Biotechnology, UCLA School of Dentistry, Box 951668 CHS B3-087, Los Angeles, CA 90095, USA

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 boney 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 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.

Keywords

Bisphosphonates; Osteonecrosis of the jaw; BRONJ; MRONJ; Drug displacement; Prophylaxis

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 BPONJ 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 ^{1}H and ^{31}P 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-alpha medium (MEM-alpha), 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 unprenylated 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 μg/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 μg/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% CO_2 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 μm–100 μm 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 μg/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 μM FAM-ZOL through the retro-orbital venous plexus. Six d later, mice received an intra-oral injection of 2-μl of 5 μM 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 μg/kg ZOL through the retro-orbital venous plexus. After 6 d, a group of mice received 2-μl of 250 μM 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 μM 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 μM 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 μMAF647-ZOL in PBS vehicle solution were IV injected into B6 mice followed by sub-periostial cranial *in situ* injection of 5 0 μM FAM-ZOL or 50 μM 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 μM 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 unprenylated 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 μM 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 μM in 100 μl 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 differentiation medium (MEM-alpha, 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). 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 μg/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, unprenylated Rap1A was detected by Western blotting after 24 h of ZOL treatment, whereas unprenylated 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 μg/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; however, mice receiving an intraoral injection of MHDP and ETI showed relatively normal oral wound healing (Fig. 5B). The area of oral mucosa swelling was significantly reduced in the MDHP and ETI intra-oral injection groups (Fig. 5C). In some sections of the ZOL-treated control group, cytokeratin 14 positive epithelium reached the necrotic bone surface and appeared to form fistulas. The lpBP intra-oral injection groups did not manifest abnormal oral epithelial hyperplasia (Fig. 5D and S3).

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-prenylation 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× 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 contra-indicated 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 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 subsituent 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 flurorescence 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 anti-microbial 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-absorbed 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH/NIDCR grants 1R43DE025524, 2R44DE025524, R01DE022552, R21DE023410, by NIH/NCRR grant C06RR014529 and by the USC College of Letters, Arts and Sciences. We thank the UCLA Translational Pathology Core Laboratory (TPCL) for histopathological specimen preparation. We also thank Dr. Matthew J. Schibler, UCLA CNSI for the technical support for confocal microscopy.

References

[1]. Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, Gagel RF, Gilsanz V, Guise T, Koka S, McCauley LK, McGowan J, McKee MD, Mohla S, Pendrys DG, Raisz LG, Ruggiero SL, Shafer DM, Shum L, Silverman SL, Van Poznak CH, Watts N, Woo SB, Shane E, B. American Society for, R. Mineral, Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research, J. Bone Miner. Res. 22 (10) (2007) 1479–1491. [PubMed: 17663640]

[2]. Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, O'Ryan F, O. American Association of, S. Maxillofacial, American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw–2014 update, J. Oral Maxillofac. Surg. 72 (10) (2014) 1938–1956. [PubMed: 25234529]

[3]. Khan AA, Morrison A, Hanley DA, Felsenberg D, McCauley LK, O'Ryan F, Reid IR, Ruggiero SL, Taguchi A, Tetradis S, Watts NB, Brandi ML, Peters E, Guise T, Eastell R, Cheung AM, Morin SN, Masri B, Cooper C, Morgan SL, Obermayer-Pietsch B, Langdahl BL, Al Dabagh R,

Davison KS, Kendler DL, Sandor GK, Josse RG, Bhandari M, El Rabbany M, Pierroz DD, Sulimani R, Saunders DP, Brown JP, Compston J, J. International Task Force on Osteonecrosis of the, Diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus, J. Bone Miner. Res. 30 (1) (2015) 3–23. [PubMed: 25414052]

[4]. McGowan K, McGowan T, Ivanovski S, Risk factors for medication-related osteonecrosis of the jaws: a systematic review, Oral Dis. 24 (4) (2018) 527–536. [PubMed: 28656643]

[5]. Jha S, Wang Z, Laucis N, Bhattacharyya T, Trends in media reports, oral bisphosphonate prescriptions, and hip fractures 1996–2012: an ecological analysis, J. Bone Miner. Res. 30 (12) (2015) 2179–2187. [PubMed: 26018247]

[6]. Tennis P, Rothman KJ, Bohn RL, Tan H, Zavras A, Laskarides C, Calingaert B, Anthony MS, Incidence of osteonecrosis of the jaw among users of bisphosphonates with selected cancers or osteoporosis, Pharmacoepidemiol. Drug Saf. 21 (8) (2012) 810–817. [PubMed: 22711458]

[7]. Lin TC, Yang CY, Yang Y.H. Kao, Lin SJ, Incidence and risk of osteonecrosis of the jaw among the Taiwan osteoporosis population, Osteoporos. Int. 25 (5) (2014) 1503–1511. [PubMed: 24515577]

[8]. Shibahara T, Morikawa T, Yago K, Kishimoto H, Imai Y, Kurita K, National survey on bisphosphonate-related osteonecrosis of the jaws in Japan, J. Oral Maxillofac. Surg. 76 (10) (2018) 2105–2112. [PubMed: 29746838]

[9]. Fedele S, Porter SR, D'Aiuto F, Aljohani S, Vescovi P, Manfredi M, Arduino PG, Broccoletti R, Musciotto A, Di Fede O, Lazarovici TS, Campisi G, Yarom N, Nonexposed variant of bisphosphonate-associated osteonecrosis of the jaw: a case series, Am. J. Med. 123 (11) (2010) 1060–1064. [PubMed: 20851366]

[10]. Hutchinson M, O'Ryan F, Chavez V, Lathon PV, Sanchez G, Hatcher DC, Indresano AT, Lo JC, Radiographic findings in bisphosphonate-treated patients with stage 0 disease in the absence of bone exposure, J. Oral Maxillofac. Surg. 68 (9) (2010) 2232–2240. [PubMed: 20728032]

[11]. Taguchi A, Uemura Y, Imai T, Tanaka S, Ohta H, Nakamura T, Orimo H, Sugimoto T, Soen S, Shiraki M, g. Adequate Treatment of Osteoporosis research, Incidence of osteonecrosis of the jaw in Japanese osteoporosis patients taking minodronic acid, J. Bone Miner. Metab. (2019), 10.1007/s00774-019-00990-5.

[12]. Schiodt M, Reibel J, Oturai P, Kofod T, Comparison of nonexposed and exposed bisphosphonate-induced osteonecrosis of the jaws: a retrospective analysis from the Copenhagen cohort and a proposal for an updated classification system, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 117 (2) (2014) 204–213. [PubMed: 24332520]

[13]. Topaloglu G, Koseoglu OT, Karaca C, Kosemehmetoglu K, The effect of chronic dental inflammation on development of stage 0 medication-related osteonecrosis of the jaw, J. Craniomaxillofac. Surg. 45 (8) (2017) 1158–1164. [PubMed: 28601298]

[14]. Aghaloo TL, Dry SM, Mallya S, Tetradis S, Stage 0 osteonecrosis of the jaw in a patient on denosumab, J. Oral Maxillofac. Surg. 72 (4) (2014) 702–716. [PubMed: 24397946]

[15]. Devlin H, Greenwall-Cohen J, Benton J, Goodwin TL, Littlewood A, Horner K, Detecting the earliest radiological signs of bisphosphonate-related osteonecrosis, Br. Dent. J. 224 (1) (2018) 26–31. [PubMed: 29192692]

[16]. Aghaloo T, Hazboun R, Tetradis S, Pathophysiology of osteonecrosis of the jaws, Oral Maxillofac. Surg. Clin. North Am. 27 (4) (2015) 489–496. [PubMed: 26412796]

[17]. Landesberg R, Woo V, Cremers S, Cozin M, Marolt D, Vunjak-Novakovic G, Kousteni S, Raghavan S, Potential pathophysiological mechanisms in osteonecrosis of the jaw, Ann. N. Y. Acad. Sci. 1218 (2011) 62–79. [PubMed: 21291478]

[18]. Allen MR, The effects of bisphosphonates on jaw bone remodeling, tissue properties, and extraction healing, Odontology 99 (1) (2011) 8–17. [PubMed: 21271320]

[19]. Hokugo A, Christensen R, Chung EM, Sung EC, Felsenfeld AL, Sayre JW, Garrett N, Adams JS, Nishimura I, Increased prevalence of bisphosphonate-related osteonecrosis of the jaw with vitamin D deficiency in rats, J. Bone Miner. Res. 25 (6) (2010) 1337–1349. [PubMed: 20200938]

[20]. Hansen T, Kunkel M, Springer E, Walter C, Weber A, Siegel E, Kirkpatrick CJ, Actinomycosis of the jaws–histopathological study of 45 patients shows significant involvement in bisphosphonate-

associated osteonecrosis and infected osteor-adionecrosis, Virchows Arch. 451 (6) (2007) 1009–1017. [PubMed: 17952459]

[21]. Russmueller G, Seemann R, Weiss K, Stadler V, Speiss M, Perisanidis C, Fuereder T, Willinger B, Sulzbacher I, Steininger C, The association of medication-related osteonecrosis of the jaw with Actinomyces spp. infection, Sci. Rep. 6 (2016) 31604. [PubMed: 27530150]

[22]. Nicolatou-Galitis O, Razis E, Galiti D, Galitis E, Labropoulos S, Tsimpidakis A, Sgouros J, Karampeazis A, Migliorati C, Periodontal disease preceding osteonecrosis of the jaw (ONJ) in cancer patients receiving antiresorptives alone or combined with targeted therapies: report of 5 cases and literature review, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 120 (6) (2015) 699–706. [PubMed: 26455289]

[23]. Aghaloo TL, Kang B, Sung EC, Shoff M, Ronconi M, Gotcher JE, Bezouglaia O, Dry SM, Tetradis S, Periodontal disease and bisphosphonates induce osteonecrosis of the jaws in the rat, J. Bone Miner. Res. 26 (8) (2011) 1871–1882. [PubMed: 21351151]

[24]. Aguirre JI, Akhter MP, Kimmel DB, Pingel JE, Williams A, Jorgensen M, Kesavalu L, Wronski TJ, Oncologic doses of zoledronic acid induce osteonecrosis of the jaw-like lesions in rice rats (Oryzomys palustris) with periodontitis, J. Bone Miner. Res. 27 (10) (2012) 2130–2143. [PubMed: 22623376]

[25]. Aftimos V, Zeinoun T, Tayeh R. Bou, Aftimos G, Bisphosphonate related osteo-necrosis of the jaw: a study of 18 cases associated with fungal infection, Int. J. Dent. 2014 (2014) 869067.

[26]. Sun Y, Kaur K, Kanayama K, Morinaga K, Park S, Hokugo A, Kozlowska A, McBride WH, Li J, Jewett A, Nishimura I, Plasticity of myeloid cells during oral barrier wound healing and the development of bisphosphonate-related osteone-crosis of the jaw, J. Biol. Chem. 291 (39) (2016) 20602–20616. [PubMed: 27514746]

[27]. van Beek ER, Lowik CW, Ebetino FH, Papapoulos SE, Binding and anti-resorptive properties of heterocycle-containing bisphosphonate analogs: structure-activity relationships, Bone 23 (5) (1998) 437–442. [PubMed: 9823450]

[28]. Diab DL, Watts NB, Bisphosphonate drug holiday: who, when and how long, Ther. Adv. Musculoskelet. Dis. 5 (3) (2013) 107–111. [PubMed: 23858334]

[29]. Bauss F, Lalla S, Endele R, Hothorn LA, Effects of treatment with ibandronate on bone mass, architecture, biomechanical properties, and bone concentration of ibandronate in ovariectomized aged rats, J. Rheumatol. 29 (10) (2002) 2200–2208. [PubMed: 12375334]

[30]. Hokugo A, Sun S, Park S, McKenna CE, Nishimura I, Equilibrium-dependent bisphosphonate interaction with crystalline bone mineral explains anti-resorptive pharmacokinetics and prevalence of osteonecrosis of the jaw in rats, Bone 53 (1) (2013) 59–68. [PubMed: 23219943]

[31]. Sun S, Blazewska KM, Kadina AP, Kashemirov BA, Duan X, Triffitt JT, Dunford JE, Russell RG, Ebetino FH, Roelofs AJ, Coxon FP, Lundy MW, McKenna CE, Fluorescent bisphosphonate and carboxyphosphonate probes: a versatile imaging toolkit for applications in bone biology and biomedicine, Bioconjug. Chem. 27 (2) (2016) 329–340. [PubMed: 26646666]

[32]. Park S, Kanayama K, Kaur K, Tseng HC, Banankhah S, Quje DT, Sayre JW, Jewett A, Nishimura I, Osteonecrosis of the jaw developed in mice: disease variants regulated By gammadelta T cells in oral mucosal barrier immunity, J. Biol. Chem. 290 (28) (2015) 17349–17366. [PubMed: 26013832]

[33]. Tseng HC, Kanayama K, Kaur K, Park SH, Park S, Kozlowska A, Sun S, McKenna CE, Nishimura I, Jewett A, Bisphosphonate-induced differential modulation of immune cell function in gingiva and bone marrow in vivo: role in osteoclast-mediated NK cell activation, Oncotarget 6 (24) (2015) 20002–20025. [PubMed: 26343372]

[34]. Kawamoto T, Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants, Arch. Histol. Cytol. 66 (2) (2003) 123–143. [PubMed: 12846553]

[35]. Coxon FP, Helfrich MH, Larijani B, Muzylak M, Dunford JE, Marshall D, McKinnon AD, Nesbitt SA, Horton MA, Seabra MC, Ebetino FH, Rogers MJ, Identification of a novel phosphonocarboxylate inhibitor of Rab geranylgeranyl transferase that specifically prevents Rab prenylation in osteoclasts and macrophages, J. Biol. Chem. 276 (51) (2001) 48213–48222. [PubMed: 11581260]

[36]. Neto N. Conte, Spolidorio LC, Andrade CR, A SB, Guimaraes M, Marcantonio E Jr., Experimental development of bisphosphonate-related osteonecrosis of the jaws in rodents, Int. J. Exp. Pathol. 94(1) (2013) 65–73. [PubMed: 23317355]

[37]. Zandi M, Dehghan A, Janbaz P, Malekzadeh H, Amini P, The starting point for bisphosphonate-related osteonecrosis of the jaw: alveolar bone or oral mucosa? A randomized, controlled experimental study, J. Craniomaxillofac. Surg. 45 (1) (2017) 157–161. [PubMed: 27919595]

[38]. Kim T, Kim S, Song M, Lee C, Yagita H, Williams DW, Sung EC, Hong C, Shin KH, Kang MK, Park NH, Kim RH, Removal of pre-existing periodontal inflammatory condition before tooth extraction ameliorates medication-related osteonecrosis of the jaw-like lesion in mice, Am. J. Pathol. 188 (10) (2018) 2318–2327. [PubMed: 30059656]

[39]. Hadaya D, Soundia A, Gkouveris I, Dry SM, Aghaloo TL, Tetradis S, Development of medication-related osteonecrosis of the jaw after extraction of teeth with experimental periapical disease, J. Oral Maxillofac. Surg. 77 (1) (2019) 71–86. [PubMed: 30218655]

[40]. Soundia A, Hadaya D, Mallya SM, Aghaloo TL, Tetradis S, Radiographic predictors of bone exposure in patients with stage 0 medication-related osteonecrosis of the jaws, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 126 (6) (2018) 537–544. [PubMed: 30266375]

[41]. Kang B, Cheong S, Chaichanasakul T, Bezouglaia O, Atti E, Dry SM, Pirih FQ, Aghaloo TL, Tetradis S, Periapical disease and bisphosphonates induce osteonecrosis of the jaws in mice, J. Bone Miner. Res. 28 (7) (2013) 1631–1640. [PubMed: 23426919]

[42]. Zustin J, Reske D, Zrnc TA, Heiland M, Scheuer HA, Assaf AT, Friedrich RE, Pseudoepitheliomatous hyperplasia associated with bisphosphonate-related osteonecrosis of the jaw, In Vivo 28 (1) (2014) 125–131. [PubMed: 24425847]

[43]. Hutcheson A, Cheng A, Kunchar R, Stein B, Sambrook P, Goss A, A C-terminal crosslinking telopeptide test-based protocol for patients on oral bisphosphonates requiring extraction: a prospective single-center controlled study, J. Oral Maxillofac. Surg. 72 (8) (2014) 1456–1462. [PubMed: 24793621]

[44]. Russell RG, Watts NB, Ebetino FH, Rogers MJ, Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy, Osteoporos. Int. 19 (6) (2008) 733–759. [PubMed: 18214569]

[45]. El-Rabbany M, Sgro A, Lam DK, Shah PS, Azarpazhooh A, Effectiveness of treatments for medication-related osteonecrosis of the jaw: a systematic review and meta-analysis, J. Am. Dent. Assoc. 148(8) (2017) (584–594 e2). [PubMed: 28527518]

[46]. de Molon RS, Shimamoto H, Bezouglaia O, Pirih FQ, Dry SM, Kostenuik P, Boyce RW, Dwyer D, Aghaloo TL, Tetradis S, OPG-fc but not zoledronic acid discontinuation reverses osteonecrosis of the jaws (ONJ) in mice, J. Bone Miner. Res. 30 (9) (2015) 1627–1640. [PubMed: 25727550]

[47]. Ebetino FH, Sun S, Hokugo A, Lundy MW, Mckenna CE, Nishimura I, Skeletal Removal of Bisphosphonates, (2017) (PCT/US2017/035169).

[48]. Yamaguchi K, Oizumi T, Funayama H, Kawamura H, Sugawara S, Endo Y, Osteonecrosis of the jawbones in 2 osteoporosis patients treated with nitrogen-containing bisphosphonates: osteonecrosis reduction replacing NBP with non-NBP (etidronate) and rationale, J. Oral Maxillofac. Surg. 68 (4) (2010) 889–897. [PubMed: 20056305]

[49]. Oizumi T, Yamaguchi K, Sato K, Takahashi M, Yoshimura G, Otsuru H, Tsuchiya M, Hagiwara Y, Itoi E, Sugawara S, Takahashi T, Endo Y, A strategy against the osteonecrosis of the jaw associated with nitrogen-containing bisphosphonates (N-BPs): attempts to replace N-BPs with the non-N-BP etidronate, Biol. Pharm. Bull. 39 (9) (2016) 1549–1554. [PubMed: 27582334]

[50]. Ebetino FH, Hogan AM, Sun S, Tsoumpra MK, Duan X, Triffitt JT, Kwaasi AA, Dunford JE, Barnett BL, Oppermann U, Lundy MW, Boyde A, Kashemirov BA, McKenna CE, Russell RG, The relationship between the chemistry and biological activity of the bisphosphonates, Bone 49 (1) (2011) 20–33. [PubMed: 21497677]

[51]. Kashemirov BA, Bala JL, Chen X, Ebetino FH, Xia Z, Russell RG, Coxon FP, Roelofs AJ, Rogers MJ, McKenna CE, Fluorescently labeled risedronate and related analogues: “magic linker” synthesis, Bioconjug. Chem. 19 (12) (2008) 2308–2310. [PubMed: 19032080]

[52]. Duan X, Xia Z, Zhang H, Quijano M, Dobson RLM, Triffit JT, The effects of pH on the relative bone mineral-binding affinities of bisphosphonates determined by hydroxyapatite-column chromatography, J. Bone Miner. Res. 25 (2010) S347 Supple 1).

[53]. Francis MD, Ferguson DL, Tofe AJ, Bevan JA, Michaels SE, Comparative evaluation of three diphosphonates: in vitro adsorption (C-14 labeled) and in vivo osteogenic uptake (Tc-99m complexed), J. Nucl. Med. 21 (12) (1980) 1185–1189. [PubMed: 6449567]

[54]. Elsayed R, Abrahama P, Awad ME, Kurago Z, Baladhandayuthamc B, Whitford GM, Pashley DH, McKenna CE, Elsalanty ME, Removal of matrix-bound zoledronate prevents post-extraction osteonecrosis of the jaw by rescuing osteoclast function, Bone 110 (2018) 141–149. [PubMed: 29408511]

[55]. Mark DB, Anstrom KJ, Clapp-Channing NE, Knight JD, Boineau R, Goertz C, Rozema TC, Liu DM, Nahin RL, Rosenberg Y, Drisko J, Lee KL, Lamas GA, Quality-of-life outcomes with a disodium EDTA chelation regimen for coronary disease, Circ. Cardiovasc. Qual. Outcomes 7 (2014) 508–516. [PubMed: 24987051]

[56]. Pratt G, Goodyear O, Moss P, Immunodeficiency and immunotherapy in multiple myeloma, Br. J. Haematol. 138 (5) (2007) 563–579. [PubMed: 17686051]

[57]. Kalyan S, Wang J, Quabius ES, Huck J, Wiltfang J, Baines JF, Kabelitz D, Systemic immunity shapes the oral microbiome and susceptibility to bisphosphonate-associated osteonecrosis of the jaw, J. Transl. Med. 13 (2015) 212. [PubMed: 26141514]

[58]. Vandone AM, Donadio M, Mozzati M, Ardine M, Polimeni MA, Beatrice S, Ciuffreda L, Scoletta M, Impact of dental care in the prevention of bisphosphonate-associated osteonecrosis of the jaw: a single-center clinical experience, Ann. Oncol. 23 (1) (2012) 193–200. [PubMed: 21427065]

[59]. Montefusco V, Gay F, Spina F, Miceli R, Maniezzo M, Ambrosini M. Teresa, Farina L, Piva S, Palumbo A, Boccadoro M, Corradini P, Antibiotic prophylaxis before dental procedures may reduce the incidence of osteonecrosis of the jaw in patients with multiple myeloma treated with bisphosphonates, Leuk. Lymphoma 49 (11) (2008) 2156–2162. [PubMed: 19021059]

[60]. Ferlito S, Puzzo S, Liardo C, Preventive protocol for tooth extractions in patients treated with zoledronate: a case series, J. Oral Maxillofac. Surg. 69 (6) (2011) e1–e4. [PubMed: 21316136]

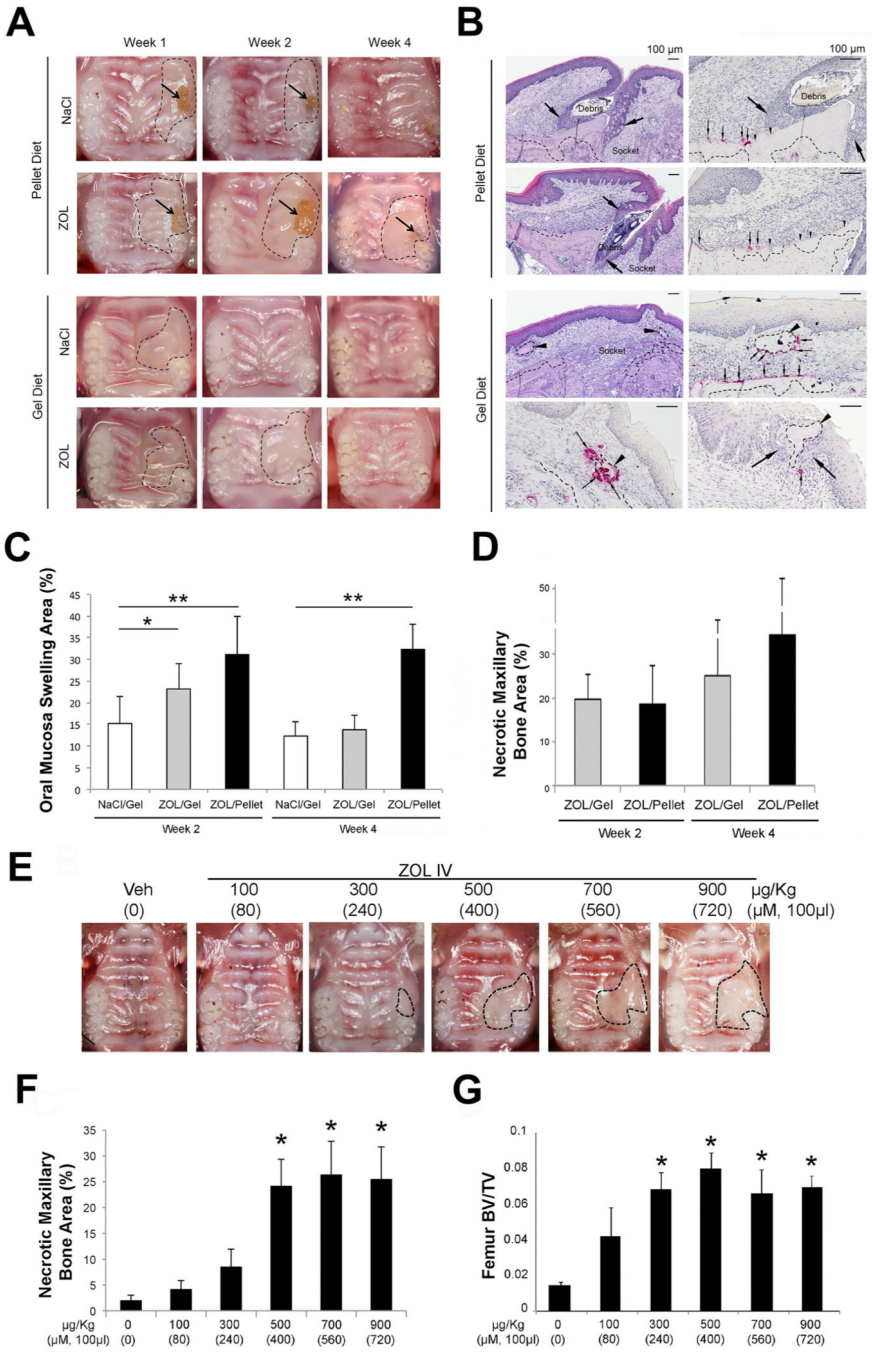

Fig. 1.

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.)

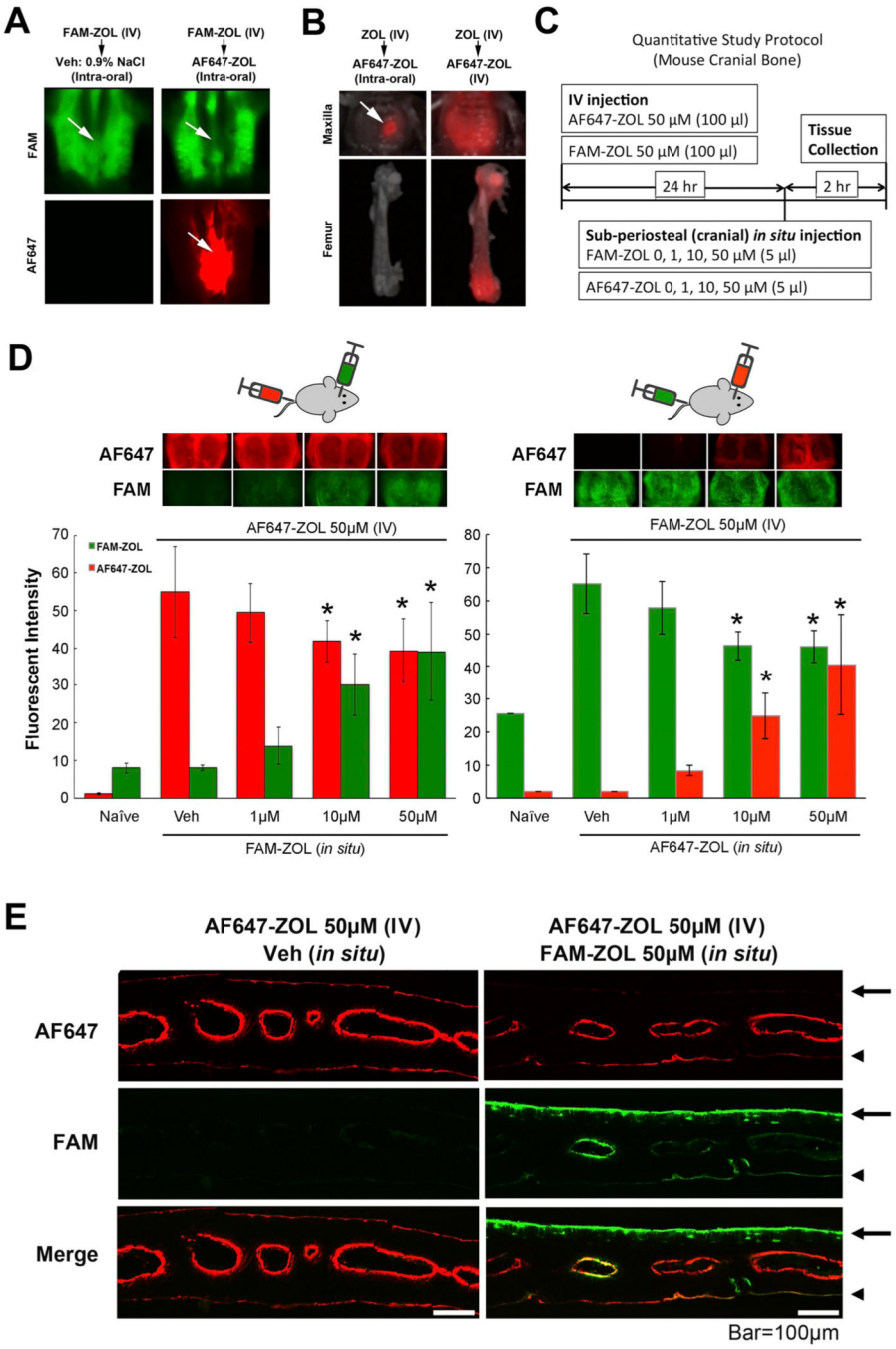

Fig. 2.

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 administrated 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.

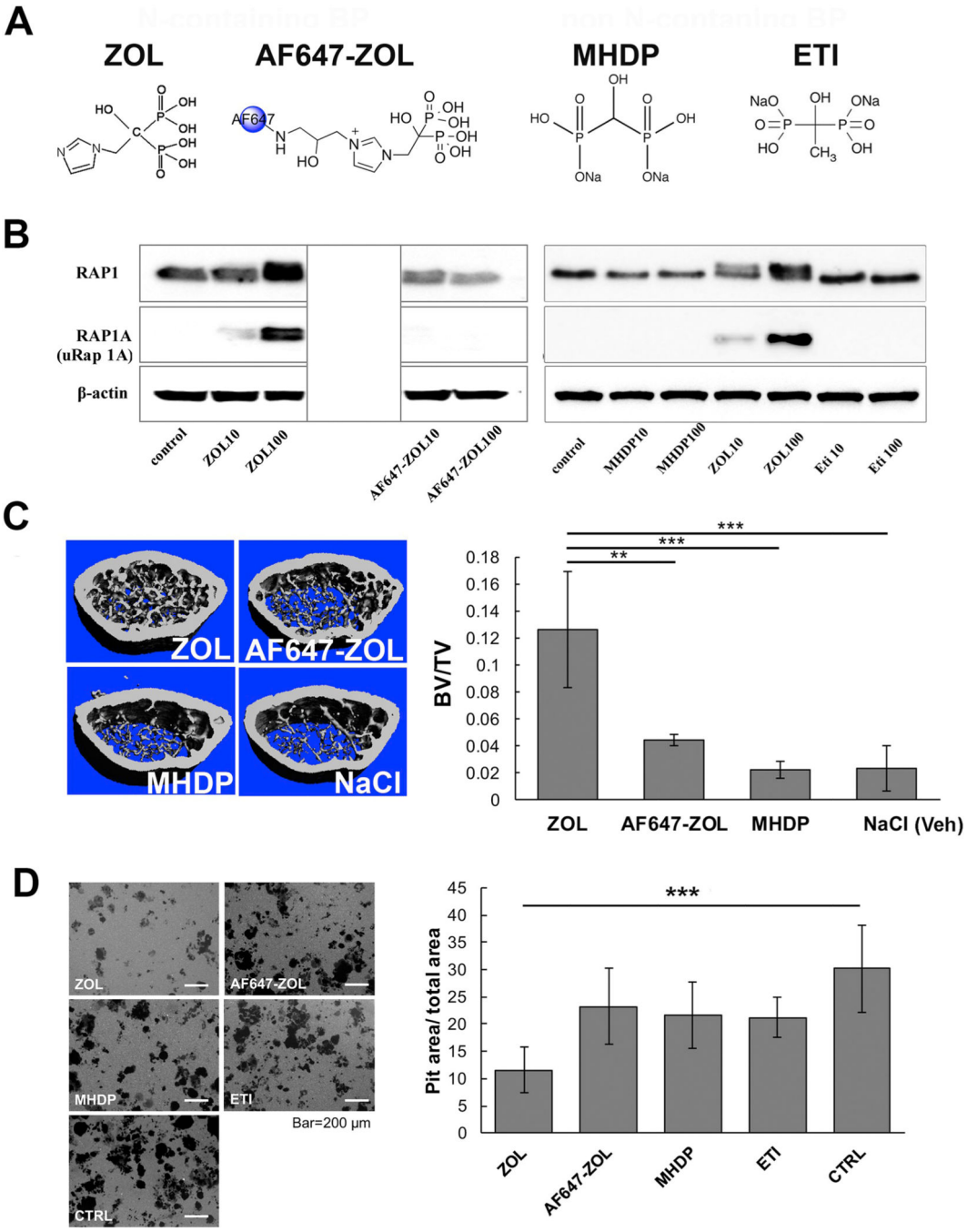

Fig. 3.

Characterization of low potency BP (lpBP). A: Chemical structure of ZOL, AF647-ZOL, MHDP and ETI. B: Western blot assays for unprenylated Rap1A (uRap1A) of J774.2 macrophages treated. ß-actin served as loading control. C: MicroCT images and relative bone volume (BV/TV) of mouse femur trabecular structure demonstrated the lack of anti-resorptive activity of AF647-ZOL and MHDP (injection of 100 µl of 400 µM of each compound). ** $P \leq .01$; *** $P \leq .001$ *vs* NaCl (Veh). D: Pit formation assay with osteoclasts differentiated from RAW264.7 cells cultured on synthetic CaP plate with medium

supplement of 10 μM of ZOL, AF647-ZOL, MHDP or ETI. Data are expressed as the mean ± SD (n = 6 per group). *** P ≤ .001 *vs* CTRL.

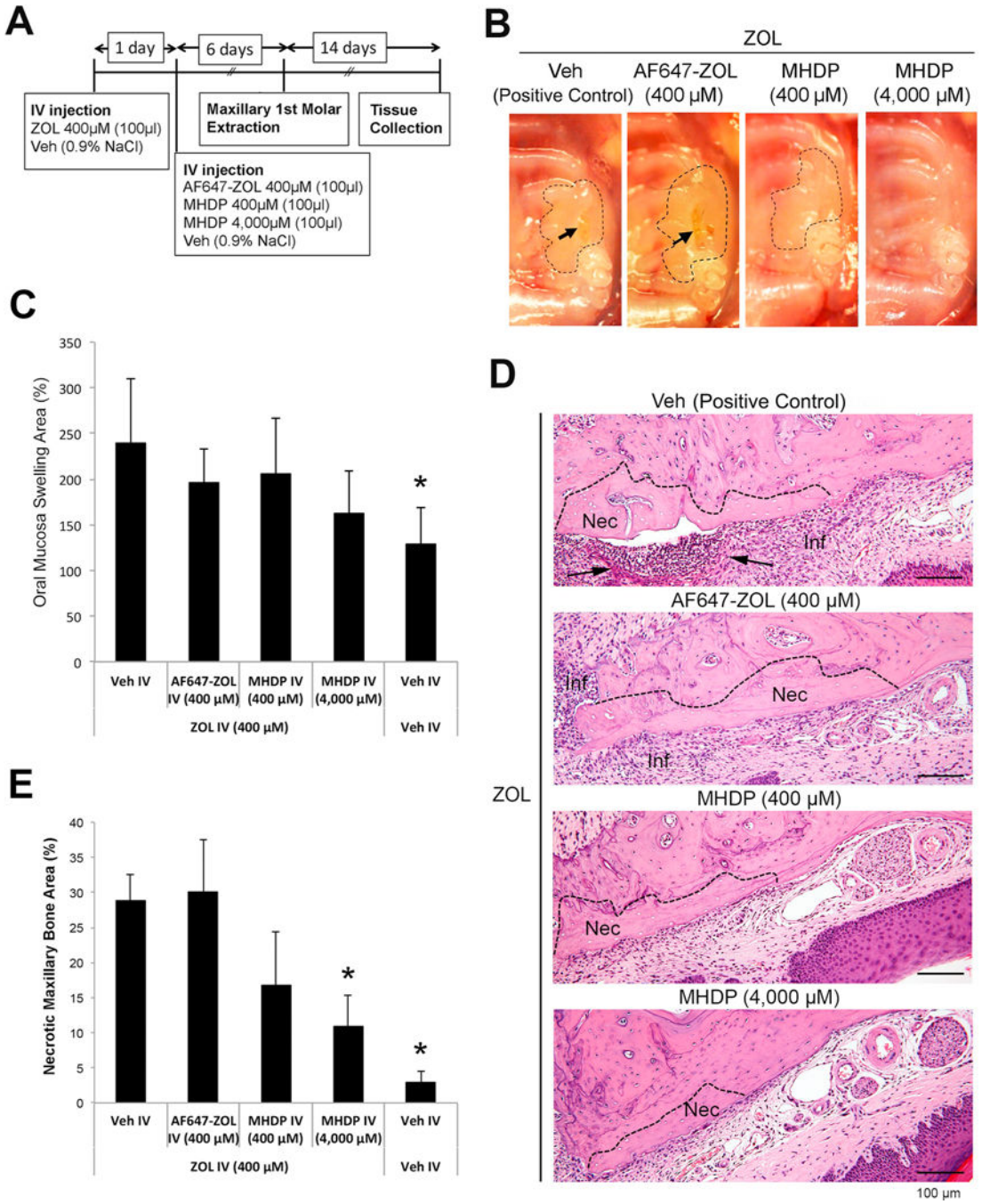

Fig. 4.

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 ± 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).

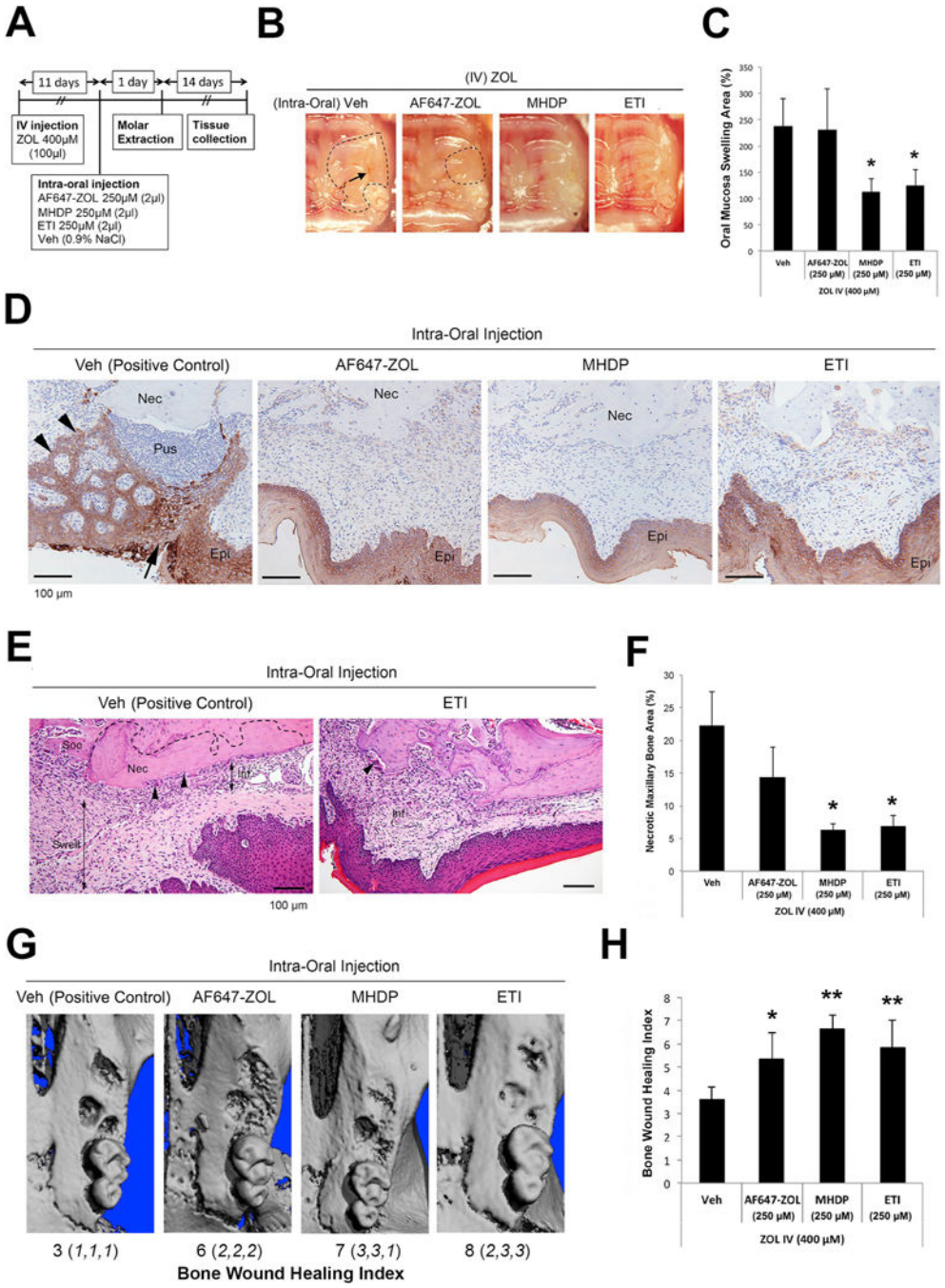

Fig. 5.

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 ± SEM (n = 6, 4, 4, and 5 for ZOL-pretreated mice with vehicle, AF647-ZOL, MHDP or ETI intra-oral injection). *, $p < .05$; **, $p < .01$.