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Release of Nitrogen-Containing Bisphosphonates (NBPs) from Hydroxyapatite by Non-NBPs and by Pyrophosphate

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Bisphosphonates (BPs) are major anti-bone-resorptive drugs. Among them, the nitrogen-containing BPs (NBPs) exhibit much stronger anti-bone-resorptive activities than non-nitrogen-containing BPs (non-NBPs). However, BP-related osteonecrosis of the jaw (BRONJ) has been increasing without effective strategies for its prevention or treatment. The release of NBPs (but not non-NBPs) from NBP-accumulated jawbones has been supposed to cause BRONJ, even though non-NBPs (such as etidronate (Eti) and clodronate (Clo)) are given at very high doses because of their low anti-bone-resorptive activities. Our murine experiments have demonstrated that NBPs cause inflammation/necrosis at the injection site, and that Eti and Clo can reduce or prevent the inflammatory/necrotic effects of NBPs by inhibiting their entry into soft-tissue cells. In addition, our preliminary clinical studies suggest that Eti may be useful for treating BRONJ. Notably, Eti, when administered together with an NBP, reduces the latter's anti-bone-resorptive effect. Here, on the basis of the above background, we examined and compared *in vitro* interactions of NBPs, non-NBPs, and related substances with hydroxyapatite (HA), and obtained the following results. (i) NBPs bind rapidly to HA under pH-neutral conditions. (ii) At high concentrations, Eti and Clo inhibit NBP-binding to HA and rapidly expel HA-bound NBPs (potency Eti >> Clo). (iii) Pyrophosphate also inhibits NBP-binding to HA and expels HA-bound NBPs. Based on these results and those reported previously, we discuss (i) possible anti-BRONJ strategies involving the use of Eti and/or Clo to reduce jawbone-accumulated NBPs, and (ii) a possible involvement of pyrophosphate-mediated release of NBPs as a cause of BRONJ.

Key words bisphosphonate; hydroxyapatite; etidronate; clodronate; pyrophosphate; osteonecrosis

INTRODUCTION

Bisphosphonates (BPs) are important anti-bone-resorptive drugs.^{1–3} BPs bind strongly to bone, especially to bones exhibiting rapid turnover or inflammation. Due to their high affinity for the hydroxyapatite (HA) of bones, BPs accumulate within bones upon repeated administration. Among the BPs, the nitrogen-containing BPs (NBPs) have anti-bone-resorptive activities that are far stronger than those of the non-nitrogen-containing BPs (non-NBPs)^{2,4} (Supplementary Fig. 1). However, NBPs are known to have inflammatory/necrotic side effects after intravenous administration, as well as various gastrointestinal side effects after oral administration. In particular, since the first reports in 2003,^{5,6} NBP-related osteonecrosis of the jaw (BRONJ) has been increasing without effective clinical strategies.⁷ In Japan, 263 BRONJ patients were reported in the period 2006–2008, but as many as 4797 during 2011–2013.⁸

Only two non-NBPs (etidronate (Eti) and clodronate (Clo)) are currently used for osteoporosis. To compensate for their weak anti-bone-resorptive activities, they are given to patients at doses much higher than those of NBPs.^{1,9} Unlike NBPs, Eti and Clo have no severe inflammatory/necrotic side effects. It is notable that Clo is a BP that can be given intramuscularly as well as orally and intravenously.¹⁰

A single injection of a BP into young mice produces a clear sclerotic band in their tibias (we call this the “BP-band”) that is detectable by radiography a few weeks after the injection, reflecting an inhibition of bone resorption. By examining the BP-band, we previously obtained the following results. Eti, when administered to mice together with an NBP reduces the anti-bone-resorptive effect of the NBP,¹¹ while such an effect of Clo, if any, is minimal.¹² Eti, when administered *after* an NBP, also reduces the anti-bone-resorptive effect of the NBP.¹³ Before obtaining those findings, we had reported that Clo can inhibit the inflammatory/necrotic effects of NBPs in mice.¹⁴ Our later pharmacological studies indicated that Eti and Clo inhibit the entry of NBPs into soft-tissue cells *via* inhibition of phosphate transporters.^{15–17} Taken together, these results suggest that (i) Eti inhibits the binding of an NBP when the two are co-administered, (ii) Eti can expel NBPs that have already accumulated within bones, and (c) Eti may inhibit the entry of the expelled NBPs into soft-tissue cells around the bones, thus preventing the inflammatory/necrotic effects of the expelled NBPs and reducing the anti-bone-resorptive activities of NBPs. Our clinical trials to examine the use of Eti as a substitution drug for NBPs suggest that Eti may be useful for reducing the signs of BRONJ.^{18,19} Here, based on the background described above, we examined and compared the *in vitro* effects of Eti and Clo on the binding

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Fig. 1. NBP-Binding to HA

(a) NBP-binding capacity of HA. An indicated amount of HA and 4 mM of a given NBP (100 μ L) were mixed by pipetting, and the mixture was left standing for 60 min and then centrifuged. Then, the NBP in the resultant supernatant was measured. (b) NBP-binding velocity to HA. HA 10 mg and 4 mM of a given N-BP (100 μ L) were mixed by pipetting, and the mixture was left standing for the indicated time before being centrifuged. Finally, the NBP in the resultant supernatant was measured. Each value is the mean $\pm$ S.D. from 4 tubes. * p < 0.05 vs. indicated group.

of NBPs to HA and on the release of HA-bound NBPs. We focused particularly on the latter because the NBPs released from NBP-accumulated jawbones may play critical roles in the development of BRONJ.

MATERIALS AND METHODS

Reagents Minodronate- H_2O (Min) and pamidronate- $2\text{Na}\cdot\text{anhydrous}$ (Pam) were from LKT Laboratories, Inc. (St. Paul, MN, U.S.A.). 2,4,6-Trinitrobenzene sulfonic acid hydrate (TNBS), alendronate- $\text{Na}\cdot 3\text{H}_2\text{O}$ (Ale), zoledronate- $2\text{Na}\cdot 4\text{H}_2\text{O}$ (Zol), risedronate- $\text{Na}\cdot 2.5\text{H}_2\text{O}$ (Ris), and etidronate- $2\text{Na}\cdot\text{anhydrous}$ (Eti) were purchased from Tokyo Kasei (Tokyo, Japan). Phosphate- $\text{Na}\cdot 2\text{H}_2\text{O}$ (Pi), dimethylsulfoxide (DMSO), $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, and $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ were from Nacalai Tesque (Kyoto, Japan). Clodronate- $2\text{Na}\cdot\text{anhydrous}$ (Clo) and hydroxyapatite (HA) (#677418: particle size ≤ 200 nm, specific surface area $\geq 9.4\text{ m}^2/\text{g}$) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). Pyrophosphate- $4\text{Na}\cdot\text{anhydrous}$ (PPi) and phosphonoformic acid (PFA) were from Alfa Aesar (Tewksbury, MA, U.S.A.). Phosphonoacetic acid (PAA) was from Wako (Osaka, Japan).

Preparation of BP Solutions BPs were dissolved in distilled water. Unless otherwise indicated, solutions were adjusted to pH 7 with NaOH solution. In some experiments, solutions were adjusted to the indicated pH values with NaOH

or HCl solutions. Then, the concentration of the BP solutions was adjusted to that required with distilled water.

Preparation of HA HA was suspended in distilled water, with the pH of the suspension being adjusted to 7 with NaOH solution or, in some experiments, adjusted to the indicated pH values with NaOH or HCl solutions. Then, the concentration of the HA suspensions was adjusted to 100 mg/mL with distilled water and used as indicated. The required amount of the HA suspension (100 μ L; *i.e.*, 10 mg HA, in most experiments) was transferred to a test tube. Then, the tube was centrifuged at $10000\times g$ for 5 min, and the supernatant was removed. The resultant precipitate was used as HA.

Binding of NBPs to HA To a tube containing HA, 100 μ L solution containing an NBP alone or NBP plus a test material(s) was added, and the contents of the tube mixed by pipetting. Then, the tube was left standing at room temperature for approx. 60 min before being centrifuged at $10000\times g$ for 5 min. The resultant supernatant was subjected to measurement of the amount of the NBP. The amount of the NBP bound to the HA was expressed as a percentage of the NBP added.

Release of an NBP from NBP-Bound HA NBP-bound HA (abbreviated "NBP-HA") was prepared by mixing 10 mg HA and 100 μ L NBP (concentration indicated) and removal of the supernatant following centrifugation. To an NBP-HA, a solution of a test material(s) (100 μ L) was added, and the

Fig. 2. Effects of pH and the Non-NBPs Eti and Clo on NBP-Binding to HA

(a) Effects of pH on the binding of NBPs to HA. HA 10 mg and 4 mM of a given NBP (100 μ L) (pH of each having been adjusted as indicated) were mixed by pipetting, and the mixture was left standing for 60 min before being centrifuged. Finally, the NBP in the resultant supernatant was measured. (b) Effects of Eti and Clo on the binding of NBPs to HA. HA 10 mg and 100 μ L of a solution containing 4 mM of an NBP and the indicated concentration of Eti or Clo were mixed by pipetting, and the mixture was left standing for 60 min before being centrifuged. Finally, the NBP in the resultant supernatant was measured. Each value is the mean $\pm$ S.D. from 4 tubes. * p < 0.05 vs. indicated group.

contents of the tube were mixed by pipetting. Then, the tube was left standing at room temperature for approx. 60 min before being centrifuged at $10000 \times g$ for 5 min. The resultant supernatant was subjected to measurement of the amount of the NBP, and the amount of the NBP released from the NBP-HA was calculated and expressed as a percentage of the NBP bound to HA.

Measurement of Pam and Ale Pam and Ale were measured spectrophotometrically (absorbance at 450 nm) after reaction of their amino group with 2,4,6-trinitrobenzene sulfonate (TNBS).²⁰⁾ To each well of a 96-well plate, 200 μ L borate buffer (0.2 M, pH 8.5), 100 μ L TNBS solution (1 mg/mL DMSO), and 10 μ L of the solution of Pam or Ale were added. After completing the TNBS reaction for 30 min at 37 $^{\circ}$ C, the absorbance of the reaction mixture at 450 nm was measured.

Measurement of Zol, Ris, and Min Zol, Ris, and Min were measured spectrophotometrically by measuring their native UV absorbance (at 220 nm for Zol and Ris and at 290 nm for Min). However, in the presence of a large amount of Eti and/or Clo, Zol and Ris could not be measured because Eti and Clo, at high concentrations, also exhibit UV absorption at around 220 nm and therefore disturb the measurement of

Zol and Ris (e.g., the absorbances at 220 nm of 4 mM Zol, 4 mM Ris, 150 mM Eti, and 150 mM Clo are 1.75, 1.9, 0.5, and 1.5, respectively). Fortunately, Min could be measured even in the presence of a large amount of Eti and/or Clo. The UV absorbance of a given solution was measured by using a UV-star 96-well plate (Greiner, Frickenhausen, Germany) and a FlexStation 3 microplate reader (Molecular Devices, CA, U.S.A.).

Statistical Analysis Experimental values are given as the mean $\pm$ standard deviation (S.D.) from 4 tubes. Sample size was based on an α error of 0.05 and a β error of 0.2 using power analysis. The statistical significance of differences was analyzed using a Bonferroni multiple comparison test after ANOVA with the aid of InStat software (InStat, Scottsdale, AZ, U.S.A.). The results shown for a given experiment were confirmed by repeating the experiment at least once more.

RESULTS

NBP-Binding to HA Figure 1A shows that in spite of structural differences, with each NBP almost 100% of 100 μ L of a 4 mM solution of the NBP binds to about 10 mg HA.

Fig. 3. Effects of Eti and Clo on NBP-Release from NBP(4mM)-HA

In these experiments, NBP(4mM)-HA was used. (a, b) Effects of concentrations of Eti and Clo. To each NBP-HA, 100 μ L of the indicated concentration of Eti, Clo, or both was added and they were mixed by pipetting, left standing for 60 min, and the mixture was centrifuged and the resultant supernatant subjected to measurement of the N-BP. (c) NBP-releasing velocity from HA. NBP(4mM)-HA and 100mM Eti (100 μ L) were mixed by pipetting. Then, the mixture was left standing for the indicate time and centrifuged, and the resultant supernatant was subjected to measurement of the NBP. (d) Effect of repeated treatment with Eti. NBP(4mM)-HA was mixed with Eti (concentration as indicated) by pipetting without standing, and the supernatant obtained by centrifugation immediately after the mixing was subjected to measurement of the NBP. This treatment was performed three times. Each value is the mean $\pm$ S.D. from 4 tubes. * p < 0.05 vs. indicated group.

This means that (i) the 10mg HA used in the present study is almost completely saturated with 400 nmols of an NBP, and thus (ii) when 10mg HA was mixed with 100 μ L of 4mM of an NBP solution, almost no free NBP (*i.e.*, not attached to HA) is present in the mixture. Figure 1B shows that most of each NBP became bound to HA during the mixing (pipetting), although Min and Zol take a somewhat longer time than Ale and Pam for complete binding. Figure 2A shows that at pH values from 3–11, all the tested NBPs bind almost completely to HA within 60min, indicating that NBPs exhibit potent affinities for HA over a wide pH range. Figure 2B shows that the non-NBPs Eti and Clo each concentration-dependently (16–128mM) reduce NBP-binding to HA for all of the N-BPs tested, indicating that NBPs and non-NBPs bind competitively to HA. In this experiment, Zol was not tested because Eti and Clo disturb the measurement of Zol (see Materials and Methods).

Effects of Eti and Clo on NBP-Bound HA (NBP-HA) If an NBP-bound HA is *not* saturated with an NBP, then when the NBP is released from the HA, the released NBP might immediately reattach to the HA. In the following experiments, to avoid that happening, we used HA saturated with an NBP, *i.e.*, each NBP-bound HA was prepared from 10mg HA and 100 μ L of 4mM NBP (abbreviated “NBP(4mM)-HA”). Thus, the HA is nearly saturated with the relevant NBP. Figure 3A

shows that Eti and Clo concentration-dependently release each NBP. For each NBP, about 80% of the amount bound to HA was released by 300mM Eti, while the release induced by 300mM Clo was smaller. As shown in Fig. 3B, a combination of Eti and Clo (Eti + Clo), each 150mM, produced no detectable additive effect on the release of N-BPs from HA, indicating affinities for HA of Eti >> Clo. Figure 3C shows that 30–40% of each NBP is released during mixing, although the release of Min is slightly slower than those of Ale and Pam. In these experiments, the mixing was carried out 20–30 times during 15–20s until each NBP-HA in the tube was completely dispersed. Such mixing with 300mM Eti two or three times in total resulted in complete release or removal of each NBP from HA (Fig. 3D).

Effects of pH on NBP-Release by Eti As shown in Fig. 2A, the *binding* of NBPs to HA was not modified by pH under the conditions tested. However, Fig. 4A shows that the *release* of NBPs bound to HA by Eti is markedly affected by pH; *i.e.*, the release of Ale and Pam increased with the increase in pH, while the release of Min was maximal at pH 5.

NBP-Binding to Non-NBP(4mM)-HA As shown in Fig. 3A, for NBP-release from NBP-bound HA, a high concentration (approx. 300mM) of non-NBP (Eti or Clo) was required. However, Fig. 4B shows that the NBPs tested, even at 4mM, almost entirely bind to non-NBP(4mM)-bound HA, indicating

Fig. 4. Additional Properties of NBP(4mM)-HA

In these experiments, NBP(4mM)-HA was used. (a) Effects of pH on Eti-induced NBP-release from HA. HA-NBP and 50mM Eti (100 μ L), with the pH adjusted as indicated, were mixed by pipetting and left standing for 60 min, and the mixture was then centrifuged and the resultant supernatant subjected to measurement of the NBP. (b) Effects of Eti or Clo bound to HA (Eti-HA or Clo-HA) on NBP-binding to HA. HA-Eti or HA-Clo was prepared from HA 10 mg and 4 mM Eti or Clo (100 μ L). They were mixed with 4 mM of a given NBP (100 μ L) by pipetting and the mixture left standing for 60 min. Then, the mixture was centrifuged and the resultant supernatant subjected to measurement of the NBP. (c) Effects of BP-related substances on NBP-release from NBP-HA. NBP(4mM)-HA and the indicated test substance (100 μ L) were mixed by pipetting and the mixture left standing for 60 min. Then, the mixture was centrifuged and the resultant supernatant subjected to measurement of the NBP. Eti, Clo, PFA, and PAA exhibited a UV spectrum at 220 nm. So, their effects on Zol could not be measured in the present study. Data were compared vs. those obtained from distilled water (data not shown). Each value is the mean $\pm$ S.D. from 4 tubes. * p < 0.05 vs. distilled water group.

that the affinities for HA are NBPs (Ale, Pam, Min) >> non-NBPs (Eti and Clo).

Effects of BP- and HA-Related Substances on NBP-Release BPs are analogs of PPI, and the BPs used in the present study are sodium salts, except Min. So, we examined whether NaCl, PPI, and PPI-related substances (Pi, PFA, and PAA) can release NBPs from NBP(4mM)-HA. Figure 4C shows that although effects of 300 and 450 mM NaCl are absent, 300 mM Pi, 150 mM PFA, and 150 mM PAA slightly but significantly release the NBPs. In this experiment, we could measure Zol, too, because PPI does not disturb the measurement of Zol. Interestingly, 150 mM PPI markedly releases all the NBPs tested. For Ale and Pam, the releasing effects are Eti > Clo > PPI. However, for Min, the releasing effects are Eti $\geq$ PPI. Ca^{2+} is a component of HA, and Mg^{2+} easily substitutes for the Ca^{2+} . However, even at 300 mM, they exhibit no significant effects on NBP-release from HA. Histamine, putrescine, and spermidine are basic substances, and their productions are known to be increased at inflammatory sites. So, they might interact with the PO_4^{3-} in HA *in vivo*. However, none of them, at 150 mM, exhibited a detectable effect (data not shown).

Effects of pH on the PPI-Induced NBP-Release Figure 5A shows that the release of Ale and Pam from HA increases as pH increases. However, the release of Min was maximal at pH 5, and the release of Zol increased with the increase in pH and reached near maximum at pH 7.

Effects of Ca^{2+} , Mg^{2+} , and Amines on the PPI-Induced NBP-Release As shown in Fig. 4C, Ca^{2+} , Mg^{2+} , and amines, by themselves, exhibited no detectable effects on NBP-release from HA. In the present experiments, however, we could not study the effects of Ca^{2+} and Mg^{2+} on PPI-induced NBP-release from HA because CaCl_2 and MgCl_2 each formed insoluble product when mixed with PPI. Concerning histamine, putrescine, and spermidine, none of them modified the PPI-induced release of Min from HA (Fig. 5B).

Experiments Using 2 mM NBPs (a Concentration That Does Not Saturate HA (10 mg)) As shown in Fig. 1B, for complete binding of 4 mM NBPs to HA, Min and Zol required a longer time than Ale and Pam. In addition, the release of Min from HA induced by Eti was slower than those of Ale and Pam (Fig. 3C). We suspected these differences might be due to steric hindrance, because the molecular sizes of Min and Zol are greater than those of Ale and Pam, and 4 mM is

Fig. 6. Properties of NBP(2mM)-HA

(a) NBP-binding velocity to HA. HA 10mg and 2mM of a given N-BP (100 μ L) were mixed by pipetting, and the mixture was left standing for the indicated time, and then centrifuged. Finally, the NBP in the resultant supernatant was measured. (b) NBP-releasing velocity. NBP(2mM)-HA and 100mM Eti (100 μ L) were mixed by pipetting. Then, the mixture was left standing for the indicated time and centrifuged, and the resultant supernatant was subjected to measurement of the NBP. Each value is the mean $\pm$ S.D. from 4 tubes. * p < 0.05 vs. indicated group. (c) Effects of Eti, Clo, and PPi on NBP-release from NBP-HA. NBP(2mM)-HA and the indicated test substance or distilled water (100 μ L) were mixed by pipetting and the mixture left standing for 60min. Then, the mixture was centrifuged and the resultant supernatant was subjected to measurement of the NBP. Each value is the mean $\pm$ S.D. from 4 tubes. * p < 0.05 vs. distilled water group (data not shown).

Fig. 7. Effects of PPi, Pi, Ca^{2+} , Mg^{2+} , and Amines on NBP-Binding to HA

HA 10mg and 100 μ L of the indicated test sample(s) were mixed by pipetting, and the mixture was left standing for 60min before being centrifuged. Then, the NBP in the resultant supernatant was measured. Each value is the mean $\pm$ S.D. from 4 tubes. * p < 0.05 vs. NBP alone.

the entry of the released NBP into soft-tissue cells might be minor, if any. However, if all bones are saturated with an NBP, entry of the released NBP into soft-tissue cells and/or

its excretion might occur to a significant extent. The ratio between these two events would differ depending on the degree of saturation with NBPs as well as on the doses of adminis-

Fig. 8. Effects of PPI Concentration on NBP-Binding to HA

HA 10mg and 100 μ L of a solution containing 4mM of an NBP and the indicated concentration of PPI were mixed by pipetting, and the mixture was left standing for 60min before being centrifuged. Then, the NBP in the resultant supernatant was measured. Each value is the mean $\pm$ S.D. from 4 tubes. * $p < 0.05$ vs. 0 (no PPI).

Table 1. Summary of the Findings in the Present Study

Test substances	Effects of test substances on	
	NBP-binding to HA	NBP-release from HA-bound NBPs
Eti, Clo, PPI	Inhibition Eti > Clo (Fig. 2B)	Promotion Eti >> Clo (Fig. 3A) Eti > Clo > PPI for Ale and Pam (Figs. 4C, 6C) Eti $\geq$ PPI > Clo for Min, Zol, Ris (Figs. 4C, 6C)
Pi, PFA, PAA	Inhibition Very weak, if any (Fig. 7)	Promotion PPI > PFA > Pi > PAA (Fig. 4C)
Ca ²⁺ , Mg ²⁺	Inhibition Very weak, if any (Fig. 7)	Promotion Very weak, if any (Fig. 4C)
Amines*	No detectable effect (Fig. 7)	No detectable effects (Fig. 5B)

NBPs used in the present study were Ale, Pam, Min, Zol, and Ris. *Histamine, putrescine, and spermidine Note: 1. NBP-releasing effects and NBP-binding inhibitory effects of PPI are greater for Min, Zol, and Ris than for Ale and Pam (Figs. 6C, 8). 2. The effects of Pi, PFA, PAA, Ca²⁺, and Mg²⁺, and amines on NBP-binding to HA and NBP-release from NBP-bound HA are minimal, if any. 3. Under neutral conditions, NBPs bind to HA and the binding is saturable (1mg HA is nearly saturated with 40 nmols of each NBP) (Figs. 1A, B). The velocities of both NBP-binding to HA and NBP-release from NBP-bound HA are very rapid at an unsaturated concentration of each NBP (Figs. 6A, B). 4. For complete binding to HA and for complete release from NBP-bound HA, Min and Zol require a somewhat longer standing time than Ale and Pam (Figs. 1B, 3C). 5. Although NBP-binding to HA was not modulated by pH under the conditions used (Fig. 2A), NBP-release from HA by Eti or PPI was modulated by pH (Figs. 4A, 5A). 6. The affinities for HA are NBPs (Ale, Pam, Min) >> non-NBPs (Eti and Clo) (Figs. 3A, 4B).

tered non-NBPs or the amount of endogenous PPI present. It should be noted that bone scintigraphy using non-NBPs (the non-NBPs Med or Oxi) (see Supplementary Fig. 1) labeled with 99mTc is a useful method for detecting the sites in HA (*i.e.*, in bones) to which 99mTc-BP attaches, including when tumors, fractures, infection, and/or inflammation are present. Oral tissues are frequently exposed to bacteria, and bacteria are thought to trigger or promote BRONJ.^{22,23} Notably, NBPs can be detected in the saliva of BRONJ patients,²⁴ indicating that jawbones are susceptible to the accumulation of BPs and may become saturated with a BP after its repeated administration. Therefore, the following scenarios need to be considered. (i) The NBPs released by PPI may enter soft-tissue cells, leading to BRONJ. (ii) The NBPs released by Eti and Clo may not enter soft-tissue cells because the co-presence of Eti and/or Clo inhibits SLC20 and/or SLC34. We discuss these ideas in the following paragraphs.

A reduction in alkaline phosphatase has been shown to result in an increase in PPI in the urine in both humans and mice.^{25,26} Alkaline phosphatase is expressed on the cell mem-

brane of osteoblasts, and NBPs are toxic to osteoblasts at 10⁻⁵ M.²⁷ Thus, if NBPs reach 10⁻⁵ M around jawbones, the number of osteoblasts might be reduced, resulting in an increase in PPI. It is also known that PPI is extracellularly produced by the hydrolysis of nucleotide triphosphates, including ATP, and ATP is released from nerve termini, immune cells, injured cells, and even from bacteria.²⁸ Interestingly, platelets store PPI in dense granules together with ATP.²⁹ A cell-wall component of Gram-negative bacteria, namely lipopolysaccharide (LPS), and the inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor α (TNF- α) are potent stimulators of platelets.³⁰ Thus, ATP and PPI may be increased at inflammatory, necrotic, and/or infected sites in the oral tissues of NBP-treated patients. Collectively, the above makes it seem likely that PPI-induced NBP-release occurs in NBP-accumulated jawbones, contributing to the development of BRONJ (Supplementary Fig. 4).

As shown in Supplementary Table 1, the non-NBPs Eti and Clo have a variety of properties in addition to their anti-bone-resorptive effects. Taking these properties and the present

findings into account, we now discuss the clinical applications of Eti and Clo.

(i) *Eti as a Substitution Drug for NBPs to Treat and Prevent BRONJ*: Once taken into cells, NBPs (dependent on their amounts) are cytotoxic for all cell types³¹⁾ because they inhibit the mevalonate pathway (common to all eukaryotic cells).³²⁾ Here, we demonstrated that Eti releases NBPs from NBP-bound HA. In addition, we previously demonstrated that NBPs enter cells *via* the phosphate transporters SLC20 and/or 34, and that Eti and Clo can inhibit this entry.^{15–17)} The anti-bone-resorptive effect of Eti is far less powerful than those of NBPs (Supplementary Fig. 1). To compensate for this weak effect, Eti is used clinically at much larger doses (200–1000 mg) than NBPs (used at 1–50 mg). Thus, such a large dose of Eti would expel bone-bound NBPs from the bone and inhibit the entry of the released NBPs into soft-tissue cells around the jawbones. Consequently, Eti would be expected to be effective as a substitution drug for an NBP in patients with BRONJ or at risk of BRONJ. Indeed, our clinical attempts support this idea.^{18,19)} Eti might also be effective for preventing BRONJ if it is given orally or intravenously just prior to invasive dental treatments.

(ii) *Eti as an Irrigating Agent during Invasive Dental Treatments in NBP-Treated Patients*: Invasive dental treatments (e.g., tooth extraction, dental implants, apical/periodontal surgery) are thought to be risk or causal factors for BRONJ.⁸⁾ Interestingly, Eti has appeared recently on the market as a root canal-irrigating agent (brand name “Dual Rinse® HEDP”),^{33,34)} with the Eti being used at a very high concentration [0.9 g/10 mL (360 mM)]. When injected into mouse ear-pinnas (20 µL/ear), Eti does not exhibit inflammatory/necrotic effects even at 100 mM.³⁵⁾ On the contrary, Eti, when injected together with an NBP, inhibits the NBP-induced inflammation by inhibiting its entry into cells. For example, 16–50 mM Eti completely prevents the inflammatory/necrotic effects of 2–4 mM Zol.^{13,17)} Thus, irrigation with a solution including a high concentration of Eti might be a safe and effective method for reducing jawbone-accumulated NBPs. As shown in Figs. 4A and 5A, release of an NBP (Ale, Pam, or Zol, but not Min) from NBP-HA is greater at higher pH. Thus, an Eti solution with a higher pH (possibly around pH 9) may be very suitable as an irrigating solution for removing jawbone-bound NBPs (Ale, Pam, or Zol). Interestingly, Hokugo *et al.* recently reported that in Zol-treated mice, injections of Eti around a tooth one day before its extraction reduced the development of jawbone necrosis.³⁶⁾ The same authors previously (2013) showed by *in vitro* experiments using fluorophore-conjugated NBPs that a BP and HA would establish an equilibrium, and thus an additional application of a BP may partially replace the BP while maintaining the equilibrium.³⁷⁾

(iii) *Clo as a Preventive Drug against BRONJ*: The affinity of Clo for HA is the lowest among BPs and lower than that of PPI (Supplementary Table 2), and its ability to release NBPs from HA is much weaker than that of Eti. In our *in vivo* studies in mice, although Eti reduces the anti-bone-resorptive activities of NBPs,^{12,35)} Clo does not reduce them.^{11,13)} Clo does, however, prevent or reduce the inflammatory/necrotic effects of NBPs by inhibiting NBPs' entry into cells *via* the phosphate transporters SLC20 and/or SLC34, and this effect is stronger than that of Eti.^{14,15,38,39)} Thus, a combination of Clo and an NBP might be effective for preventing BRONJ while

preserving the powerful anti-bone-resorptive effect of the NBP. Moreover, Clo and Eti have analgesic effects that are independent of their anti-bone-resorptive effects and are derived from inhibition of the phosphate transporter SLC17.^{15,40–45)} The analgesic effect of Clo is also greater than that of Eti.^{40,43)}

The affinities for HA are NBPs (Ale, Pam, Min) >>> non-NBPs (Eti and Clo) (Figs. 3A, 4B). So, the results shown in Fig. 4B suggest that if these NBPs were given to patients who had previously been treated with non-NBPs for the removal of NBPs (to prevent or treat BRONJ), the non-NBPs may then be replaced with NBPs, *i.e.*, restoring the potent anti-bone-resorptive effects of NBPs.

Eti and Clo were the first BPs reported as possible drugs following collaborations between Fleisch and Francis.⁴⁶⁾ Eti (synthesized as early as 1897) has recently undergone reappraisal because it has various properties that appear unique *vs.* NBPs and it has a host of “off-label” uses.⁴⁷⁾ Clo has also been reappraised because of its variety of effects (including its analgesic effect) and because of the availability of various administration routes (including intramuscular injection) and its tolerability or safety.^{10,48,49)}

Within the last few years, NBPs have been studied as drugs for preventing the non-gravity-induced osteoporosis that occurs during a prolonged stay in space, supporting NBPs being very important drugs available for preventing osteoporosis. Therefore, establishing methods for their safe use is critical. In conclusion, our findings may contribute to this. Although NBPs have been thought to undergo stable binding to bone, and thus to have a long half-life in bone, the present results suggest that NBPs might be released from NBP-accumulated jawbones by PPI produced in the environment of the jawbones, leading to the development of BRONJ.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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