Regular Article

Inflammatory Effects of Nitrogen-Containing Bisphosphonates (N-BPs): Modulation by Non-N-BPs

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Bisphosphonates (BPs) are used against diseases with enhanced bone resorption. Those classed as nitrogen-containing BPs (N-BPs) exhibit much stronger anti-bone-resorptive effects than non-nitrogen-containing BPs (non-N-BPs). However, N-BPs carry the risk of inflammatory/necrotic side effects. Depending on their side-chains, BPs are divided structurally into cyclic and non-cyclic types. We previously found in mice that etidronate and clodronate (both non-cyclic non-N-BPs) could reduce the inflammatory effects of all three N-BPs tested (cyclic and non-cyclic types), possibly by inhibiting their entry into soft-tissue cells [via SLC20 and/or SLC34 phosphate transporters]. Tiludronate is the only available cyclic non-N-BP, but its effects on N-BPs’ side effects have not been examined. Here, we compared the effects of etidronate, clodronate, and tiludronate on the inflammatory effects of six N-BPs used in Japan [three cyclic (risedronate, zolendronate, mimodronate) and three non-cyclic (pamidronate, alendronate, ibandronate)]. Inflammatory effects were evaluated in mice by measuring the hind-paw-pad swelling induced by subcutaneous injection of an N-BP (either alone or mixed with a non-N-BP) into the hind-paw-pad. All of six N-BPs tested induced inflammation. Etidronate, clodronate, and the SLC20/34 inhibitor phosphonoformate inhibited this inflammation. Tiludronate inhibited the inflammatory effects of all N-BPs except ibandronate and mimodronate, which have higher molecular weights than the other N-BPs. The mRNAs of SLC20a1, SLC20a2, and SLC34a2 (but not of SLC34a1 and SLC34a3) were detected in the soft-tissues of hind-paw-pads. These results suggest that etidronate, clodronate, and phosphonoformate may act non-selectively on phosphate transporter members, while tiludronate may not act on those transporting N-BPs of higher molecular weights.

Key words bisphosphonate; side effect; inflammation; phosphate transporter; phosphonoformate; tiludronate

Bisphosphonates (BPs) are analogs of pyrophosphoric acid (Fig. 1). Instead of the latter’s hydrolysable P-O-P bond, BPs possess a P-C-P bond that is resistant to biological destruction.1,2) R1 and R2 substitutions on the central carbon allow these BPs to possess a P-C-P bond that is resistant to biological destruction.1,2) Instead of the latter’s hydrolysable P-O-P bond, BPs allow stronger anti-bone-resorptive effects than the non-nitrogen-containing BPs (non-N-BPs).1,2) Those classed as nitrogen-containing BPs (N-BPs) have much stronger anti-bone-resorptive effects than the non-nitrogen-containing BPs (non-N-BPs).1,2) However, inflammation and/or necrosis reportedly occur in tissues exposed to N-BPs, such as esophagus, stomach, and jawbones.3,4) Many cases of BP-related osteonecrosis of the jaw (BRONJ) have been reported among patients given intravenous N-BPs, while oral administration of N-BPs to patients with osteoporosis can also reportedly cause BRONJ.5) Notably, the ratio of the number of BRONJ cases among osteoporotic patients receiving oral N-BPs to that in patients receiving intravenous N-BPs is higher in Japan than in the U.S.A. and EU.6)

The cytotoxicity of N-BPs has been shown to be due to the intracellular inhibition of farnesyl pyrophosphate synthase (FPPS) in the pathway of cholesterol synthesis.2) The pathway of cholesterol synthesis exists widely in eukaryotic cells. Indeed, N-BPs are cytotoxic not only to osteoclasts, but also to various other cell-types.7) However, it is poorly understood how N-BPs enter cells at a neutral pH. We previously found in mice that the non-cyclic non-N-BPs etidronate (Eti, used in Japan) and clodronate (Clo, not used in Japan) can reduce or prevent the inflammatory side effects of both the non-cyclic N-BP alendronate (Ale) and the cyclic N-BPs zolendronate (Zol) and mimodronate (Min).8,9) Concerning the mechanism underlying such interesting effects of Eti and Clo, our pharmacological studies have suggested that they can competitively inhibit the entry of N-BPs into soft-tissue cells, and that this possibly occurs via SLC20 and/or SLC34 phosphate transporters.10) Tiludronate (Til, not used in Japan) is a cyclic non-N-BP. Thus, it might be expected that tiludronate would be an effective inhibitor of the entry of cyclic N-BPs into soft-tissue cells, thereby reducing or preventing their inflammatory side effects. However, there are no published reports concerning any modulating effects of Til on the inflammatory effects of N-BPs.

Here, we examined in mice the effects of Eti, Clo, and Til on the inflammatory effects of six N-BPs [three cyclic N-BPs (Ris, Zol, and Min) and three non-cyclic N-BPs (Pam, Ale,
and Iba)] (Fig. 1), all of which are used in Japan. In this study, unlike in previous studies, inflammatory effects were evaluated by measuring the swelling induced in hind-paw-pads by subcutaneous injection of test reagents into them. Such swelling is more precisely and objectively measurable than the inflammation/necrosis in ear-pinnas, on which the previous method depended.

MATERIALS AND METHODS

Mice and Reagents  ddY mice were obtained from SLC (Hamamatsu, Japan). All experiments complied with Regulations for Animal Experiments and Related Activities at Tohoku University. Min was synthesized for basic studies by Chengdu D-Innovation Pharmaceutical Co., Ltd. (Chengdu, China). Zol and Clo were from Toronto Research Chemicals Inc. (North York, ON, Canada) and Sigma (St. Louis, MO, U.S.A.), respectively. Iba was provided by Boehringer Mannheim (Mannheim, Germany).11) Ale, Pam, and Ris were from LKT Laboratories, Inc. (St. Paul, MN, U.S.A.), and Til was from Sigma. Phosphonoformate (PFA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The above drugs were dissolved in sterile saline, with the pH of the solutions being adjusted to 7 with NaOH. Experimental protocols are described in the text or in the legend to the Figure relating to each experiment.

Evaluation of Inflammatory Effects of N-BPs  Each BP solution was injected subcutaneously into the left hind-paw-pads (20 µL each pad), and the changes in the volume of the hind-paw-pads distal to the ankle was measured by the use of a plethysmometer (MK-101P; Muromachi Kikai, Co., Ltd., Tokyo, Japan).

Evaluation of Anti-bone-Resorptive Effects  A clear sclerotic band is detectable in tibias by radiography a few weeks after a single injection of a BP into mice (tentatively called the BP-band), reflecting an inhibition of bone resorption.12–14) Hence, we estimated the anti-bone-resorptive effects of BPs by using the BP-band as a marker. Briefly, each BP solution was intraperitoneally injected (0.1 mL/10 g body weight) into young male ddY mice (5 weeks old). The mice were decapitated 10 d later, and tibias (both sides) were removed and subjected to X-ray analysis for the detection and quantification of the BP-bands. To this end, soft X-ray radiographs were taken using SOFTEX and Fuji Industrial X-ray film, the conditions being 80 V, 1 mA, duration 55 s,12) and the BP-bands were quantitatively analyzed using NIH Image software. In this analysis, we recorded a value derived by multiplying the “mean gray value” (average gray value of pixels within a selected band) by the area (mm²) of that BP-band. In each experiment, a value obtained from a corresponding area of a control tibia excised from a mouse given no BP was subtracted from the above experimental value.

Detection of mRNAs of Phosphate Transporters  Total RNA was extracted from kidney and from soft tissues taken from hind-paw-pads using ISOGEN II (Nippon Gene, Toyama, Japan) according to the manufacturer’s protocol. cDNA synthesis was carried out using a Transcriptor First Strand cDNA synthesis kit (Roche Diagnostic, Indianapolis, IN, U.S.A.). One milligram of total RNA was applied to cDNA synthesis. PCR mixtures contained 0.25 mL of cDNA mixture, 0.8 mM of each primer, and 0.625 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster, CA, U.S.A.) in a total volume of...
25 mL. The primer sequences were as listed in Table 1. The PCR condition was 40 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. PCR products were electrophoresed using agarose gel and stained with ethidium bromide.

**Data Analysis** Experimental values for inflammation are given as the mean±standard deviation (S.D.). The statistical significance of the difference between two means was evaluated using a Bonferroni multiple-comparison test after ANOVA. Data were analyzed using Instat software (GraphPad Software Inc., La Jolla, CA, U.S.A.).

**RESULTS**

**Inflammatory Effects of N-BPs** As shown in Fig. 2, all the N-BPs tested induced swelling in the hind-paw-pads into which they were injected. Min and Zol induced swelling at 1 and/or 2 mM, while the inflammatory effects of other N-BPs were evident at 16 mM. The relative potencies to induce inflammation seem to be Min>Zol>lba>Pam>Ale>Ris. A similar order was noted for four N-BPs in a previous study in which we measured ear-pinna inflammation: namely, Zol>Pam>Ale>Ris.8) It should be noted that in each study, the inflammatory effect of Ris was smaller than those of Pam and Ale, although the relative potencies of their anti-bone-resorptive effects are Ris>Ale>Pam (Fig. 1).

**Anti-bone-Resorptive Effects of N-BPs** The relative potencies shown in Fig. 1 for the anti-bone-resorptive effects of BPs were estimated in experiments on rats.11 Here, when we compared the anti-bone-resorptive effects of four N-BPs (Fig. 3) by examining BP-bands formed in young mice, we found their anti-bone-resorptive potencies to be Min=Zol>Iba=Ale. This rank order is similar to that shown in Fig. 1, and suggests that N-BPs (except Ris) with strong anti-bone resorptive effects tend to have strong inflammatory effects too.

**Modulating Effects of Non-N-BPs on the Inflammatory Effects of N-BPs** As described in Introduction, the non-cyclic non-N-BPs Eti and Clo can reduce or prevent the inflammatory effects of both the non-cyclic N-BP Ale and the cyclic N-BPs Zol and Min in mouse ear-pinna.8,9 Here, we tested the effect of Til, a cyclic non-N-BP, in mouse hind-paw-pads. Against our expectation, under the condition shown in Fig. 4, Til, at 50 mM, did not reduce the inflammatory swelling induced by Min (the cyclic N-BP with the greatest inflammatory effect). Likewise, Til did not reduce the inflammatory effect of Iba, which had the greatest inflammatory effect among the non-cyclic N-BPs tested (Fig. 2). However, like Eti and Clo, Til did reduce the inflammatory effects of the other N-BPs tested, irrespective of whether they were cyclic (Zol

### Table 1. Primer Sequences for Phosphate Transporters

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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<tbody>
<tr>
<td>SLC20a1</td>
<td>5'-GTG GGA GAC TGC ATG GGA GAT TC-3'</td>
<td>5'-TAT GGG TGT TGC CGC TTT TGT AGA-3'</td>
</tr>
<tr>
<td>SLC20a2</td>
<td>5'-AGC GGA CCG GAC GAC CTC-3'</td>
<td>5'-GCC CCC AGC AGC ACA GA-3'</td>
</tr>
<tr>
<td>SLC34a1</td>
<td>5'-GCA GGC AGG GGA CAG GAC-3'</td>
<td>5'-GCC AGG GGA CAG GAC-3'</td>
</tr>
<tr>
<td>SLC34a2</td>
<td>5'-TCA GCG GCC CAG AAC AAG AG-3'</td>
<td>5'-GAT GGG CAG ACG GGT GAA TG-3'</td>
</tr>
<tr>
<td>SLC34a3</td>
<td>5'-TGG CGG GCT TGG TCA TTG-3'</td>
<td>5'-CTT CCC TGG GGC GTC TCC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-CGTGCACATCCGTAAGACCTC-3'</td>
<td>5'-AGCCACCGATCCCACAGA-3'</td>
</tr>
</tbody>
</table>

![Fig. 2. Inflammatory Effects of N-BPs in Hind-Paw-Pad](image)

An N-BP solution with the indicated concentration was injected subcutaneously into a hind-paw-pad, and the volume of the footpad was measured as described in Materials and Methods. n=5 mice.
and Ris) or non-cyclic (Pam and Ale) N-BPs. These results are summarized in Table 2. It should be noted that Til (50 mM) was not effective at reducing the inflammatory effects of 32 mM of Ris or Pam (data not shown).

Comparison of the Effects of PFA, Eti, and Clo

PFA is an inhibitor of SLC20 and/or SLC34 phosphate transporters, and Eti and Clo also inhibit phosphate transporters. We previously found that PFA can inhibit the inflammatory effect of Zol in mouse ear-pinnas. Here, we compared the effects of 50 mM PFA, Eti, and Clo on the inflammatory effects of other N-BPs. As shown in Fig. 5, under the present condition, all three of these were able to reduce the inflammatory effects of all the N-BPs tested, their potencies being Clo>Eti>PFA.

Comparison of the Effects of Eti, Clo, and Til on Zol-Induced Inflammation

As described above, we found that Eti and Clo could reduce the inflammatory effects of all of the N-BPs tested, while Til did not reduce those of Min and Iba (Table 2). In our experiments, Zol was the N-BP with the most potent inflammatory effect that could be reduced by Eti, Clo,
Table 2. Effects of PFA, Eti, Clo, and Til on the Inflammatory Effects of N-BPs (Zol, Ris, Min, Ale, and Iba)

<table>
<thead>
<tr>
<th>Cyclic N-BPs</th>
<th>Non-cyclic N-BPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zol (272) 2 mM</td>
<td>Pam (235) 16 mM*</td>
</tr>
<tr>
<td>Ris (283) 16 mM</td>
<td>Ale (249) 16 mM</td>
</tr>
<tr>
<td>Min (322) 2 mM</td>
<td>Iba (319) 32 mM or 16 mM*</td>
</tr>
</tbody>
</table>

50 mM of PFA or non-N-BPs

PFA (126)

Eti (206)

Clo (245)

Til (319)

( ) molecular weight (salt-free); ↓ inhibition; — not reduced. * PFA, Eti, and Clo can also reduce the inflammation induced in ear-pinnas by these N-BPs. † Til was not effective at reducing the inflammatory effects of 32 mM of Ris or Pam (data not shown). a) PFA, Eti, and Clo reduced the inflammatory effect of 32 mM of Iba. b) Til did not reduce the inflammatory effect of 16 mM of Iba.

Fig. 5. Effects of Clo, Eti, and PFA on the Inflammatory Effects of N-BPs

A solution of Min, Zol, Iba, Ale, or Ris, each alone or in a mixture with Clo, Eti, or PFA (at the indicated concentrations), was subcutaneously injected into a hind-paw-pad. The volume of the footpad was measured as described in Materials and Methods. n = 5 mice.

Fig. 6. Comparison of the Effects of Eti, Clo, and Til on Zol-Induced Inflammation

Zol alone or in a mixture with Eti, Clo, or Til (at the indicated concentrations) was subcutaneously injected into a hind-paw-pad, and the volume of the footpad was measured as described in Materials and Methods. n = 5 mice.
or Til. We therefore compared the inhibitory effects of 10 and 50 mM of these three non-N-BPs on the inflammatory effect of 4 mM Zol. As shown in Fig. 6, their inhibitory effects on Zol-induced inflammation were dose-related. The inhibitory effect of 10 mM Clo was greater than that of 50 mM Eti, and the inhibitory effect of 10 mM Eti was greater than that of 50 mM Til. These results indicate that the rank order of potencies for these effects is Clo>>Eti>>Til.

Detection of mRNAs of Phosphate Transporters Two and three members are known to exist of the phosphate-transporter families SLC20 and SLS34, respectively. SLC20a1 and SLC20a2 are known to be present ubiquitously throughout the body.16,19 The mRNAs of SLC34a1 and SLC34a3 are reportedly expressed at highest levels in the kidney, while SLC34a2 mRNA is widely expressed in various tissues.19 We confirmed in mice the presence (a) of the mRNAs of SLC20a1 and SLC20a2, and also of SLC34a2 (but not a1 and a3 in the soft-tissues of hind-paw-pads and (b) of all members of SLC20 and SLC34 in the kidney (Fig. 7).

DISCUSSION

Modulating Effects of Non-N-BPs on the Inflammatory Effects of N-BPs When taken into cells, non-N-BPs (Eti, Clo, and Til) are converted into cytotoxic ATP analogs.20 However, it should be noted that Eti and Clo are not retained at all within soft-tissues after their intravenous injection into mice, although a small quantity of Pam (N-BP) is retained for a few days.25 These results suggest that non-N-BPs are hardly taken at all into soft-tissue cells, while N-BPs are taken into such cells, even if in smaller amounts than in bone. In our previous studies in which we used ear-pinnas for examining inflammation, Eti and Clo were found to reduce the inflammatory effects of both the non-cyclic N-BP Ale and the cyclic N-BPs Zol and Min.8,9 Our pharmacological studies suggest that Eti and Clo inhibit the entry of Zol into soft-tissue cells via SLC20 and/or SLC34 phosphate transporters.10 The results obtained in the present study (summarized in Table 2) indicate that the non-cyclic non-N-BPs Eti and Clo, like the SLC20/34 inhibitor PFA, non-selectively reduce the inflammatory effects of all the N-BPs tested, while the cyclic non-N-BP Til either does not reduce or only weakly reduces the inflammatory effects of Iba (non-cyclic N-BP) and Min (cyclic N-BP). Although the explanation for Til having such weak effects is unclear, it should be noted that (a) the molecular weights of Til (319), Iba (319), and Min (322) are higher than those of the other N-BPs (each below 290), and (b) the molecular weight of phosphoric acid is only 98. Thus, the “hole size” of phosphate transporters may be narrow for BPs, especially for Til, Iba, and Min. Since the SLC20 and 34 families include two and three members, respectively,21 we might further speculate that (i) different members of the SLC20 and SLC34 families may transport different molecular sizes of N-BPs (irrespective of their possession of cyclic or non-cyclic side-chains), and (ii) Clo, Eti, and PFA may non-selectively inhibit these transporter members, while Til may inhibit the members that transport smaller N-BPs, but not those that transport larger N-BPs. Whether these ideas are correct remains to be clarified in future studies.

Mechanisms Underlying the Inflammatory/Necrotic Side Effects of N-BPs Having been taken into cells, N-BPs exhibit cytotoxicity not only against osteoclasts, but also against other cell-types,20 by inhibiting FPPS in the pathway of cholesterol synthesis.20 Indeed, (i) N-BPs carry the risk of directly injuring esophageal and gastrointestinal epithelial tissues,27 (ii) N-BPs, when injected topically, induce inflammation and necrosis at the injection sites,8,28 and (iii) bone-bound Zol can inhibit the growth of adjacent non-bone-cells in vitro.29 Moreover, N-BPs (as well as non-N-BPs) have high affinities for bone hydroxyapatite, so that repeated administrations result in them accumulating within bones.1,30 This accumulation is augmented in inflamed bones (as in periodontitis), in which hydroxyapatite is exposed. Indeed, bone scintigraphy using 99mTc-labeled BPs clearly indicates such an augmented accumulation.31 It is likely that inflammation induced by infections (i.e., periodontitis) or tooth extraction acts together with the inflammatory effects induced by a given N-BP itself to promote the accumulation of the N-BP in bones around the inflamed tissues. Importantly, N-BPs can be detected in the saliva of patients treated with N-BPs who have developed BRONJ,32 indicating that bone-bound N-BPs are released from jawbones in some situations, such as tooth extraction and/or infection. Thus, the release of N-BPs from jawbones and their entry into soft-tissue cells surrounding the jawbones via phosphate transporters may be the critical steps in the development of BRONJ.

Comparison of the Effects of N-BPs The rank orders of (a) the anti-bone-resorptive effects of N-BPs and (b) their inhibitory effects on FPPS are the same (Table 3), supporting the idea that inhibition of FPPS in osteoclasts is the key mechanism underlying N-BPs’ anti-bone-resorptive effects.21 Although the anti-bone resorptive effect of Ris is greater than those of Ale and Pam, the affinity of Ris for hydroxyapatite is weaker than those of Ale and Pam. Thus, although such
affinity is indispensable for the anti-bone-resorptive activities of N-BPs; its contribution to the rank order of their anti-bone-resorptive activities may not be so important. It should also be noted that the rank order of the inflammatory effects of N-BPs is similar to, although not exactly the same as, that of their inhibitory effects on FPPS, supporting the idea that the inflammatory effects of N-BPs depend both on their abilities to enter cells and on their abilities to inhibit FPPS. For example, the inhibitory effect of Ris on FPPS is greater than those of Ale and Pam, but its potential to enter cells may be much smaller than those of the other two N-BPs, with the overall result being that the inflammatory effect of Ris is weaker than those of Pam and Ale.

Possible Clinical Applications of Eti, Clo, and Til

There are only a few reports of inflammatory/necrotic side effects occurring in patients treated with either Eti or Clo,33) and there is no clinical report of Til-associated inflammatory or necrotic side effects. Of the BPs shown in Table 3, the anti-bone-resorptive effect of Eti is the smallest. Thus, the clinical oral dose of Eti (several hundreds ca. 1000mg) is very high compared with those of N-BPs (several mg or several tens of mg). In contrast, the affinity of Eti for hydroxyapatite is compared with those of N-BPs (several mg or several tens of mg). In contrast, the affinity of Eti for hydroxyapatite is middling among BPs (Table 3). Therefore, it seems possible that Eti, at a large dose, might substitute (at least partly) for, or eliminate, an N-BP that has already accumulated within bones, suggesting that Eti might be useful or eliminate, an N-BP that has already accumulated within bones, suggesting that Eti might be useful as a substitution drug for N-BPs with the aim of reducing the risk of BRONJ.9) Indeed, we have reported just such an effect in the clinical setting.31,34) In contrast, because the bone affinity of Clo is the lowest as shown in Table 3, Clo might have a very weak inhibitory effect of that type, if it has any at all. Indeed, Clo does not impair the anti-bone-resorptive effects of N-BPs (including Ale, Ris, and Zol).8) However, Clo has the strongest anti-inflammatory effect against N-BPs (i.e., the ability to inhibit the entry of N-BPs into cells) (Table 3). Thus, Clo might be useful as a combination drug with an N-BP with the aim of preventing the inflammatory/necrotic side effects of the N-BP while retaining the potent anti-bone-resorptive effect of the same N-BP.9) On the other hand, the usefulness of Til may be limited, because it was not effective at reducing the inflammatory effects of Iba or Min. Bone-joint-muscle pain is commonly experienced by elderly patients with osteoporosis and/or osteoarthritis. Interestingly, in patients35) as well as in mice36) Eti and Clo have recently been shown to possess potent analgesic effects that are independent of their anti-bone-resorptive activities. Clo and Eti exert analgesic effects via inhibition of phosphatases transporters of the SLC17 family.37) Those findings, together with the ones made in the present study, may suggest that Eti and Clo should be reappraised as safe and useful drugs for osteoporosis.

Perspectives

It remains to be identified which members of the SLC20 or SLC34 families serve to transport which types of N-BPs. It would also be of interest to examine the effects of PFA against the anti-bone-resorptive effects of N-BPs. It is of even more interest to know whether PFA itself exhibits an anti-bone-resorptive effect. Incidentally, it has been reported that a carboxylate analog of Ris (with a P-C-COOH structure instead of P-C-P) reduces experimental bone-resorption in vitro, although its activity is 8000-fold less potent than that of Ris itself.38,39) Thus, it also remains to be clarified whether such analogs can exert anti-bone-resorptive effects in vivo.

CONCLUSION

All six of the N-BPs used in Japan have inflammatory effects, with their relative potencies to induce inflammation being Min>Zol>Iba>Pam>Ale>Ris. The non-cyclic non-N-BPs Eti and Clo and the SLC20/34 inhibitor PFA can reduce or prevent the inflammatory effects of all these non-N-BPs (their potencies being Clo>Eti>PFA), possibly by inhibiting their entry into cells via the phosphate transporters SLC20 and/or SLC34. The cyclic non-N-BP Til reduced the inflammatory effects of Zol, Pam, Ale, and Ris, irrespective of whether they were cyclic or non-cyclic types, but not those of Iba or Min (N-BPs with higher molecular weights). Our results support Eti being useful as a substitution drug for N-BPs to reduce the risk of BRONJ, and Clo being useful as a combination drug with an N-BP for preventing the inflammatory/necrotic side effects of the N-BP while retaining its potent anti-bone resorptive effects.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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