Phosphonocarboxylates Can Protect Mice against the Inflammatory and Necrotic Side Effects of Nitrogen-Containing Bisphosphonates by Inhibiting Their Entry into Cells via Phosphate Transporters

Tomomi Kiyama, Masahiro Tsuchiya, Satoru Okada, Takefumi Oizumi, Kouji Yamaguchi, Keiichi Sasaki, Shunji Sugawara, and Yasuo Endo

Biphosphonates (BPs) are used against diseases involving increased bone-resorption. Among BPs, nitrogen-containing BPs (N-BPs) have much stronger anti-bone-resorptive effects than non-nitrogen-containing BPs (non-N-BPs). However, N-BPs carry the risk of inflammatory/necrotic effects, including osteonecrosis of jawbones. When injected into mouse ear-pinnas, N-BPs induce inflammatory/necrotic effects within the ear-pinna. We previously found that (a) the non-N-BPs clodronate and etidronate can reduce such side effects of N-BPs, and (b) phosphonoformate (an inhibitor of the phosphate transporters SLC20 and SLC34) can reduce the inflammatory/necrotic effects of zoledronate (the N-BP with the highest reported risk of side effects). However, it is not clear (i) whether phosphonoformate can reduce the side effects of other N-BPs, too, and (ii) whether other phosphonocarboxylates have such inhibitory effects. Here, using the mouse ear-pinna model, we compared the effects of etidronate, clodronate, and four phosphonocarboxylates on the inflammatory/necrotic effects of N-BPs of the alkyl type (alendronate) or cyclic type (zoledronate and minodronate). Like phosphonoformate, the other three phosphonocarboxylates protected against the inflammatory/necrotic effects of all the N-BPs. The protective potencies were clodronate > etidronate > phosphonoacetate > phosphonoformate > phosphonopropionate > phosphonobutyrate. With a similar order of potencies, these agents reduced the amount of $^3$H-alendronate retained within the ear-pinna after its injection therein. The mRNAs of SLC20 and SLC34 were detected in untreated ear-pinnas. These findings suggest that the inhibition of phosphate transporters by phosphonocarboxylates, as well as by etidronate and clodronate, might be a useful preventive strategy against the side effects of both alkyl- and cyclic-type N-BPs.

Key words phosphonocarboxylate; bisphosphonate side effect; phosphate transporter; phosphonoacetate; phosphonoformate
dronate (a cyclic-type N-BP that has the highest reported risk of side effects among N-BPs). However, it is not clear (i) whether phosphonoformate can reduce the side effects of other N-BPs, too, and (ii) whether other phosphonocarboxylates can exhibit an inhibitory effect such as that of phosphonoformate. Here, we focused on these questions.

MATERIALS AND METHODS

Mice and Reagents  BALB/c mice were bred in our laboratory. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University. Minodronate was synthesized for basic studies by Chengdu D-Innovation Pharmaceutical Co., Ltd. (Chengdu, China). Zoledronate and clodronate were from Toronto Research Chemicals Inc. (North York, ON, Canada) and Sigma (St. Louis, MO, U.S.A.), respectively. Etidronate, phosphonoformate, phosphonacetate, phosphonopropionate, and phosphonobutyrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2,3-3H-Alendronate (20–40 Ci/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, U.S.A.). 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.

Inflammatory and Necrotic Effects of N-BPs  Female mice (6–8 weeks of age) were anesthetized with ethyl ether, and a BP solution was injected subcutaneously into both the right and the left pinna (inside) near the root of the ear (20µL each ear). The concentrations used are indicated in the relevant experiments. As described below, the inflammatory and necrotic actions of BPs were evaluated daily. All experiments were terminated on day 7.

(a) Inflammation
The length and width of the area of inflammation (Fig. 2) at the back of the ear (detectable as a red area) were recorded, and length×width (mm²) was used as an indicator of inflammation.

(b) Necrosis
After maximum inflammation (estimated as described above) had been attained, the center of the inflammatory site became necrotic [detectable as a change of color from red to dark brown (or black) or as a tissue defect] (Fig. 2). At the start of the necrosis, we stopped measuring inflammation, and in each group of mice we recorded the number of ears with and the number without necrosis [expressed as the incidence of necrosis (e.g., maximum incidence is 8 in a group of 4 mice)].

Fig. 1. Structures of Substances Tested in the Present Study

Bisphosphonates (BPs), with a non-hydrolysable P-C-P structure, are the analogs of pyrophosphate (PPi), which has a hydrolysable P-O-P structure. There are two types of BPs, the nitrogen-containing BPs (N-BPs) and the non-nitrogen-containing BPs (non-N-BPs). Alendronate, zoledronate, and minodronate are N-BPs, while etidronate and clodronate are non-N-BPs. Four phosphonocarboxylates are also shown. Among them, phosphonoformate (PFA) is a well-known inhibitor of the phosphate transporter SLC34, although at higher concentrations it inhibits SLC20, too. The relative potencies of the anti-bone-resorptive effects of BPs are shown within parentheses.

Fig. 2. Inflammation and Necrosis Induced by an NBP
Zoledronate (2 mM) was subcutaneously injected into an ear-pinna (inside) near the root of the ear (20µL each ear).
Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis of Phosphate Transporters mRNA Using Trizol reagent (Invitrogen, CA, U.S.A.), total RNA was extracted from the ear-pinnas of mice. cDNA was prepared with the aid of a Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, U.S.A.) and subjected to quantitative real-time PCR analysis using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, U.S.A.). Gene-specific primers (Table 1) were designed using DNAStar software (DNASTAR, Inc., Madison, WI, U.S.A.). The internal control primers for EF1α were F5’-ATT CCG GCA AGT CCA CCA CAA-3’/uni2032 and R5’-CAT CTC AGG AGC CTC CTT CTC AAA C-3’. Quantitative real-time PCR was performed using the iQ5 real-time detection system (Bio-Rad). The PCR profile was 3 min at 95°C for initial melting; 20 s at 95°C, 30 s at 58°C for 50 cycles; 30 s at 95°C for 1 cycle; and then 1 min at 55°C followed by stepwise temperature increases from 55 to 95°C to generate the melt curve. Standard curves and PCR efficiencies were determined for each primer-set using control cDNA and a 10-fold dilution series ranging from 1000 to 1 ng/mL. Relative expression levels of phosphate transporters were calculated as a function of EF1α expression.17)

Measurement of 2,3-[^3]H-Alendronate in Ear-Pinna Tissues First, [^3]H-alendronate (0.2 μCi), either alone or in a mixture with a test substance, was injected into both ear-pinnas (20 μL each ear) in mice anesthetized with diethylether. Then, the ear-pinnas were taken at the indicated time and the amount of [^3]H-alendronate retained within them was counted as follows. Each ear-pinna was homogenized in a tube containing 1.5 mL of solubilizer (Soluen-350: PerkinElmer, Inc., Waltham, MA, U.S.A.). The homogenate was solubilized by keeping the tube at 50°C for 3 h. The solubilized sample was put into a glass vial containing 10 mL scintillation cocktail (Pico-Fluor 40: PerkinElmer, Inc.), and radio-activity was counted using a scintillation counter.

Tissue Preparation and Immunohistochemistry Auricles were resected and immediately fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C overnight. After being dehydrated using a graded series of ethanol solutions and infiltrated with xylene, specimens were embedded in paraffin. Serial sections of 5-μm thickness were cut and used for immunohistochemistry, which was performed by following a previously reported protocol.18) After deparaffinization, antigen retrieval was performed using proteinase K solution (Roche; 20 μg/mL in Tris-ethylenediaminetetraacetic acid (TE) buffer, pH 8.0) for 5 min at 37°C, and tissue sections were blocked for endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 15 min at room temperature (r.t.). Sections were incubated at 4°C overnight with the primary antibody for SLC20a1 (dilution 1:1000; abcam, Cat. #: ab104687) or SLC20a2 (dilution 1:200; Bioss, Cat. # bs-8610R) diluted with 5% normal goat serum in phosphate buffered saline (PBS) containing 0.05% Triton-X and 5% bovine albumin. Next, the sections were incubated with the secondary antibody (1:1000, biotinylated goat anti-rabbit antibody; Vectafor) for 1 h at r.t., and then visualized using a Vectastain ABC kit (Vector Laboratories, Funakoshi Co., Tokyo, Japan). Sections were counterstained with methyl green solution.

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. For differences in incidence between 2 experimental groups at a given time, analysis was by the Fisher exact probability test. p Values less than 0.05 were considered to be significant. Data were analyzed using Instat software (GraphPad Software Inc., La Jolla, CA, U.S.A.).

RESULTS

Effects of Etidronate and Clodronate (Non-N-BPs) on Inflammatory/Necrotic Effects of N-BPs We used alendronate, zolendronate, and minodronate as the test N-BPs (Fig. 1). Alendronate is an alkyl N-BP, while minodronate and zolendronate are cyclic N-BPs. Minodronate’s anti-bone-resorptive effect and inflammatory/necrotic effects in mice are similar to, or greater than, those of zolendronate.19) As shown in Fig. 3, minodronate and zolendronate induced inflammatory/necrotic effects at 2 μM, while alendronate did so at 32 μM. The non-N-BPs etidronate and clodronate were each able to reduce or prevent the inflammatory/necrotic effects of all three of the N-BPs. The main features of the data shown in Fig. 2 may be summarized as follows. (i) The relative potencies for the induction of inflammatory/necrotic effects by the N-BPs were

<table>
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<th>Target gene</th>
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minodronate ≥ zoledronate > alendronate. (ii) The relative potencies with which the non-N-BPs reduced the inflammatory/necrotic effects of the N-BPs were clodronate > etidronate. (iii) Higher concentrations of clodronate and etidronate may be required to reduce the inflammatory/necrotic effects of alendronate (which here were induced by a concentration higher than those used for zoledronate and minodronate). The inhibitory effects of etidronate and clodronate on the inflammation and necrosis were essentially the same. Thus, the data relating to inflammation have been omitted from the following sections.

Effects of Phosphonocarboxylates on the Necrotic Effect of 2 mM Zoledronate As shown in Fig. 4, all four of the phosphonocarboxylates tested in the present study reduced or prevented the necrotic effects of 2 mM zoledronate. Phosphonoformate at 20 mM completely prevented the necrotic effect of zoledronate. Unexpectedly, phosphonoacetate at 5 and 10 mM almost completely prevented the necrotic effect of zoledronate, indicating that its protective effect against the necrotic effects of zoledronate may be greater than that of phosphonoformate, which is a well-known inhibitor of SLC20/34. Complete prevention of the necrosis induced by zoledronate could also be achieved with phosphonopropionate and phosphonobutyrate, but in each case at a higher concentration (50 mM). These results indicate that the relative potencies with which phosphonocarboxylates reduce or prevent the necrotic effects of zoledronate are phosphonoacetate > phosphonoformate > phosphonopropionate ≥ phosphonobutyrate (Table 2).

Effects of Phosphonocarboxylates on the Necrotic Effect of 2 mM Minodronate As shown in Fig. 5, we found that all four of the phosphonocarboxylates tested in the present study reduced or prevented the necrotic effects of 2 mM minodronate. Phosphonoformate at 20 mM completely prevented the necrotic effect of minodronate. Unexpectedly, phosphonoacetate at 5 and 10 mM almost completely prevented the necrotic effect of minodronate, indicating that its protective effect against the necrotic effects of minodronate may be greater than that of phosphonoformate, which is a well-known inhibitor of SLC20/34. Complete prevention of the necrosis induced by minodronate could also be achieved with phosphonopropionate and phosphonobutyrate, but in each case at a higher concentration (50 mM). These results indicate that the relative potencies with which phosphonocarboxylates reduce or prevent the necrotic effects of minodronate are phosphonoacetate > phosphonoformate > phosphonopropionate ≥ phosphonobutyrate (Table 2).
Thus, the relative potencies of phosphonocarboxylates against the necrotic effect of minodronate were similar to those found against the necrotic effect of zoledronate (Table 2).

Effects of Phosphonocarboxylates on the Necrotic Effect of 24 mM Alendronate

As shown in Fig. 6, the necrotic effects of 24 mM alendronate were reduced or tended to be reduced by the phosphonocarboxylates tested. Among these agents, only phosphonoacetate (at 80 mM) completely prevented the necrotic effect of alendronate, while the prevention by phosphonoformate was partial even at 100 mM. No significant preventive effects were detected with either phosphonopropionate or phosphonobutyrate. Thus, the relative antinecrotic potencies of phosphonocarboxylates were similar against alendronate as against zoledronate and minodronate (Table 2).

Effects of Etidronate, Clodronate, Phosphonoformate, and Phosphonoacetate on the Retention of $^{3}$H-Alendronate within Ear-Pinnas

2,3-$^{3}$H-alendronate was the only radio-isotope-labeled N-BP we could obtain commercially. As shown in Fig. 7A, the $^{3}$H-alendronate (2 $\mu$Ci) injected into ear-pinnas was largely lost within 1 h, although a low level was retained even 24 h after its injection, possibly reflecting the amount that had entered cells in the ear-pinna. The necrotic effects of N-BPs, including alendronate, are not detectable at one or two days after their injection (see Figs. 3 to 6). Therefore, we examined whether etidronate, clodronate, phosphonoformate, or phosphonoacetate might reduce the amount of $^{3}$H-alendronate still present within ear-pinnas at 24 h after its injection. As shown in Fig. 7B, each of these agents did so, with their relative effects being clodronate > etidronate > phosphonoacetate > phosphonoformate. This order is similar to that obtained for their abilities to reduce the inflammatory/necrotic effects of N-BPs (see above).

Table 2. Minimum Concentrations (mM) of Non-N-BPs [Clodronate (Clo) and Etidronate (Eti)] and of Phosphonocarboxylates [Phosphonoformate (PFA), Phosphonoacetate (PAA), PhosphonoPropionate (PPA), and Phosphonobutyrate (PBA)] That Completely Protected against the Necrotic Effects of N-BPs in Mouse Ear-Pinnas

<table>
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<tr>
<th></th>
<th>Zol (2 mM)</th>
<th>Min (2 mM)</th>
<th>Ale (24 or 32 mM)*</th>
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<tbody>
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<td>Clo</td>
<td>8</td>
<td>&gt;10</td>
<td>&gt;32</td>
<td>Fig. 2</td>
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<tr>
<td>Eti</td>
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<tr>
<td>PFA</td>
<td>20</td>
<td>50</td>
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<td>Fig. 3–5</td>
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<tr>
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<td>PBA</td>
<td>50</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>Fig. 3–5</td>
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</table>

* In the experiments involving non-N-BPs and phosphonocarboxylates, the concentrations of Ale were 32 and 24 mM, respectively. The data indicate that the relative potencies of the protective effects are Clodronate > Etidronate > Phosphonoacetate > Phosphonoformate > Phosphonobutyrate.
Detection of Phosphate Transporters within Ear-Pinnas

Among the SLC transporter families, three families (SLC17, SLC20, and SLC34) are known to be phosphate transporters. The SLC17 family includes 9 members, while the SLC20 family has 3 members (one pseudo) and the SLC34 family also has 3 members.20–22) Finally, therefore, we examined whether these transporters might be present within the untreated ear-pinnas of mice. As shown in Fig. 8, we could detect the mRNAs of all these phosphate transporter families in mouse ear-pinnas. Among these phosphate transporter families, SLC20 and/or SLC34 have been suggested to be involved in the transport of N-BPs.26) Among the antibodies that would be required to detect them by immunostaining, antibodies against only SLC20a1 and SLC20a2 are commercially available. Figure 9 shows immunostaining results obtained using those two antibodies. The findings indicate that SLC20a1 and SLC20a2 are distributed widely in ear-pinnas. Actually, the mRNAs of SLC20 family members can be detected ubiquitously among various human tissues.23)

DISCUSSION

Summary of the Findings

The main findings made in the present study may be summarized as follows. Like etidronate and clodronate, some phosphonocarboxylates protected against the inflammatory/necrotic effects of N-BPs (zoledronate, minodronate, and alendronate) in mouse ear-pinnas. The rank order of protective potencies was clodronate > etidronate > phosphonoformate > phosphonoacetate > phosphonobutyrate. With a similar rank order of potencies, the first four in that list reduced the amount of 3H-alendronate retained within ear-pinnas after its injection therein. The mRNAs of SLC20 and SLC34 were detected in mouse ear-pinnas. These findings support the hypotheses that (i) N-BPs may enter soft-tissue cells via SLC20 and/or SLC34 transporters, and (ii) inhibition of these phosphate transporters might be a useful strategy in future attempts to prevent and/or treat various side effects of N-BPs. However, it should be noted that phosphonoformate (also known as “foscarnet”) has activities...
other than its inhibition of SLC34 and SLC20. These include: (i) anti-viral activity via an inhibition of viral DNA synthesis due to its interaction with viral DNA polymerase or reverse transcriptase\(^2\) and (ii) inhibition of calcium deposition.\(^2\) Thus, we cannot rule out the possibility that these effects might also contribute to the results observed in the present study via mechanisms whose details remain unknown.

**Significance of the Experimental System** Oral N-BPs directly injure esophageal and gastrointestinal epithelial tissues,\(^2\) and N-BPs (as well as non-N-BPs) accumulate within bones,\(^1\) especially in bones exhibiting inflammation.\(^2\) Thus, it is very likely that if jawbone-accumulated N-BPs are released from the bone, they will directly injure the surrounding soft-tissue cells.\(^2\) It is conceivable that such release of N-BPs from jawbones results from the bone destruction caused by tooth extraction and/or infection and when osteoclasts are killed by N-BPs. Support for such release into soft tissues comes from the detection of zoledronate in the saliva of zoledronate-treated patients.\(^2\) In the present study, injection of N-BPs into mouse ear-pinnas directly induced inflammation and necrosis at the injection site. Since the mevalonate pathway exists widely in eukaryotic cells, and if an N-BP is able to enter cells, it is likely to exhibit cytotoxicity in a wide variety of cell types, not just in osteoclasts. Thus, experiments like ours may be considered a convenient way to obtain information concerning the inflammatory and/or necrotic profiles of N-BPs in the soft tissues that surround bone tissues.

**Mechanism Underlying the Side Effects of N-BPs** In addition to ONJ, N-BPs have several undesirable side effects (such as influenza-like fever, increase in acute-phase proteins, gastrointestinal lesions, and ophthalmic inflammation).\(^3\) The inhibition by N-BPs of farnesyl pyrophosphate synthase results in (a) a decrease in cholesterol, which is an essential structural component of cell membranes, (b) decreases in isoprenoids, which are necessary for the prenylation of small GTPases, leading to the functional disruption of these signaling proteins, (c) increases in the substrates for that enzyme (isopentenyl pyrophosphate and dimethylallyl pyrophosphate), which are converted into cytotoxic ATP analogs within cells, and (d) activation of a human population of γδ T cells (V\(γ\)9V\(δ\)2 T cells) via stimulation by isopentenyl pyrophosphate.\(^2,3\) Moreover, N-BPs induce an enhanced production of interleukin (IL)-1β,\(^3\) an effect that is mediated by mechanism (b).\(^3\) Thus, we suppose that all four of the above effects (a–d) might be causally involved in the inflammatory and/or necrotic effects of N-BPs. Although denosumab [a monoclonal antibody against the receptor activator of nuclear factor-κB ligand (RANKL)] lacks such inflammatory and cytotoxic effects, this potent inhibitor of osteoclasts has also been reported to cause ONJ,\(^3\) suggesting that decreased bone-resorption may be an essential cause of BRONJ. However, reduced osteoclastic activity cannot be the only cause of ONJ, because BRONJ-like ONJ has not been described in osteopetrosis, in which osteoclasts are naturally inhibited.\(^3\) Thus, other factors—possibly, infection or impairment of the defense mechanisms against infection—might be the common, and most important, factor leading to the induction of ONJ by N-BPs and denosumab.\(^2,3,3\) We previously reported that lipopolysaccharide (LPS), a potent inflammatory component of the cell walls of Gram-negative bacteria, augments both the production of the inflammatory cytokine IL-1 and the induction of the histamine-forming enzyme histidine decarboxyl-
culture system and/or a
that (i) specialized techniques (such as an appropriate cell-
hibit the transport of N-BPs. This limitation is due to the fact
clodronate, and phosphonocarboxylates can competitively in -
SLC20 and/or SLC34 families can transport N-BPs and the
effects of N-BPs might involve not only use of etidronate and
or prevent the inflammatory/necrotic side effects of N-BPs.
and moreover that it may be in this way that they can reduce
phosphate transporters in a competitive inhibitory manner,
via
SLC34 and/or SLC20
phosphonoformate), clodronate, and etidronate may inhibit the
phosphonocarboxylates (especially phosphonoacetate and
side effects of N-BPs, the mechanism by which phosphono-
carboxylates and non-N-BPs protect against these side effects
in mice seems to be very simple. Thus, our present findings
suggest that inhibition of the uptake of N-BPs by soft-tissue
cells might become a basis for a useful strategy aimed at pre-
venting and/or treating BRONJ, as well as other inflammatory
side effects of N-BPs.

CONCLUSION

The present findings support the hypothesis that in mice,
phosphono-carboxylates (especially phosphonoacetate and
phosphonoforate), clodronate, and etidronate may inhibit the
entry of N-BPs into soft-tissue cells via SLC34 and/or SLC20
phosphate transporters in a competitive inhibitory manner,
and moreover that it may be in this way that they can reduce
or prevent the inflammatory/necrotic side effects of N-BPs.
We propose that future strategies for the prevention of the side
effects of N-BPs might involve not only use of etidronate and
clodronate, but also appropriate use of phosphono-carboxylates
or their analogs.

PERSPECTIVES

The present study does not provide direct evidence (i) that
SLC20 and/or SLC34 families can transport N-BPs and the
non-N-BPs etidronate and clodronate, or (ii) that etidronate,
clodronate, and phosphono-carboxylates can competitively in-
hbit the transport of N-BPs. This limitation is due to the fact
that (i) specialized techniques (such as an appropriate cell-
culture system and/or a Xenopus oocytes expression system)
are needed for direct measurement of the transportation of
these substances, and such techniques were not available to
us, and (ii) the only commercially available isotope-labeled
bisphosphonate is ³H-alendronate. However, we hope to be
able to perform the necessary experiments in the near future.

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Conflict of Interest The authors declare no conflict of
interest.

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