

# Bone-Specific Alkaline Phosphatase Activity Is Inhibited by Bisphosphonates

## *Role of Divalent Cations*

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### ABSTRACT

Bisphosphonates (BPs) are drugs widely used in the treatment of various bone diseases. BPs localize to bone mineral, and their concentration in resorption lacunae could reach almost milimolar levels. Bone alkaline phosphatase (ALP) is a membrane-bound exoenzyme that has been implicated in bone formation and mineralization. In this study, we investigated the possible direct effect of three N-containing BPs (alendronate, pamidronate and zoledronate) on the specific activity of bone ALP obtained from an extract of UMR106 rat osteosarcoma cells. Enzymatic activity was measured by spectrophotometric detection of *p*-nitrophenol product and by *in situ* visualization of ALP bands after an electrophoresis on cellulose acetate gels. Because ALP is a metalloprotein that contains  $\text{Zn}^{2+}$  and  $\text{Mg}^{2+}$ , both of which are necessary for catalytic function, we also evaluated the participation of these divalent cations in the possible effect of BPs on enzymatic activity. All BPs tested were found to dose-dependently inhibit spectrophotometrically measured ALP activity (93–42% of basal) at concentrations of BPs between  $10^{-5}$  M and  $10^{-4}$  M, the order of potency being zoledronate  $\equiv$  alendronate > pamidronate. However, incubation with excess  $\text{Zn}^{2+}$  or  $\text{Mg}^{2+}$  completely abolished this inhibitory effect. Electrophoretic analysis rendered very similar results: namely a decrease in the enzymatic activity of the bone-ALP band by BPs and a reversion of this inhibition by divalent cations. This study shows that N-containing BPs directly inhibit bone-ALP activity, in a concentration range

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to which this exoenzyme is probably exposed *in vivo*. In addition, this inhibitory effect is most possibly the result of the chelation of  $\text{Zn}^{2+}$  and  $\text{Mg}^{2+}$  ions by BPs.

**Index Entries:** Bisphosphonates; bone-specific alkaline phosphatase; magnesium; zinc; chelation; osteoblasts.

## INTRODUCTION

Bisphosphonates (BPs) are widely used drugs in the treatment of bone diseases such as osteoporosis, Paget's disease, and tumor-associated osteolysis and hypercalcemia (1). BPs have a similar chemical structure to that of inorganic pyrophosphate, in which the P-O-P bond, which links the two phosphate groups, is replaced by a carbon atom (P-C-P structure). This phospho-ether bond is highly resistant to hydrolysis, making these compounds extremely stable under acidic or alkaline conditions. BPs have a structural motif in common, the so-called bone hook, which consists of the two phosphonate groups that facilitate binding to bone mineral (2): the ability to chelate divalent cations and to inhibit the growth of calcium crystal (2). The exact molecular targets and mechanisms of action of BPs have remained unclear for more than 30 yr. Current evidence suggests that BPs not only act on the osteoclasts, inhibiting bone resorption, but also have direct effects on osteoblasts, osteocytes, and macrophages, thus regulating the function of different skeletal cells. Recent studies implicate diverse possible targets for the action of these drugs.

Alkaline phosphatase (ALP) is a membrane-bound metallo-enzyme anchored to the cell membrane (3). Skeletal ALP has been implicated in the processes of bone formation and mineralization (4). Although the physiological role of this enzyme is still unclear, three functions have been proposed for human ALP *in vitro*: phosphohydrolysis of organic phosphomonoesters, phosphotransferase activity, and protein phosphatase activity. ALP catalytic activity depends on a multimeric configuration and requires  $\text{Mg}^{2+}$  as a cofactor (5). Each monomer has one active site and contains two  $\text{Zn}^{2+}$  ions that stabilize its tertiary structure (6).

Because ALP is an exoenzyme that faces the extracellular compartment, it is conceivable that its activity and function could be modulated by environmental conditions. For instance, we have recently provided evidence for the alteration of ALP activity and structure as a consequence of its *in vitro* glycation (7). BPs are widely used *in vivo*, and it has been shown that these compounds selectively locate on the exposed bone mineral surface of resorption lacunae, where their local concentration can reach several hundred micromoles per liter (2). In the present study, we have investigated the direct effect of BPs on osteoblastic-derived ALP activity as well as their possible mechanisms of action by  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  chelation.

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## MATERIALS AND METHODS

### Materials

Alendronate [1-hydroxy-3-aminobutylidene-1,1-bisphosphonic acid] was provided by Elea Laboratories (Argentina); pamidronate [1-hydroxy-3-aminopropylidene-1,1-bisphosphonic acid, disodium salt] and zoledronate [2-(imidazol-1-yl)-hydroxyethylidene-1,1-bisphosphonic acid, disodium salt] were obtained from Novartis (Basel, Switzerland). All BPs were dissolved in water and stored at  $-20^{\circ}\text{C}$ . Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and fetal bovine serum (FBS) were from Gibco, (Life Technology, Buenos Aires, Argentina) and tissue culture disposable material was from Nunc (Buenos Aires, Argentina). Gelatinized cellulose acetate (Cellogel) was from Chemetron (Milano, Italy). Fast blue RR,  $\alpha$ -naphthyl phosphate, *p*-nitrophenyl phosphate (*p*-NPP), and other reagents of analytical degree were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### Cell Culture

UMR106 rat osteosarcoma-derived cells were obtained from American Type Culture Collection (ATCC) (Rockville, MI, USA). Cells were grown in 75-cm<sup>2</sup> flasks at  $37^{\circ}\text{C}$  in a humidified 5% CO<sub>2</sub> atmosphere in DMEM (Gibco, Gaithersburg, MD, USA), supplemented with 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin and 10% FBS. This cell line has been shown to conserve certain characteristics of differentiated osteoblastic phenotype and has been used extensively in our laboratory (8,9). When 70–80% confluence was reached, cells were subcultured using 0.1% trypsin–1 mM EDTA in Ca<sup>+2</sup>–Mg<sup>+2</sup>-free phosphate-buffered saline (PBS). After cells reached confluence, the monolayer was washed with PBS and solubilized in 0.1% Triton X-100. Aliquots of total cell extract were used for protein determination by the Bradford technique (10).

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### Characterization of Bone-Specific ALP in the UMR106 Extract

Aliquots of 0.1% Triton-X100 osteosarcoma extract were preincubated at  $56^{\circ}\text{C}$  for 10 min (11). After this incubation period, ALP activity was assayed as described next. Bone-specific ALP was estimated as a percentage of remaining activity compared to total ALP (samples without heating) in the cell extract.

### Alkaline Phosphatase Activity Assay

The initial rate of ALP activity in the UMR106 cell extract was carried out by spectrophotometric determination of hydrolysis of *p*-NPP to *p*-nitrophenol (*p*-NP) in a glycine–Mg buffer, pH 10.5, at  $37^{\circ}\text{C}$  for 10 min. Formation of the product was assessed by absorbance at 405 nm. Under

Table 1  
Effect of BPs on ALP Activity

Concentration [M]	Pamidronate	Zoledronate	Alendronate
$10^{-5}$	$93 \pm 5$	$90 \pm 2$	$93 \pm 1$
$5 \cdot 10^{-5}$	$71 \pm 1$	$75 \pm 3$	$68 \pm 1$
$10^{-4}$	$65 \pm 4$	$51 \pm 5$	$42 \pm 2$

*Note:* The ALP activity was evaluated after 10 min preincubation with the indicated doses of BPs. Data are expressed as percent of basal  $\pm$  SEM ( $n = 3$ ). The control value was  $40.6 \pm 0.7$  nmol *p*-NP/tube.

these conditions, the reaction proceeds linearly for 30 min. The effect of different bisphosphonates and other agents was tested after a 10-min preincubation with the osteoblastic extract. The inhibitory effect of BPs was determined as a percentage of the basal initial rate of ALP activity.

### ***Separation of ALP Isoforms by Electrophoretic Methods***

To evaluate possible changes in the electrophoretic mobility of ALP after incubation with BPs, aliquots corresponding to 2  $\mu$ g protein were electrophoresed on gelatinized cellulose acetate, at a constant current of 1 mA/cm, for 1 h (7). The enzymatic reaction was then performed by placing the gel on an agar phase containing  $\alpha$ -naphthyl phosphate as the substrate at 37°C for 30 min. The product formed on the cellulose acetate gel,  $\alpha$ -naphthol, was then allowed to react with Fast Blue RR (also in an agar phase) for 5 min at 25°C. The intensity of the ALP-specific bands was quantified by densitometry after scanning of cellulose acetate strips, and images were analyzed using the Scion-beta 2 program.

### ***Statistical Analysis***

For each experimental condition, at least three separate experiments were performed. Data are expressed as the mean  $\pm$  SEM. Statistical differences were analyzed using Student's *t*-test.

## **RESULTS**

The ALP in the UMR106-cell extract was characterized as a bone-specific isoenzyme by a method that utilizes heat inactivation. In the Triton X-100 extract of osteosarcoma cells, the bone isoform represented about 90% of the total ALP.

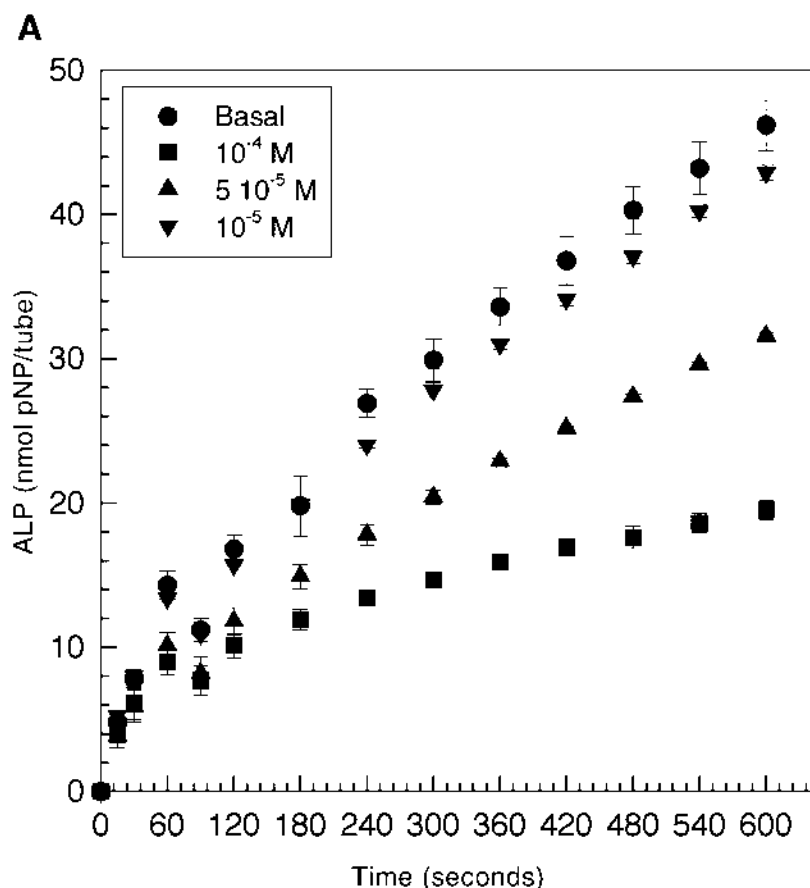


Fig. 1. Dose-dependent effect of BPs on the initial rate of bone-ALP activity. Increasing concentrations of alendronate (a), pamidronate (b), or zoledronate (c) were preincubated with bone-ALP extracts for 10 min. ALP activity was measured as indicated in the Materials and Methods section. Results are expressed as nanomoles of *p*-NP/tube and represented as mean  $\pm$  SEM ( $n = 3$ ). (Figure continues)

Next, we investigated the effect of different BPs on the activity of ALP. Under the conditions tested, the initial velocity of *p*-NPP hydrolysis was inhibited by all BPs. After 10 min of incubation, a significant decrease in ALP activity was observed, the order of potency of BPs being zoledronate  $\cong$  alendronate  $>$  pamidronate (Table 1). As can be seen in Figs. 1a–1c, all BPs assayed inhibited ALP activity in a time- and dose-dependent manner, in the concentration range  $10^{-5}$ – $10^{-4}$  M.

Because ALP is a zinc- and magnesium-dependent metalloenzyme, we investigated if the BP-induced ALP inhibition could be the result of metal chelation by the phosphonate groups of the drugs. To address this question, we examined the effect of  $10^{-4}$  M zoledronate on the ALP activ-

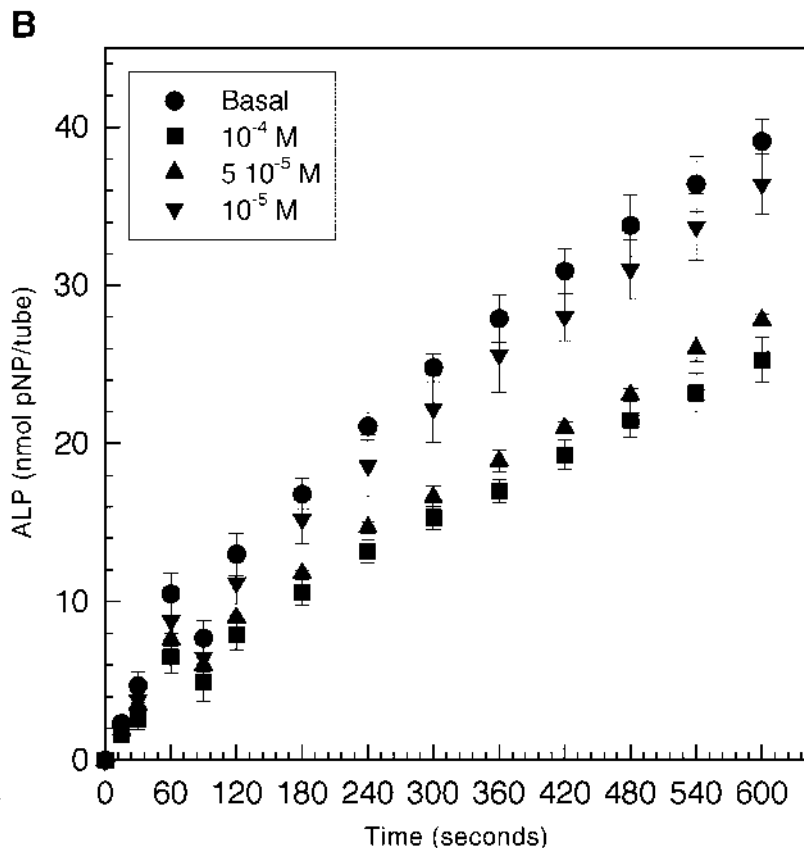


Fig. 1. (Continued)

ity in the presence of an excess of  $\text{Zn}^{2+}$  or  $\text{Mg}^{2+}$ . As can be seen in Fig. 2, ALP inhibition by zoledronate was completely abolished in the presence of  $\text{Zn}^{2+}$  or  $\text{Mg}^{2+}$ .

We next evaluated other possible changes in bone ALP that could have occurred as a consequence of its interaction with BPs. Samples preincubated for 10 min with  $10^{-4}$  M zoledronate with or without  $10^{-6}$  M  $\text{Zn}^{2+}$  or control samples (no additions) were electrophoresed on cellulose acetate gels. The bands of ALP activity were visualized by an *in situ* colorimetric reaction as described in the Materials and Methods section. Figure 3 shows the bone-ALP bands of different samples. A decrease in the intensity of the band, without changes in its mobility, can be seen in the sample incubated with  $10^{-4}$  M zoledronate (lane 3 of Fig. 3). The area under each peak was integrated to estimate ALP activity. These values were in general agreement with the ALP assay using *p*-NPP as a substrate. On the other hand, preincubating the osteoblastic extract with zoledronate

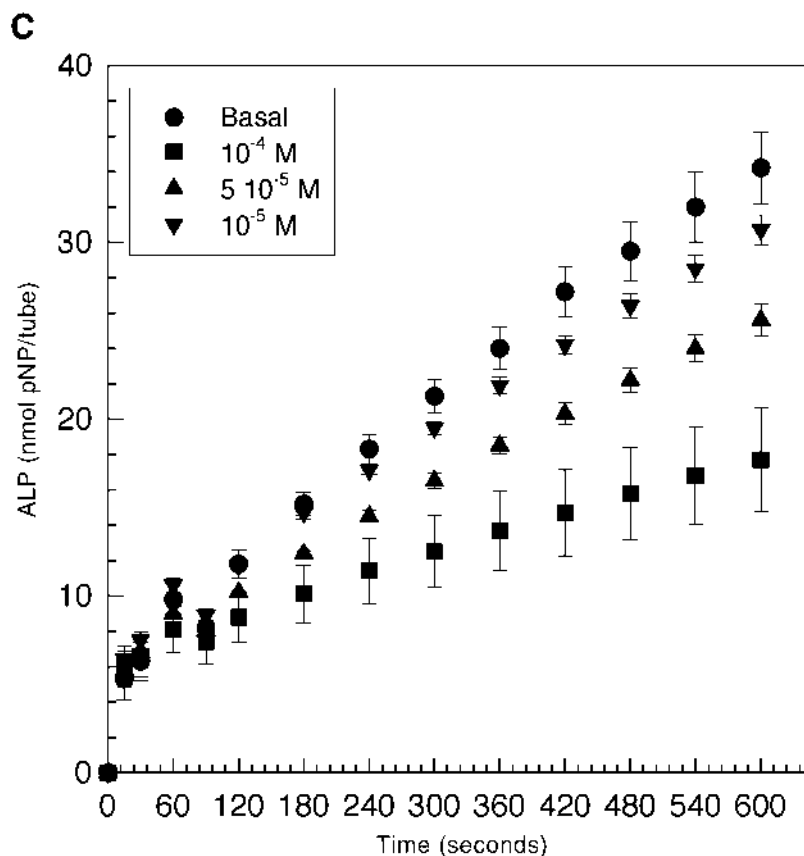


Fig. 1. (Continued)

and zinc prevented the BP-induced inhibition of ALP activity, as can be observed in lane 2 of Fig. 3. These results are also similar to the observations presented in Fig. 2.

## DISCUSSION

Recent reports have shown that BPs not only act on osteoclast bone resorption but also affect the behavior and metabolism of other bone-related cells, such as osteoblasts, osteocytes, and macrophages (1,2). Several authors have reported that BPs affect proliferation and differentiation of osteoblasts, partially by the macrophage-activated protein kinase (MAPK) pathway and through the regulation of different enzymes (12–16). However, few studies have reported the direct effect of BPs on specific osteoblast-derived phosphatases.

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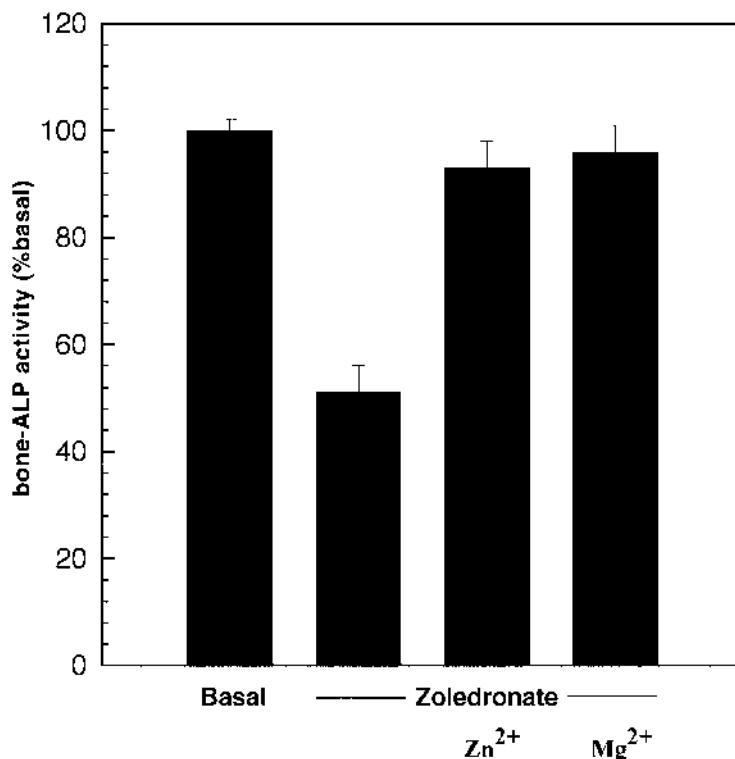


Fig. 2. Reversion of BP-induced inhibition of ALP activity by divalent cations. Aliquots of bone-ALP extracts were preincubated for 10 min with no addition (basal), with  $10^{-4}$  M zoledronate alone, with zoledronate plus  $1 \mu\text{M}$   $\text{Zn}^{2+}$ , or with zoledronate plus  $100 \mu\text{M}$   $\text{Mg}^{2+}$ . After this incubation period, ALP activity of samples was measured as indicated in the Materials and Methods section. Results are expressed as percent basal and represent the mean  $\pm$  SEM ( $n = 3$ ).

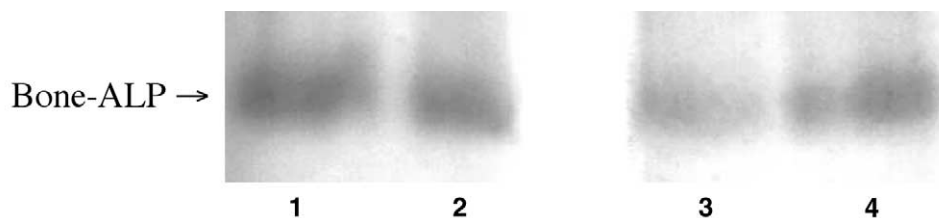


Fig. 3. Bone ALP extracts were preincubated with  $10^{-4}$  M zoledronate (lane 3), zoledronate plus  $1 \mu\text{M}$   $\text{Zn}^{2+}$  (lane 2), or no addition (lanes 1 and 4) for 10 min. Samples were submitted to electrophoresis on gelatinized cellulose acetate, and bone-ALP bands were evidenced as described in the Materials and Methods section.



In this study, we have shown that three nitrogen-containing BPs exerted a direct inhibition of bone ALP. This effect was time and dose dependent. The concentrations tested were chosen based on our previous studies on the effects of these compounds on UMR106 cells (17), in which we reported a biphasic effect of BPs on osteoblastic differentiation, as assessed by ALP specific activity. Low doses of BPs ( $10^{-10}$ – $10^{-5}$  M) stimulated ALP activity, whereas high concentrations ( $10^{-4}$  M) inhibited it. In addition, levels of  $10^{-5}$ – $10^{-4}$  M are estimated to be found in vivo in the resorption lacunae of experimental animal models. Thus, our present observations are physiologically relevant in the context of a local action of BPs used in the treatment of different bone diseases.

It is also interesting to compare the relative in vivo antiresorption potency of the BPs with their in vitro capacity to inhibit bone ALP. It is known that increasing the length of the  $R^2$  side chain attached to the geminal carbon increases the BP's potency to inhibit bone resorption, whereas the -OH group in the  $R^1$  position maximizes its affinity for bone mineral (1). This is possibly the reason for the lower potency of pamidronate (three-carbon  $R^2$  side chain) in comparison with alendronate (four-carbon  $R^2$  side chain) in the present study. In addition, the antiresorptive potency of the amino-alkyl bisphosphonates has been found to be further increased by substitution of the primary amine in the  $R^2$  side chain, with a tertiary nitrogen in a ring structure, which is the case for zoledronate. However, in our present report, alendronate and zoledronate had a similar effect on ALP inhibition.

Mammalian tissue nonspecific ALPs are strongly activated by  $Mg^{2+}$  and also require  $Zn^{2+}$ , whereas the intestinal and placental enzymes are less influenced by these cations (18). The BPs used in the present study have a structural motif in common, the so-called "bone hook," which consists of the two phosphonate groups that bind to mineral. Our present observations suggest that BPs inhibited the activity of ALP through chelation of divalent cations with their bone hook structure. This suggestion is based on the finding that (1) ALP inhibition could be reverted by an excess of  $Zn^{2+}$  or  $Mg^{2+}$ , and (2) zoledronate inhibited the enzymatic activity of the bone-ALP band of electrophoresed samples, an effect that was also prevented by zinc. This mechanism of action could at least partially explain the inhibitory effect of BPs on bone ALP.

In conclusion, this study demonstrates a direct effect of N-containing BPs on bone ALP that is most probably the result of a chelation of divalent cations.

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