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Structurally Different Bisphosphonates Exert Opposing Effects on Alkaline Phosphatase and Mineralization in Marrow Osteoprogenitors

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Abstract Bisphosphonates (BPs) are inhibitors of bone resorption and soft tissue calcification. The biological effects of the BPs in calcium-related disorders are attributed mainly to their incorporation in bone, enabling direct interaction with osteoclasts and/or osteoblasts through a variety of biochemical pathways. Structural differences account for the considerable differences in the pharmacological activity of BPs. We compared the effects of two structurally different compounds, alendronate and 2-(3′-dimethylaminopyrazinio)ethylidene-1,1-bisphosphonic acid betaine (VS-6), in an osteoprogenitor differentiation system. The BPs were examined in a bone marrow stromal-cell culture system, which normally results in osteoprogenitor differentiation. The drugs were present in the cultures from days 2 to 11 of osteogenic stimulation, a period estimated as being comparable to the end of proliferation and the matrix-maturation stages. We found that the two different BPs have opposing effects on specific alkaline phosphatase (ALP) activity, on stromal-cell proliferation, and on cell-mediated mineralization. These BPs differentially interact with cell-associated phosphohydrolysis, particularly at a concentration of 10^{-2} of ALP K_m, in which alendronate inhibits whereas VS-6 did not inhibit phosphatase activity. VS-6 treatment resulted in similar and significantly increased mineralization at 10 and 1 μM drug concentrations, respectively. In contrast, mineralization was similar to control, and significantly decreased at 10 and 1 μM drug concentrations, respectively, under alendronate treatment. J. Cell. Biochem. 68:186–194, 1998.

Key words: osteoprogenitors; marrow-stroma; alkaline phosphatase; bisphosphonates; cell proliferation; mineralization

Bisphosphonates (BPs) are stable analogs of pyrophosphate, a physiological regulator of calcification and bone resorption [Fleisch, 1989]. A number of such geminal BPs have been approved for clinical use in Paget's disease, hypercalcemia of malignancy, and tumor osteolysis [Averbuch, 1993; Blomqvist and Elomaa, 1996; Body et al., 1996; Delmas and Meunier, 1997; Siris, 1997] and, recently, alendronate, in osteoporosis [Fleisch, 1997; Liberman et al., 1995; Raisz, 1997]. BPs, like other bone-seeking agents, are irreversibly trapped with calcium in sites of new bone formation, a property that underlies their use as bone-scanning agents. The biological effects of the BPs in calcium-related disorders are attributed mainly to their incorporation in bone, enabling direct interaction with osteoclasts and/or osteoblasts through a variety of biochemical pathways [Fleisch, 1993; Sahni et al., 1993]. The most significant effects of the BPs is inhibition of bone resorption and calcification of soft tissue [Van Gelder et al., 1995; Van Gelder and Golomb, 1995]. The increase of the gap between the dose eliciting antiresorptive and antimineralization activities on bone, of the second and third generation BPs, allowed the relatively safe utilization of novel compounds in bone resorption disorders.

It is believed that structural differences due to the side-chain composition account for the considerable differences in the pharmacological activity and potency of these compounds [Geddes et al., 1994; Muhlbauer, 1995], but a precise structure-activity relationship as well as mechanism of drug action has not been established. Inhibition of osteoclast-stimulating factors released from osteoblasts results in inhibition of

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bone resorption [Nishikawa et al., 1996; Sahni et al., 1993; Vitte et al., 1996]. Another possibility is a direct effect on osteoclasts' function by toxic effects to cells that ingest the compounds [Fleisch, 1993; Rodan and Balena, 1993; Sato et al., 1991]. On the other hand, it is clear that the high accumulation in bone, due to the high affinity to hydroxyapatite (HAP) through bidentate or tridentate binding with the phosphonic functions and the geminal hydroxyl, is essential for mediating in vitro and in vivo activity [Flanagan and Chambers, 1991; Fleisch, 1993; Geddes et al., 1994; Muhlbauer, 1995]. This type of uptake, and the direct effect on osteoblasts, might mediate actions other than inhibition of bone resorption, like osteomalacia or changes in the quantity and quality of the newly formed bone [Bijvoet et al., 1993]. Structure-activity relationship studies reveal that the hydroxyl group on the geminal carbon atom increases the affinity to HAP [Sunberg et al., 1991; Van Beek et al., 1994]. Derivatives with an amino group on the side chain, or cyclic geminal BPs, especially those containing a nitrogen atom in the ring, are highly active [Geddes et al., 1994].

2-(3′-dimethylaminopyrazinio)ethylidene-1,1-bisphosphonic acid betaine (VS-6) is a novel, cyclic, nitrogen-containing BP (see Fig. 1) that has been shown to be an effective antiresorptive and anticalcification agent [Cohen et al., 1996]. In contrast to alendronate, a clinically used BP in osteoporosis, VS-6 doesn't contain a hydroxyl group on the geminal carbon, and it bears a permanent positive charge on a heterocyclic nitrogen. Therefore, in the present study we compared the effects of these different compounds in an osteoprogenitor differentiation system. To this end, both BPs were examined in a bone marrow stroma-cell culture system, which normally results in osteoprogenitor differentiation [Bellows et al., 1987; Kamalia et al., 1992; Klein et al., 1993a; Leboy et al., 1991]. The drugs were present in the cultures from days 2 to 11 of osteogenic stimulation, the period in which osteoprogenitor cells are selectable by Krebs cycle inhibition. This period was defined by nitropropionate [Klein et al., 1996] and by malonate [Klein et al., 1993a] used to inhibit succinate dehydrogenase, resulting in relative enrichment of the cultures with ALP expressing cells and increasing mineralization. This period is estimated as being comparable to the end of proliferation and to the matrix-maturation stages as defined by others [Pockwinze et al., 1992; Stein et al., 1990]. We found that the two different BPs have opposing effects on specific alkaline phosphatase (ALP) activity, on stroma-cell proliferation, and on cell-mediated mineralization.

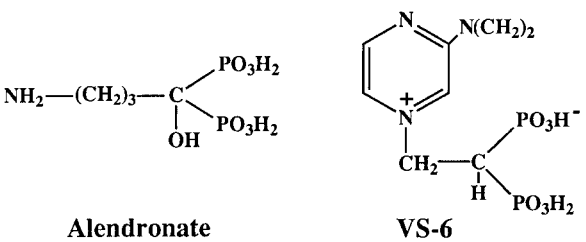

Fig. 1. Chemical structures of tested bisphosphonates.

MATERIALS AND METHODS

Reagents and Drugs

ALP kit 104 LL, dexamethasone, ascorbate, and β-glycerophosphate were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Grand Island Biological Company (Grand Island, NY). Alendronate was synthesized by reacting 4-aminobutyric acid with phosphorus trichloride and phosphorous acid. VS-6[2-(′-dimethylaminopyrazinio)ethylidene-1,1-bisphosphonic acid betaine] was synthesized by reacting 2-dimethylaminopyrazine with vinylidene-1,1-bisphonic acid [V. Solomon, Thesis, Hebrew University, 1997].

Stromal Cell Culture

Bone marrow cell suspensions were obtained from femurs and tibiae of female Sabra rats, weighing 60–80 g. Rats were killed by cervical dislocation, dipped in ethanol, and their femora and tibiae removed by dissection of their joint capsules and ligaments and placed in a sterile hood in medium containing antibiotics at ten-fold concentration. The bones were transferred 4 times every 10 min to antibiotic-rich fresh medium; subsequently, epiphyses were cut to expose the medullary canal. The marrow was ejected from the medullary canal with growth medium using a syringe with a 20-gauge needle. Marrow cells were seeded in 25 cm^2 flasks, 10^8 cells/flask as described in Maniatopoulos et al. [1988]. Stromal cells were obtained by removing the non-adherent hematopoietic cells after the first 10 days of culture. Adherent stromal cells were propagated in maintenance medium, consisting of DMEM (Dulbecco modified Eagle's

medium) supplemented with 15% FCS and antibiotics, at 37°C in a humid 9% CO_2 atmosphere. For osteoprogenitor cell (OPC), stimulation cells were removed 7 days later by trypsinization and plated in 96-well plates, 3,000 cells/well, and grown in ordinary medium or OPC stimulation medium. This consisted of maintenance medium containing 10^{-8} M dexamethasone (DEX), 50 μg/ml ascorbate, and 10 mM β-glycerophosphate, designated DEX-medium.

Alkaline Phosphatase Activity Assay

ALP activity was measured in situ in microtiter plates. On day 11 of dexamethasone stimulation, ALP assay and cell counting [Klein et al., 1993a] was performed after washing the cells twice in situ with 0.2 ml TBS (Tris-buffered saline, 50 mM Tris, 150 mM NaCl, pH 7.6). ALP substrate, p-nitrophenyl phosphate (pNPP) in TBS, 0.665 mg/ml, was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min and optical density of the hydrolyzed pNPP was measured in a multichannel optical densitometer at 405 nm wavelength. This protocol enables long incubation as hydrolysis is 30 times slower than the usual protocol [Klein et al., 1993b]. ALP specific activity was calculated as nMol/90 min/50,000 cells. Lineweaver Burk plots for analysis of ALP inhibition by BPs were generated under similar conditions except that cultures were incubated 45 min with serially diluted pNPP. These reciprocal plots were generated by a Macintosh computer, using the Cricket graphic software.

Quantitative Cell Staining

Cells at the lower half of the 96-well plates were stained using the methylene blue (MB) method. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with distilled H_2O, and air dried over night. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well, was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water, and air dried. The MB was eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min and measured at 620 nm.

Measurement of In Vitro Precipitated Calcium

OPC stimulation medium, as opposed to ordinary medium, induces cell-mediated calcifications in uninhibited cultures after 3 weeks [Klein et al., 1993b]. To measure the precipitates, plates were washed twice with TBS and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by the arsenazo-III metallochromic method adapted to microplates [Klein et al., 1995] against samples of known calcium concentrations. Mineralization was calculated as μg calcium/well and expressed as indices relative to controls.

Statistical Analysis

The significance of the BP effect relative to untreated controls was determined using the paired sample test of Wilcoxon.

RESULTS

BP Dose-Response of Cell Proliferation, ALP Activity, and Mineralization

Cultures grown from day 2 to 11 of DEX stimulation, in the presence of BPs showed a biphasic specific ALP activity (Fig. 2A). At a concentration of 1 μM, alendronate significantly increased specific ALP activity in contrast to VS-6, which slightly decreased it, in comparison to untreated cultures. Alendronate at 1 μM did not change cell counts (Fig. 2B), indicating that cellular ALP expression increased. In contrast, VS-6 increased cell counts proportionally more than decreasing ALP expression, suggesting that VS-6 induced proliferation of cells, whether or not they expressed ALP. This effect of VS-6 on proliferation not discriminating between cells with or without ALP expression was seen also at 10 and 0.1 μM concentrations. Alendronate at 0.1 and 0.01 μM, induced proliferation of cells, which exhibit ALP activity, in addition to increasing their ALP specific activity itself (at 0.01 μM). Thus, the highest increase in ALP expression was exhibited after the cultures have been exposed to alendronate at a concentration of 1 μM. At this concentration, alendronate and VS-6 showed opposing effects on cell proliferation and on ALP and, for each, effects on these parameters showed a reciprocal pattern.

Figure 3 shows the effect of these BP on mineralization relative to the controls. Alendronate at 10 and 1 μM suppressed mineralization. In contrast, VS-6 effect was biphasic; it increased mineralization at 1 μM, and had no effect at all other concentrations used.

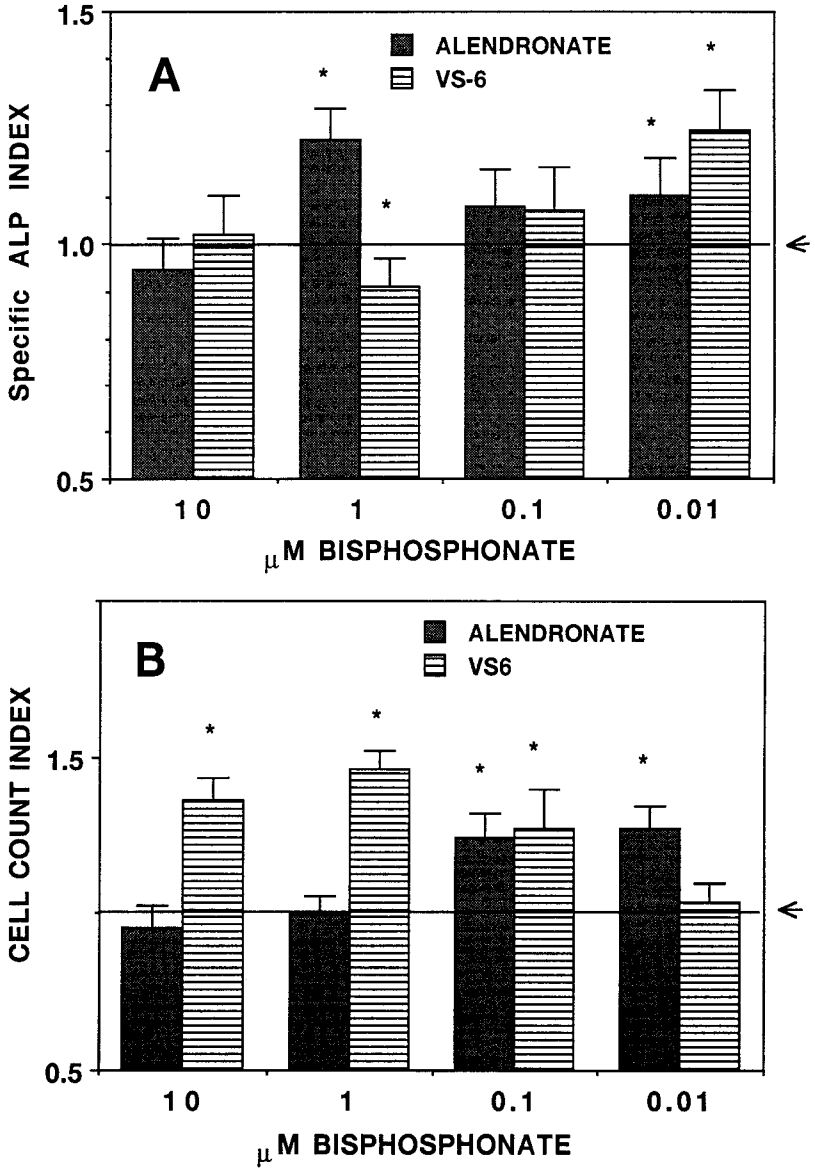

Fig. 2. Effect of bisphosphonates (BPs) on ALP activity and cell counts of differentiating stromal cells. BPs were added on day 2 of DEX stimulation to stromal cell cultures, at indicated concentrations. On day 11 of DEX stimulation, cultures were washed twice with TBS; ALP activity and cell counts were measured. **A:** Specific ALP activity, calculated as nMol/90 min/50.000 cell, is expressed as mean ± SE index relative to respective untreated controls. **B:** Cell counts, measured by methylene blue (Experimental) and expressed as mean ± SE index relative to controls. For each drug concentration, n = 20, arrows point to the unity line indicating no change; asterisks indicate significant BP effect.

Analysis of the Inhibitory Effect of BPs on the Hydrolysis of pNPP

In order to examine to what extent the discrepancy between increased ALP activity and decreased mineralization, following alendronate treatment, is related to inhibition of ALP activity, the inhibitory capacity of ALP by BPs was analyzed. Kinetics of cell-associated phosphatase, hydrolysing pNPP were measured, in the presence of different alendronate and VS-6 concentrations, under conditions described in Figure 2, using Lineweaver-Burk plots. Table I summarizes ALP kinetic under different concentrations of alendronate or VS-6, which were calculated from reciprocal plots described in Figure 4. The plots in Figure 4 represent reciprocals of V/min and [S] data derived from Table II.

Figure 4A shows that 10 and 0.1 μM alendronate were equally efficient in inhibiting pNPP hydrolysis by the cultures, exhibiting a pattern

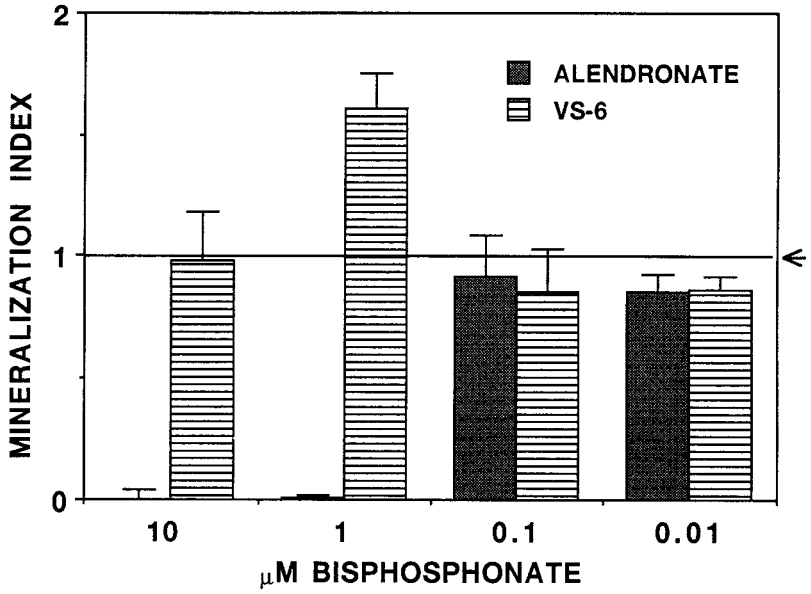

Fig. 3. Effect of BPs on stromal cell-mediated mineralization. Stromal cell cultures were treated as described in Figure 2 legend, and were cultured without BPs from day 11 to 21. On day 21, cultures were washed with TBS and water-insoluble calcium was measured.

TABLE I. Data Derived From Reciprocal Plots of ALP Inhibition by BPs

I[a]	μM[I]	$1/V_{max}$	Vmax[b]	%SEM[c]	Km/Km_{app}[d]	Ki[e]	r[f]	*P**	Inhibition type[g]
None	0	467.17	0.0021	2.8	0.096	0	0.9586	<0.025	None
Alendronate	0.1	456.55	0.0021	3.28	0.202	2×10^{-3}	0.9685	<0.025	Competitive
Alendronate	1.0	508.54	0.0019	2.5	0.102	1×10^{-2}	0.9969	<0.0025	Noncompetitive
Alendronate	10.0	469.36	0.0021	3.14	0.197	2×10^{-1}	0.9989	<0.0001	Competitive
VS-6	0.1	≫0.00002	≪0.0001	90	n.d.[†]	n.d.	n.d.[h]	n.d.	n.d.[i]
VS-6	1.0	405.04	0.0024	2	0.183	1.8×10^{-2}	0.9005[j]	<0.05**	n.d.[k]
VS-6	10.0	501.75	0.0020	3.7	0.073	7×10^{-2}	0.9246	<0.05	n.d.

[†]Not determined.
[a]Inhibitor.
[b]V = velocity in A_{405} U of hydrolysed pNPP/min.
[c]% standard error for V at 1/[S] = 0.4.
[d]Km_{app} = Km of inhibited reaction.
[e]Enzyme-inhibitor dissociation constant derived from "$Intersept_{1/[S]} = 1/Km(1 + [I]/Ki)$."
[f]"r" for regression of mean (n = 20)1/V at 4 different 1/[S].
[g]From geometrical features of reciprocal plots characterizing types of inhibition.
[h]No regression for interpolated curve.
[i]Similar to the pattern of substrate or product interaction [Cleland, 1977 pp 306–8; Mahler and Cordes, pp 251–2].
[j]Using all 80 components of 4 means of this curve resulted in r = 0.5559 for binomial regression.
[k]Probably allosteric cooperatively.
*From tables of critical values of Pearson "r."
**n = 80, $P \ll 0.001$.

characteristic of competitive inhibition, whereas 1 μM alendronate showed a mixed noncompetitive reciprocal plot [Segel, 1976]. Figure 4B shows the analysis for VS-6 interaction with cell-associated phosphatase. Plots indicate complex effects of VS-6 on phosphohydrolysis, which was slightly inhibited by 10 and 0.1 μM. Note that under maximal substrate concentration inhibition, VS-6 at 0.1 μM surged abruptly, resulting in an unusual reciprocal plot pattern. A similar pattern of reciprocal plots was presented as inhibition by substrate or product interaction (Fig. 1 in Cleland, 1977, pp 306–8). The intermediate concentration of VS-6 (1 μM) either activated or did not inhibit phosphohydrolysis in the presence of low and high sub-

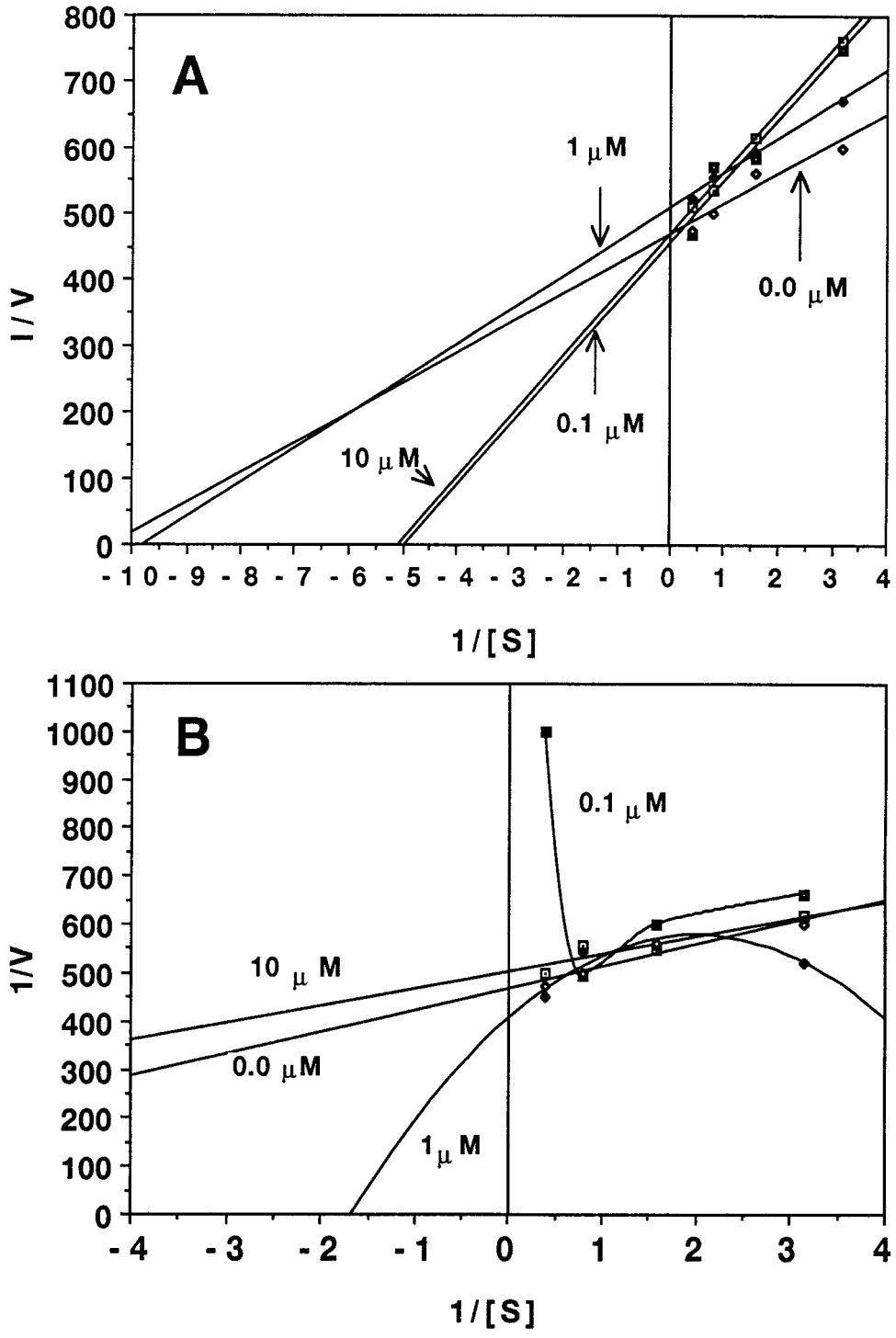

Fig. 4. Lineweaver-Burk plots for alendronate type of inhibition of cell-associated phosphatase. Stromal cell cultures on day 11 of DEX stimulation were washed with TBS. Alendronate **(A)** or VS-6 **(B)** at indicated concentrations was added to separate groups of cultures. Each group was divided into 4 subgroups to which serial dilutions of a saturating dose (2.5 mM) of substrate (S), pNPP, were added. The substrate (S) is represented as its reciprocal (1/S). After a 45-min incubation at pH 7.6 (equivalent to 1.5 min at pH 10.6) [Klein et al., 1993b] supernatant light absorption was measured at 405 nm. Reaction velocity (V) was calculated as accumulated A405 nm units of hydrolytic product/45 min, and is represented as reciprocal velocity (1/V). Each point represents the mean ± SEM of 20 wells. Km = 95 μM for uninhibited plots.

TABLE II. Rate of pNPP Hydrolysis Under Different Inhibiting ([I]) BP Concentrations*

Inhibitor	μM[I]	V[S] = 2.5 mM[a]	V[S] = 1.25 mM	V[S] = 0.612 mM	V[S] = 0.306 mM
None	0	0.095 ± 2.7	0.090 ± 5.4	0.080 ± 3.7	0.075 ± 4.6
Alendronate	0.1	0.096 ± 3.4	0.079 ± 3.5	0.077 ± 7.0	0.060 ± 5.6
Alendronate	1.0	0.086 ± 2.4	0.081 ± 3.9	0.076 ± 3.1	0.067 ± 3.7
Alendronate	10.0	0.088 ± 3.1	0.084 ± 3.4	0.073 ± 4.5	0.059 ± 3.8
VS-6	0.1	0.001 ± 90.0	0.091 ± 2.9	0.075 ± 3.0	0.068 ± 5.3
VS-6	1.0	0.100 ± 2.0	0.083 ± 4.5	0.081 ± 6.1	0.086 ± 6.7
VS-6	10.0	0.090 ± 3.7	0.081 ± 4.4	0.082 ± 4.1	0.073 ± 4.9

*Values represent ± % standard error of the mean for n = 20.
[a]Mean velocity at 2.5 mM pNPP expressed as A_{405} U/45 min.

strate concentrations, respectively, which may represent an allosteric cooperativity pattern [Segel, 1976]. Note that the non-inhibitory effect of 1 μM VS-6 at high and low substrate concentration is consistent with opposing effects of VS-6 on ALP activity (Fig. 2A) vis-a-vis mineralization (Fig. 3), which in turn are reciprocal to the respective effects of alendronate. VS-6 interaction with pNPP hydrolysis indicates allosteric inhibition or activation [Segel, 1976], depending on substrate and inhibitor concentrations and by their effect on enzyme cooperativity. The K_M derived from non-inhibited reciprocal plots was 96 μM.

DISCUSSION

In the present study, we explored the ability of two structurally distinct BPs to affect bone-forming cells in the absence of osteoclastic activity. The 3 parameters, representing 3 phases of growth and differentiation of bone-forming cells [Pockwinze et al., 1992; Stein et al., 1990], showed differential responses to the two tested BPs. The Km of pNPP hydrolysis derived from the reciprocal plots for uninhibited cultures was 96 μM, close enough to the 105 μM found by others [Anagnostou et al., 1996] under similar conditions. The Km of ALP at a more alkaline pH and of detergent-solubilized enzyme was found to be 2.78 mM [Felix and Fleisch, 1979], almost 30 times higher than the Km found in the present study. This difference closely matches with our previous finding that at a high alkaline pH the rate of pNPP hydrolysis was 30 times faster than at pH 7.6 [Klein et al., 1993b]. The pattern of competitive inhibition of ALP by 10 and 0.1 μM alendronate is associated with the insignificant changes in specific ALP activity on day 11, whereas the noncompetitive inhibitory plot for 1 μM alendronate is associated with increased specific ALP after long exposure to the drug. Other BP compounds were shown to increase ALP activity in cultured cells [Felix and Fleisch, 1979; Tenenbaum et al., 1992]. This phenomenon is mechanistically unclear, and yet it is not surprising since levamisole, which is an uncompetitive inhibitor of ecto-ALP [Cyboron et al., 1982], can similarly cause increased ALP activity in long-term cultures [Klein et al., 1993b]. High ALP activity, resulting from prolonged exposure of osteoprogenitor cells to levamisole, was shown to be accompanied by decreased mineralization [Klein et al., 1993b]. Dexamisole, the dextro-isomer of levamisole, is a poor inhibitor of ALP [Nijweide et al., 1981] and yet it decreases mineralization as well [Fallon et al., 1980]. It thus follows that phosphatase inhibitors may decrease mineralization by more than one mechanism. Indeed, inhibition of mineralization by levamisole via ALP takes place during a late (matrix-mineralization) stage, whereas ALP-independent inhibition of mineralization is induced by levamisole, earlier, during matrix-maturation [Klein et al., 1993b]. It is thus possible that alendronate, like levamisole, may inhibit cell-mediated mineralization by another mechanism, unrelated to its competitive and noncompetitive inhibition of ecto-ALP.

Long-term incubation with VS-6, at 10, 1, and 0.1 μM, did not increase specific ALP, but rather a slight decrease of ALP inhibition was observed. Upon long exposure at 1 μM, both drugs showed opposing effects for all 3 parameters, proliferation, ALP activity, and mineralization, and at this concentration each drug induced outstanding effects (other than the simple competitive inhibition) regarding interaction with the enzyme. According to the reciprocal plots, whatever effect by VS-6 on phosphatase (inhibitory or activating), substrate concentration influenced phosphatase activity

by mechanisms distinctly different from simple competition. This implies that VS-6 probably formed complexes with pNPP or its pNP product or with both. Such complexes might interact with the phosphatase, resulting in a mixture of effects, among them allosteric cooperativity [Segel, 1976]. Inhibition by other BPs required inhibitory concentrations at least a hundredfold higher than those used in our study [Shinozaki and Pritzker, 1996]. This discrepancy could reflect the difference between free vs. membrane-integrated enzyme, although for intestinal ALP lack of such difference was demonstrated [Chappelet-Tordo et al., 1974]. Based on the matching between both BPs[1] short- and long-term effects at 1 μM range, we surmise that, during their long-term presence in culture, the BPs interacted with natural ALP substrates and/or products at a similar intensity as they did with pNPP or pNP during the short competition analysis.

Additional mechanisms by which these BPs affect osteoblasts may be related to their ability to enter the cells [Felix et al., 1984]. Clodronate, at 2.5 μM, was shown to diminish glucose utilization and lactate production in calvarial osteoblasts, close to the concentration at which VS-6 induced mineralization. This biochemical features of glycolytic inhibition characterize mineralizing cells [Shapiro and Lee, 1978]. BPs were also shown to slightly inhibit succinic dehydrogenase (SDH) [Rosen and Klotz, 1957], which is consistent with our experience with the SDH inhibitory effect of malonate [Klein et al., 1993a] and of nitropropionate [Klein et al., 1996], resulting in increased mineralization.

Opposing effects exerted by these amino BPs could be attributed to the difference in their structure. VS-6 having a hydroxyl on the geminal carbon and/or the higher affinity of alendronate to Ca^{++} and other metal cations [Cohen et al., 1996], essential for ALP activity, could account for the differential effects.

The higher inhibitory effect of alendronate on mineralization in comparison to VS-6 (Fig. 3) could be due, in part, to its increased chelation properties with Ca^{++} at the extracellular milieu. The complete inhibition of mineralization by alendronate at the higher concentrations (1 and 10 μM) could also result from disruption of the cell membrane, a known side effect of geminal BPs, such as pamidronate and alendronate [de Groen et al., 1996; Twiss et al., 1994]. In addition, the opposing effects of the two BPs on mineralization could be due to difference(s) in intracellular phosphatase activity. Indeed, a recent study suggests that tyrosine kinase phosphatase activity plays an important role in osteoclast formation and function and is a putative molecular target of alendronate action [Schmidt et al., 1996]. It should, therefore, be of considerable interest to compare the effects of these BPs on the osteoblastic phenotype.

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