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Bisphosphonates and Tetracycline: Experimental Models for Their Evaluation in Calcium-Related Disorders

Hagit Cohen,[1] Vered Solomon,[2] Ivan S. Alferiev,[2] Eli Breuer,[2] Asher Ornoy,[3] Natan Patlas,[3] Naomi Eidelman,[4] Gerhard Hägele,[5] and Gershon Golomb[1,6]

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Purpose. This work was aimed at synthesizing novel bisphosphonates (BPs) and examining them in comparison to clinically used BPs such as pamidronate and alendronate, and to tetracycline, in order to evaluate their potential as anticalcification and antiresorption agents. The correlation between the various models was examined in order to establish facile experimental models for pre-screening of potential compounds.
Methods. Nitrogen-containing heterocyclic, novel BPs such as 2-(3-methylimidazolio) ethylidene-1,1-bisphosphonic acid betaine (**VS-5b**), 2-(2-dimethylamino-4-pyrazinio)ethylidene-1,1-bisphosphonic acid betaine (**VS-6b**), and 2-(2-α-pyridylethylthio) ethylidene-1,1-bisphosphonic acid (**ISA-225**), were synthesized and evaluated in comparison to clinically used BPs, in various experimental models of resorption and calcification.
Results. The physicochemical properties of the novel compounds are slightly different than the BPs in clinical use: the pKa values are lower, the affinity for hydroxyapatite is lower and the solubilities of the calcium salts are higher. The anticalcification potencies of the novel compounds were high and ranked as follows: alendronate = pamidronate > **VS-6b** = **VS-5b** = **ISA-225** > **tetracycline**. The in vivo antiresorption activity of **VS-5b** and **VS-6b** in comparison to that of the clinically employed, pamidronate, was shown to be similar and higher, respectively.
Conclusions. The anticalcification activity of the novel compounds as well as that of tetracycline was lower than that of alendronate. The antiresorption activity of **VS-6b** was similar to that of pamidronate. A good correlation between the different models was found, enabling the facile screening of novel compounds. The activities of tetracycline and EDTA highlight the distinct behavior of BPs as "crystal poison." In addition, tetracycline was found to be a potent anticalcification agent in the ectopic calcification model.

KEY WORDS: bisphosphonates; tetracycline; calcification; hydroxyapatite; bone resorption; synthesis.

INTRODUCTION

Bisphosphonates (BPs) have been shown to be effective in the inhibition of bone resorption, inhibition of mineralization of soft tissues, and, at high doses, inhibition of bone mineralization (1–4). Several BPs have been licensed as drugs for the treatment of various calcium-related disorders such as Paget's disease, hypercalcemia of malignancy, tumor osteolysis and, most recently, osteoporosis (5–7).

BPs are irreversibly trapped by calcium of hydroxyapatite (HAP) in the sites of new bone formation, a characteristic 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 (8–10). The inhibition of ectopic calcification is attributed mainly to a physicochemical mechanism (3,4,11,12).

It is clear that structural differences in the side-chains are the cause for the considerable differences in the pharmacological activity and potency of these compounds(13–15). From examining the structure of the newest and most active BPs, such as risedronate (16), and zolendronate (17), it appears that the incorporation of nitrogen-containing heterocyclic rings into the side-chains of the BPs increases their pharmacological activity. We used a facile approach to BPs synthesis involving nucleophilic additions to the double bond of vinylidenebisphosphonic acid which was previously developed by a member of our group (18). We synthesized several nitrogen-containing heterocyclic BPs (Fig. 1). The new BPs obtained contain no OH group on the geminal carbon, resulting in lower affinities to calcium and a better solubility of their calcium/salts complexes, since bidentate, rather than the less soluble tridentate binding with metal cations is expected (11). The latter characteristic could contribute to better compatibility with food and increased oral absorption, well-recognized drawbacks of the BPs currently in clinical use (19,20).

In this report we focus on the physicochemical, anticalcification, and antiresorption effects of the novel compounds in comparison to the clinically used BPs, pamidronate and alendronate. In order to understand the unique action of BPs, and to examine whether the correlations between the various models are valid for other type of compounds, we compared the BPs to the calcium chelators, EDTA and tetracycline.

EXPERIMENTAL

General

The structures of the novel compounds tested are presented in Fig. 1. All reagents used in the characterization of BPs activity were of analytical grade (Sigma, St. Louis, Missouri, USA). HAP (fast-flow grade) was obtained from Fluka (Buchs, Switzerland). Atomic absorption spectroscopy was performed on a Perkin-Elmer 403 (Norwalk, Connecticut, USA) and UV/VIS spectrophotometry was performed on a Uvikon 930 (Kontron Instruments, Zurich, Switzerland).

Elemental analyses were performed by the Analytical Laboratories of the Hebrew University of Jerusalem (Givat Ram, Jerusalem). Infrared spectra were determined on an Analect

[1] Dept. of Pharmaceutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, PO Box 12065, Jerusalem 91120, Israel.
[2] Dept. of Pharmaceutical Chemistry, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, PO Box 12065, Jerusalem 91120, Israel.
[3] Dept. of Anatomy, Faculty of Medicine, The Hebrew University of Jerusalem, PO Box 12272 Jerusalem 91120, Israel.
[4] The American Dental Association Health Foundation, Paftenbarger Research Center, National Institute of Standards and Technology, Gaithersburg, Maryland.
[5] Institut für Anorganische Chemie und Strukturchemie, Heinrich Heine Universität, Düsseldorf, Germany.
[6] To whom correspondence should be addressed. (e-mail: golomb@cc.huji.ac.il)

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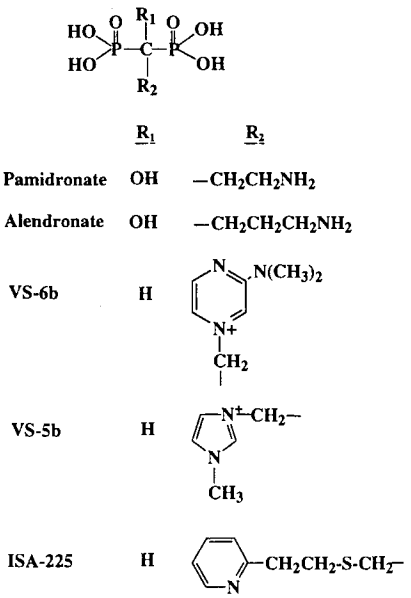

Fig. 1. Structures of BPs examined in this study (pamidronate and alendronate are in clinical use).

FTIR spectrometer (Analect Instruments FX-6160, Irvine, California). Nuclear magnetic resonance spectra were determined on a Varian VXR-300S (Palo Alto, California) instrument; ^1H NMR and ^{31}P NMR were recorded in deuteriochloroform or in deuterium oxide solutions. Chemical shifts are reported as parts per million from tetramethylsilane (TMS) as internal standard in ^1H NMR and from 10% H_3PO_4 as external standard in ^{31}P NMR; positive chemical shifts are at low field with respect to the standards.

1. Syntheses

All reagents used were of analytical grade. 1-Methylimidazole and chloropyrazine were purchased from Aldrich Chemical Company, Milwaukee, WI, USA. Alendronate, [^{14}C]-alendronate (Sp.Act. 267μCi/mg), and pamidronate were synthesized as reported (21).

2-(3-Methylimidazolio)ethane-1,1-bisphosphonic Acid Inner Salt (***VS-5b***)

A mixture of vinylidenebisphosphonic acid (18) (1.98 g, 9.9 mmol), 1-methylimidazole (1.46 g, 17.8 mmol) and water (1 ml) was heated at 98–100°C, and stirred with a glass rod until the mixture became homogeneous. After 10 minutes the mixture solidified. Heating was continued for 80 min. and the solid obtained was dissolved in water (120 ml). The solution was passed through a Dowex-50 cation exchange column, H^+-form. The column was washed with water until the pH of the eluate was 4.5 (approx. 800 ml of water). The eluate was evaporated nearly to dryness. The residue was dissolved in methanol and the solid was collected by filtration, washed with methanol (30 ml) and dried in vacuum. The product was purified by dissolving in water (90 ml) at 70–80°C and concentrating the solution *in vacuo*. The crystals were filtered, washed with water (2 × 0.5 ml) and with methanol (20 ml) and dried *in vacuo*. Yield 2.54 g, 94%. M.p. 264°C (dec.). NMR (D_2O + Na_2CO_3, pH = 9.5): ^1H, 2.16 (tt 1 H $^3J_{HH}$ = 7 Hz, $^2J_{HP}$ = 20.5 Hz), 3.67 (s 3 H), 4.33 (dt, 2H, $^3J_{HH}$ = 7 Hz, $^3J_{HP}$ = 13.0 Hz), 7.1 (d, 1 H, J = 2.1 Hz), 7.34 (s, 1 H, J = 2.1 Hz), 8.55 (s, 1 H); ^{31}P: 14.3 (dt, ^2J = 20.7 Hz, ^3J = 13 Hz). Anal. Calc. for $C_6H_{12}N_2O_6P_2$: C, 26.68; H, 4.48; N, 10.37. Found: C, 26.80; H, 4.44; N, 10.44.

2-(3-Dimethylaminopyrazinio)ethane-1,1-bis-phosphonic Acid Inner Salt (***VS-6b***)

A mixture of vinylidenebisphosphonic acid (2.01 g, 10.05 mmol), 2-dimethylaminopyrazine (2.6 g, 21.11 mmol) and water (1 ml) was heated at 98–100°C, and stirred with a glass rod until homogeneous. Heating was continued for 30 min. and the yellow solid obtained was dissolved in water (100 ml). The pH of the solution was adjusted to 5.5 by the addition of sodium bicarbonate powder, the volume was adjusted to 120 ml and the solution was passed through a Dowex-50 cation exchange column, H^+-form. The column was washed with water until the pH of the eluate was 4.5. The eluate was evaporated nearly to dryness. The residue was dissolved in methanol and the crystals were collected by filtration, washed with methanol (30 ml) and dried in vacuum. The product was purified by dissolving in water (200 ml) at 50–60°C and concentrating the solution *in vacuo*. The crystals were filtered, washed with water (4 × 0.5 ml) and with methanol (20 ml) and dried *in vacuo*. Yield 2.7 g, 86%. M.p. 225°C (dec.). NMR (D_2O + Na_2CO_3, pH ∂ 5.5): ^1H, 2.72 (m 1 H), 3.23 (s 6 H), 7.69 (d, 1 H, J = 3 Hz), 8.15 (s, 1 H), 8.38 (d, 1 H, J = 3 Hz).^{31}P: 13.1 (dt, ^2J = 21.4 Hz, ^3J = 12.8 Hz). Anal. Calc. for $C_8H_{15}N_2O_6P_2$: C, 30.88; H, 4.86; N, 13.50. Found: C, 30.70; H, 4.87; N, 13.48.

2-[2-(2-Pyridyl)ethylthio]ethane-1,1-bisphosphonic Acid (***ISA-225***)

2-(S-Isothiuronio)-ethane-1,1-bisphosphonic acid betaine: A mixture of water (3.0 ml), vinylidenebisphosphonic acid hydrate (2.26 g, containing 2.18 g of anhydrous acid, 11.6 mmol) and thiourea (2.15 g, 28 mmol) was heated in a bath at 106°C for 30 min, diluted with methanol (10 ml) and water (2 ml). The crystals were filtered, washed with methanol water and dried to give 3.06 g 2-(S-isothiuronio)-ethylidene-1,1-bisphosphonic acid betaine which was used in the next step without further purification.

2-[2-(2-pyridyl)ethylthio]-ethane-1,1-bisphosphonic acid: 2-(S-isothiuronio)-ethane-1,1-bisphosphonic acid betaine (6.1 g, 25 mmol) was added in one portion to a mixture of 10.9 moles of aqueous methylamine solution (25 ml) and water (5 ml) cooled in an ice bath. The mixture was stirred until homogenicity. Stirring was then continued at ambient temperature overnight in an inert atmosphere. The excess methylamine was removed by vacuum, and the residue was diluted to 100 ml by water and passed through a Dowex 50 cation exchange column (H^+ form) which was then eluted by water until it reached neutrality. The eluate (ca. 200 ml) containing 2-mercaptoethane-1,1-bisphosphonic acid was neutralized with triethylamine (7 ml, 50 mmol) and immediately (to prevent oxidation) concentrated in vacuum to a syrup which was mixed under argon with freshly distilled 2-vinylpyridine (2.7 ml, 25 mmol) and allowed to stand at ambient temperature for 4 days. The mixture was diluted with water to 200 ml and passed through

a cation exchange column (H^+) and washed with water. A concentration was formed from the eluate (1.1 liter), and the crystals were filtered off and washed successively with a small amount of water, ethanol and acetone. Finally, they were air-dried to give a product of 7.89 g, (96%). An analytical sample was purified by dissolving in water and triethylamine and reprecipitation by 35% hydrochloric acid. NMR (D_2O + Na_2CO_3, pH = 7): ^1H, 2.05 (tt, 1H, J = 20 Hz, J = 7 Hz); 2.90 (4H, m); 2.98 (td, 2H, J = 15 Hz, J = 7 Hz) 7.1–7.4 (m, 2 H) 7.65 (td, 1H, J = 8 Hz, J = 2 Hz); 8.30 (d, 1H, J = 5 Hz). ^{31}P, 22.67 ppm (dt, ^2J = 20.8 Hz, ^3J = 14.5 Hz). Anal: Calc. for $C_9H_{15}NO_6P_2S$: C, 33.03; H, 4.62; S, 9.80. Found: C, 33.12; H, 4.86; S, 9.86.

2. Characterization of the Physicochemical Properties of the Novel BPs

2.1. Dissociation Constants (pKa Values)

The dissociation constants of the novel compounds were determined by potentiometric titration with NaOH [G. Hägele, R. Classen, B. Drenker, and E. Breuer, in preparation]

2.2. Solubility of Ca-bisphosphonate Complexes/Salts

Solutions containing BPs at different concentrations (0.1–10 mM) and calcium at physiological concentration (2.5 mM) were dissolved in Tris buffer pH 7.4, shaken (100 rpm) and incubated at 37°C overnight. After centrifugation, the amount of calcium in the filtrate was determined by atomic absorption spectroscopy. In some cases a constant BP concentration (5 mM) and different concentrations of calcium (0.1–5 mM) were used and the amount of BP in solution was determined as above.

2.3. Adsorption of BPs to HAP

HAP (150 mg) was equilibrated in 50 ml of 0.05 M Tris buffer for 24 h at 37°C. The BPs tested (at concentrations of 0.1–1 mg/ml) were dissolved and the solutions were shaken at 37°C. After 24 h aliquots of 1 ml were centrifuged and the concentrations of the BPs were determined by a UV spectrophotometer as follows: **ISA-225, VS-5b, VS-6b** at λ = 262, 215, 263 nm, respectively. For [^{14}C]-alendronate, the amount of drug was determined by adding 5 ml of a liquid scintillation cocktail (Atomlight, NEN, Boston, MA) and counts per minute (cpm) were measured in a Kontron-Beta V liquid scintillation counter (Kontron Instruments, Zurich, Switzerland) with a suitable calibration curve.

3. Evaluation of an Antimineralization and Anticalcification Activity

3.1. Inhibition of HAP Formation

The inhibition of HAP formation in the presence of the tested compounds was studied in a supersaturated calcium phosphate solution, as described previously (11). In brief, the HAP concentration product of calcium ($CaCl_2 \cdot 2H_2O$) and phosphate (K_2HPO_4) in the incubated solutions was 9 mM^2, calcium 3.87 mM and phosphate 2.32 mM, yielding a molar ratio of $Ca/PO_4 = 1.67$, as in HAP. Each salt solution was prepared in 0.05 M Tris buffer, pH 7.4, and the tested drug was dissolved in the phosphate solution. After 24 h of incubation, calcium concentrations in the filtrates were determined by atomic absorption spectroscopy (22). Representative solid phases were washed with dd H_2O, lyophilized and characterized by FTIR spectroscopy (Nicolet Magna-IR™ FT-IR Spectrometer 550, KBr pressed pellet technique, 0.6–0.9 mg sample/400 mg KBr).

3.2. Inhibition of Ectopic Calcification

The anticalcification effect of the BPs *in vivo* was studied by examining the inhibition of bioprosthetic tissue calcification implanted subdermally in rats (3,4,11,12,22). Bioprosthetic heart valve tissue cusps were prepared from bovine pericardium treated with glutaraldehyde, as described previously (22). Mini osmotic pumps (ALZET 2001, Alza, Stanford, CA) containing 0.014 M drug solution, delivering 1.68 μmol/kg/day for 14 days of the tested compound, were placed next to subcutaneous bioprosthetic tissue cusps (1 × 1 cm) implanted in the dorsal part of each ether-anesthetized rat. One tissue cusp, implanted subcutaneously in the abdominal wall of each animal, served as a paired control. An additional group of rats receiving bioprosthetic tissue implants without treatment, served as an unpaired control. Euthanasia was carried out by ether 14 days after implantation, and the amount of calcium in the retrieved tissues was determined by atomic absorption spectroscopy on aliquots of HCl hydrolyzates. The amount of calcium was expressed as microgram calcium per milligram dry tissue weight. The control values represent the average of the data of both control groups since there was no difference between these groups.

4. Evaluation of Effects on HAP and Bone

4.1. Inhibition of HAP Dissolution

HAP tablets (300 mg, 0.5% magnesium stearate) were prepared in a carver press. Their size (10 mm in diameter) was selected following preliminary experiments. The tablets were covered with beeswax, leaving one surface exposed. The tablets were incubated at 37°C and stirred overnight with 10 ml Tris buffer, pH 7.4, containing the tested drug (0.24 or 0.048 mM). The HAP tablets with the adsorbed drugs were transferred to 10 ml acetate buffer, pH 5 and stirred at 37°C. At each time point, aliquots of 1 ml were collected and analyzed for dissolved calcium by atomic absorption spectroscopy. In order to maintain constant HAP:solution ratio, the dissolution medium was replenished with 1 ml of acetate buffer after each sampling.

4.2. Effects on Bone Development

Three-week-old male rats (Hebrew University Sabra strain) were treated by daily intramuscular injections for 14 days with the following drugs: **VS-5b, VS-6b** at a dosage of 0.1 mg P/kg/day and were compared to pamidronate. The control group received normal saline. The weight of the animals was monitored over the experimental period and the animals were examined for general signs of toxic effects. After 15 days the animals were euthanized by an overdose of ether and both tibiae were removed for examination. One tibia was fixed in 10% buffered formalin, partially decalcified in formic acid, and embedded in glycol methacrylate. Using computerized histomorphometry, we studied the structure of 2–3 μm thick longitu-

dinal sections from the upper tibiae, concentrating especially on the amount of bone and cartilage in the upper metaphysis (23). The other tibia was cleaned of excess tissue, dried overnight at 100°C, weighed and ashed after which the calcium, phosphate and magnesium content were determined (24). Additionally, blood samples from the abdominal aorta were collected and centrifuged. Following the centrifuging, the amounts of calcium, magnesium and phosphorus, and alkaline phosphatase activity were determined (24).

Statistics

The various parameters measured were expressed as mean ± SD and were statistically evaluated according to the student's t-test or paired t-test ($p < 0.05$).

RESULTS

Physicochemical Properties

Table I shows the dissociation constants of the novel BPs in comparison to clinically employed BPs. The various BPs (except for **VS-5b**) have 5 dissociation constants. All BPs have four ionizable P-OH groups. In contrast to pamidronate and alendronate in which the highest pKa was attributed to the loss of proton from the NH_3+ group (19), the situation is different for the two novel bisphosphonates. Titration of **VS-5b** in aqueous medium revealed only four pKas since the second nitrogen in the imidazol ring is not sufficiently basic to be protonated under these conditions. In **VS-6b** the pKa of N-4 can be estimated by comparing it to the two reported pKa values of pyrazine ($pKa_1 = -5.78$, for pKa_2 the values reported range from 0.5 to 1 (25) and to that of 2-dimethylaminopyrazine ($pKa_2 = 3.24$). The pKa of N-4 in fully protonated **VS-6b** should be compared to the pKa_1 (value not available) of 2-dimethylaminopyrazine (both containing doubly positively charged rings). The approximate value of this pKa may be estimated as being less than 0, based on the pKa_1 of pyrazine ($= -5.78$) by adding the effect of the 2-dimethylamino group (which raises the pKa_2 of pyrazine from 1 to 3.24). In summary, the first pKa of **VS-6b** corresponds to the loss of proton from the protonated N-4 nitrogen, while the other pKa's correspond to successive ionization of the two phosphonic groups.

Figure 2 presents the solubility of the calcium-BPs salts/complexes. At a concentration of 1 mM a decrease of 50% in calcium concentration in the filtrate was observed for alendronate and pamidronate. In contrast, the **Ca-VS-5b** and **Ca-VS-6b** complexes were soluble at this concentration. The **Ca-ISA-225** complex exhibited a bi-phasic solubility pattern with a low solubility at lower concentrations and high solubility at higher concentrations of the bisphosphonate.

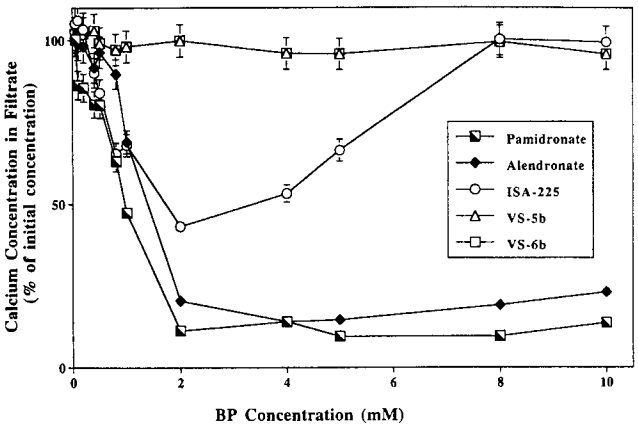

Fig. 2. The solubilities of bisphosphonate-calcium complexes at different bisphosphonate concentrations in a 2.5 mM calcium chloride solution at pH = 7.4 (n = 4).

Fig. 3 shows the adsorption isotherms of the BPs tested to HAP. These adsorption isotherms are based on the equation C/Q = C/N + 1/Kn, where C (mol/liter) is the equilibrium concentration of the solution, Q (mol/gram) is the amount adsorbed of the tested compound, and N (mol/gram) is the amount adsorbed at saturation. These curves reached a plateau upon saturation of the adsorption site. The N values were obtained from the slope of the Langmuir plot (N= 1/slope), and the affinity of the various BPs to HAP was ranked as follows: alendronate > **ISA-225** > **VS-5b** > **VS-6b.**

The compounds were found stable for weeks in aqueous solutions of pH 7–8 as shown by ^1H and ^{31}P NMR spectroscopy. In addition, the compounds retained full stability and activity after 14 days in pH of 7.4, at 37°C *in vivo*, as evidenced from anticalcification studies (see below).

Anticalcification Effects

Table II presents the effects of the various BPs on the extent of HAP formation. All BPs tested significantly inhibited HAP formation, both at low and high concentrations (0.1 and 1 M). The anticalcification activity of alendronate and pamidro-

Table I. The pKa Values of Various BPs

Compound	pKa_1	pKa_2	pKa_3	pKa_4	pKa_5
Pamidronate[a]	< 1	2.55	5.83	9.90	10.84
Alendronate[b]	0.80	2.20	6.30	10.90	12.20
VS-6b	0.37	1.48	1.83	5.75	10.00
VS-5b	–	1.36	1.77	5.93	10.36
ISA-225	0.50	1.62	2.36	5.74	7.10

[a] Kabachnik et al. (42).
[b] Lin et al. (19).

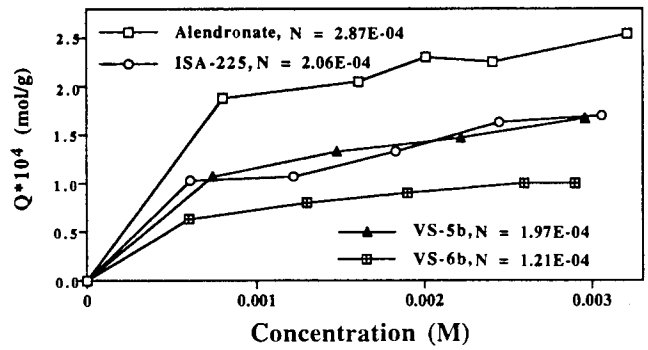

Fig. 3. Adsorption curves of the BPs tested to HAP (upper) and Langmuir isotherm of the BPs. The N value (maximum concentration of adsorption sites is calculated from the Langmuir isotherms by linear regression analysis, n = 5).

Table II. The Inhibition of Hydroxyapatite (HAP) Formation Extent in a 9mM^2 Calcium Phosphate Metastable Solution by Bisphosphonates, and the Inhibition of HAP Dissolution *In Vitro* (At Steady State) by Adsorbed Bisphosphonate. The Amount of Calcium Dissolved in the NaCl Group was Termed as 100%

	Inhibition of HAP formation (% of initial concentration)		Inhibition of HAP dissolution (% of control)	
Compound	1.0 (mM)	0.1 (mM)	0.24 (mM)	0.048 (mM)
NaCl	18.0 ± 0.5	17.0 ± 2.1	100.0 ± 6.2	100.0 ± 3.6
Alendronate	55.2 ± 1.2[a]	85.3 ± 6.8[a]	44.9 ± 3.8[a]	82.0 ± 8.3[a]
Pamidronate	46.9 ± 1.8[a]	63.8 ± 1.9[a]	55.7 ± 2.6[a]	80.5 ± 4.9[a]
VS-5b	88.9 ± 4.9[a]	75.5 ± 2.3[a]	58.6 ± 6.1[a]	84.4 ± 8.4[a]
VS-6b	88.6 ± 4.8[a]	82.5 ± 5.3[a]	62.0 ± 8.1[a]	84.0 ± 4.0[a]
ISA-225	67.3 ± 0.8[a]	73.0 ± 7.1[a]	71.6 ± 10.6[a]	85.2 ± 3.4[a]
Tetracycline	76.5 ± 1.2[a]	80.6 ± 8.8[a]	94.3 ± 12.6[a]	85.2 ± 3.4[a]
EDTA	93.3 ± 1.5[a]	37.7 ± 3.2[a]	—	—

[a] Differences were termed statistically significant by the student's t test (n = 10, mean ± SD, $p < 0.05$).

nate was found to decrease at the higher concentration and was lower than those of the novel compounds. This apparent inactivity was due to the low solubility of the calcium salts/complexes of the former two drugs, rather than increased HAP formation (11). EDTA and tetracycline at 0.1 mM concentrations significantly inhibited HAP formation (Table II). Increasing the concentration of EDTA to 1 mM resulted in increased inhibition of HAP formation. In contrast, the effect exhibited by tetracycline was similar at 0.1 mM and 1 mM concentrations.

FT-IR spectra of the solid phases collected from the experiments carried out with 0.1 mM **VS-6b, ISA-225,** pamidronate, and saline (control) are shown in Fig. 4. The IR spectra confirmed that the calcium phosphate phase formed in the control and in the BP-containing experiments was apatite. The main characteristic IR absorptions of the PO_4 group of apatite can be seen in the 600 and 1000 cm^{-1} regions: 4 PO_4 bands at 564 and 602 cm^{-1} and 3 PO_4 stretch bands at 961–962 and 1034–1037 cm^{-1}. These bands can be seen in the spectra of the experiments done with the tested BPs as well as in the control. The characteristic absorptions of CO_3 can be seen at 1422 and 1455 cm^{-1} in the control, and with lower intensities in the pamidronate-containing spectrum (1420 and 1457 cm^{-1}, see insert in Fig. 4). This carbonate which incorporated into the formed apatite is most probably due to dissolved CO_2 in the buffer solution. Additional absorptions that are not seen in the control appear especially in the 1400–1700 cm^{-1} region in the spectra of the precipitates collected from the experiments with both **ISA-225** and **VS-6b** (insert, Fig. 4). The absorptions at 1630 and 1627 cm^{-1} in the spectra of **ISA-225** and **VS-6b,** respectively, are the stretching mode of the C=N bonds in the aromatic rings of both BPs. The absorptions at 1600 and 1554 cm^{-1} in the **ISA-225** spectrum are the quadrant stretch bands of the monosubstituted pyridine rings and the absorptions at 1477 and 1463 cm^{-1} are the semicircle stretch bands of the monosubstituted pyridine rings. The absorption at 1553 cm^{-1} in the **VS-6b** spectrum is the quadrant stretch band, and those at 1457 and 1432 cm^{-1} are the semi circle stretch bands of the ring diazines. Additional aryl-type CH stretch bands can be seen in the 3000 cm^{-1} regions of the spectra of **ISA-225** and **VS-6b.** No conclusive additional absorptions could be noticed in the apatite containing pamidronate.

Figure 5 depicts the inhibition of bioprosthetic heart valve tissue calcification by the various compounds. All BPs significantly inhibited tissue calcification in the following order: alen-

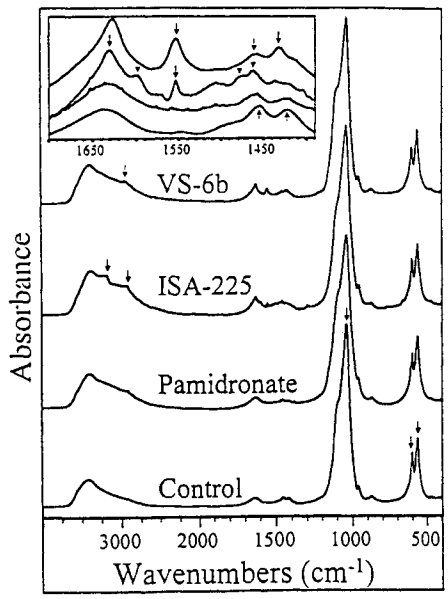

Fig. 4. FT-IR spectra of HAP precipitates form in 9mM^2 calcium-phosphate solution in the presence and absence (control) of 0. 1 mM BPs (400–4000 cm^{-1}, scans = 256, resolution = 2). Insert: zoom to the 1390–1700 cm^{-1} range, spectra appear in the same order as in the 400–4000 cm^{-1} range.

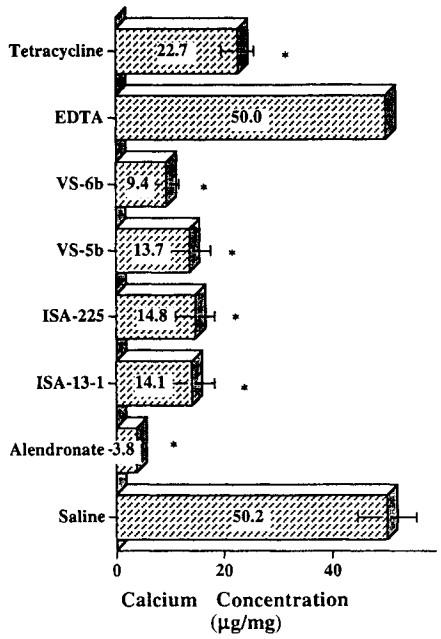

Fig. 5. The effect of bisphosphonates, EDTA and tetracycline (0.014 M) delivered by miniosmotic pumps on bioprosthetic heart valve tissue calcification implanted subcutaneously in rats for 14 days. *Differences were termed statistically significant by the paired t test (n = 6, mean ± SD, $p < 0.05$).

dronate = pamidronate > **VS-6b = VS-5b = ISA-225** > tetracycline. EDTA was found inactive as saline. The anticalcification effects of the various compounds were not associated with adverse effects on somatic growth, as evidenced by the normal weight gain of the treated animals.

Effects on HAP Dissolution and Bone

Table II shows the effect of BPs on dissolution of HAP tablets at pH 5. At a drug concentration of 0.24 mM, **VS-5b** and **VS-6b** were found to be as effective as pamidronate, and slightly less effective than alendronate, in inhibiting HAP dissolution in this *in vitro* model. At a drug concentration of 0.048 mM, no significant differences were found between the compounds examined. Both chelators, EDTA and tetracycline were found inactive in the HAP dissolution model.

Table III and Fig. 6 show the effects of daily IM injection for 14 days on bone development. After 14 days of treatment, no significant differences in weight gain were found between the control and the experimental groups. In addition, no significant effect on alkaline phosphatase activity was observed in the experimental groups, indicating normal bone turnover. Similarly, no significant differences in serum calcium, magnesium and phosphorus levels were found between the control and experimental groups (data not shown). Following treatment with **VS-5b** and **VS-6b,** the bone weight and ash quantity increased in comparison to the control group (Table III). Treatment with pamidronate caused an increase in bone weight and an insignificant increase in ash quantity. Bone volume was positively affected (Table III) by the drug tested in the following rank: **VS-6b** > **VS-5b** = pamidronate. Histological examination of longitudinal sections through the upper tibial metaphysis of control and treated rats revealed relatively thin longitudinally oriented metaphyseal bone trabeculae in the control group. In the pamidronate treatment group, the metaphysis was partially occupied by calcified cartilage/chondrocytes, whereas wide metaphyseal bone trabecula was observed in rats treated with **VS-5b** and **VS-6b** (Fig. not shown).

DISCUSSION

Physicochemical Properties

In order for a bisphosphonate to be biologically active, it has to be able to bind to HAP and accumulate in bone (8), even though the cellular effects probably dominate the potency (26, 27). Alendronate was found to have the highest chemiadsorption to HAP (Fig. 3). Alendronate contains a hydroxyl group in R_1 which contributes a third binding site (in addition to the two phosphonate groups) that permits tridentate binding and increases the binding strength (13,16,28). Binding strength of BPs to HAP has been correlated with activity (29, 30). The heterocyclic N-containing novel BPs exhibited lower binding affinity than alendronate, therefore, a lower pharmacological potency could be expected due to decreased drug concentrations at the action site. Since the ionizability at physiological pH directly affects the ability of the compound to bind to the bone, it could also affect activity. However, the requirement for activity of at least three ionized oxygens at physiological pH (3,11,31) is fulfilled by all examined BPs. Thus, the small differences in the pKa values between the compounds are not likely to be the cause of the differences in activity.

Better solubility of BPs and their metal salt/complexes could enhance oral absorption by increasing the concentration of available drug for permeation as well as by decreasing incompatibility with food in the GI tract. The Ca salts/complexes of **VS-5b** and **VS-6b** exhibited markedly better solubility than those of alendronate and pamidronate (see Fig. 2). This is most probably because bidentate complexes, rather than the less soluble tridentate ones with metal cations were formed (11). The Ca-salt of **ISA-225** was found insoluble at relatively low concentrations and soluble at relatively higher concentrations. It has been reported that such bi-phasic solubility behaviour of complexes could be a function of the number of the ligands bound (32). Moreover, it has been reported that BPs, in particular, form different complexes regarding size and structure with Ca (33).

Anticalcification Effects

In order to evaluate the anticalcification activity of the novel BPs we used *in vitro* and *in vivo* models. Our compounds were found to be as effective as alendronate in inhibiting HAP formation in the *in vitro* model (Table II). The presence of IR bands that are attributed to the substituted aromatic rings of **VS-6b** and **ISA-225** in the FTIR spectra (Fig. 4), may indicate that these BPs were adsorbed or incorporated into the apatite. The characteristic aromatic absorption bands that appeared in the IR spectra of HAP formed in the presence of **VS-6b** and **ISA-225** confirmed the incorporation of these BPs into apatite. It was also found that the BPs tested inhibited the air carbonate uptake during the HAP formation and slightly impaired the crystal quality. Further investigations on the influence of BPs on the uptake of known carbonate amounts during the HAP formation, might clarify the importance of this observation. Preliminary X-ray diffraction analysis of the precipitates also has shown differences in the crystal quality (data not shown). It is suggested that examination of the FT-IR spectra of HAP- adsorbed BPs could be an additional tool for studying the interaction between

Table III. The Effect of Daily IM Injections (14 days) of Pamidronate, VS-6b, and VS-5b (0.1 mg P/kg/day), on Bone Chemistry, and on the Upper Metaphysis Measured by Computerized Histomorphometry

Compound	Bone weight (mg)	Ash content (%)	Ca in ash (%)	Cartilage (%)	Bone volume (%)	Bone marrow (%)
Saline	219.8 ± 31.8	45.1 ± 1.8	33.5 ± 2.4	15.4 ± 0.69	16.72 ± 0.78	57.25 ± 2.05
Pamidronate	294.8 ± 37.7[a]	47.0 ± 4.5	31.3[a] ± 1.4	27.22 ± 3.70	24.30 ± 1.25[a]	38.60 ± 2.45[a]
VS-5b	290.1 ± 41.5[a]	55.0 ± 2.9[a]	35.4 ± 2.7	27.25 ± 1.55[a]	25.52 ± 2.02[a]	36.90 ± 1.97[a]
VS-6b	289.4 ± 30.0[a]	55.4 ± 2.1[a]	34.8 ± 2.6	18.67 ± 0.49[a]	31.92 ± 0.98[a]	38.85 ± 1.30[a]

[a] Differences were termed statistically significant by the paired t test ($n = 8$, $p < 0.05$, mean ± SD).

BPs and HAP/bone. Good inhibition of HAP formation *in vitro* implies high activity as a crystal poison *in vivo* (4).

Indeed, all BPs tested inhibited significantly the calcification of tissue implanted subdermally in rats. The BPs were ranked as follows (Fig. 5): alendronate = pamidronate > the N-containing group. Cellular factors involved in normal mineralization are not implicated in this model of ectopic calcification, in general, and cardiovascular, in particular (3,4,11,12,34). The mechanism of action of BPs in inhibiting BHV tissue calcification is mainly thought to be a direct physicochemical effect on crystal growth and dissolution ("crystal poisoning") effect (35). It can be assumed that the greater the affinity of the bisphosphonate to the crystal, the greater its "crystal poisoning" effect. Indeed, alendronate showed higher affinity to HAP than the novel compounds (Fig. 3), apparently contributing to the best inhibition of tissue calcification.

Effects on HAP Dissolution and Bone

The experimental conditions employed, using a HAP tablet and two concentrations of BPs, made it possible to detect small differences between the activities of the various compounds. Since adsorption to HAP affects activity in this model, it was expected that alendronate and pamidronate would be slightly more active than the other compounds. The inhibition potency decreased at low concentrations (Table II), probably because of incomplete adsorption of the newer exposed tablet surface, during the dissolution period, by the BP. The results of this model should be able to predict, to some extent, the antiresorption effects of the compounds.

Complete turnover of the metaphysis in young rats occurs in a 14-day period (36) and, therefore, the influence of the BPs on bone development was examined in this model as reported earlier for both clinically used and novel BPs (3,11,12). Marked changes after treatment with BPs were noted in the upper metaphysis and thus, histological measurements focused on this part. The treatment by BPS belonging to the novel N-containing group resulted in a positive effect on bone development (Table III and Fig. 6). After treatment with pamidronate, the ash minerals decreased, but the total ash and bone amount increased. This suggests that calcification of cartilage, rather than bone formation, occurred. Pamidronate increased the amount of cartilage in the metaphysis. This correlates with one of the well-known dose-dependent side effects of pamidronate namely, rickets, resulting from inhibition of normal mineralization (11,37,38). **VS-6b** was found to be more effective than pamidronate. The high activities of **VS-5b** and **VS-6b** can be attributed to the imidazole and pyrazine rings, resembling the highly potent bisphosphonates with similar structures, risedronate (16), and zolendronate (17), respectively. The high activity of **VS-6b,** the permanent positive charge, as well as the solubility of its Ca complex, warrant further consideration as a potential drug.

Correlations Between Models and Mechanism

A good correlation within the two pairs of *in vitro* and *in vivo* models was found: between the inhibition of HAP formation (*in vitro*) and inhibition of ectopic calcification (*in vivo*), and between the inhibition of HAP dissolution (*in vitro*) and bone resorption (*in vivo*). It is important to emphasize that no compound found to be active *in vivo* was inactive in the *in vitro* models.

In order to understand the unique action of BPs, and to examine whether the correlations between the various models are valid for other types of compounds, we compared the BPs to EDTA and tetracycline. EDTA is a known metal chelator, and the antibiotic tetracycline is also known as a metal chelator with high affinity to bone, with a moderate and transient antiresorbing activity (39). Furthermore, tetracycline, in contrast to another compound of this family (40) has been used in various studies as a marker for bone metabolism since it does not affect bone turnover (24,41). As could be expected from its chelating properties, EDTA had a stochiometric effect on HAP formation, but no effects were observed in the other models. EDTA does not act as a "crystal poison" and therefore it is inactive as an antiresorption agent. Tetracycline, similar to the BPs, was shown to inhibit HAP formation to a greater extent than EDTA at low concentration, and was found to inhibit BHV calcification. However, tetracycline is not a potent "crystal poison" and therefore, had no effect on HAP dissolution and consequently a mild effect on bone resorption. Thus, the anticalcification activity of novel compounds can be predicted by the facile, economic, *in vitro* model of HAP formation. A potential for antiresorption activity, but not the extent of potency, can be predicted from the *in vitro* model of HAP dissolution. A potent antiresorption agent could be predicted from effective inhibition of HAP formation and dissolution *in vitro*, and anticalcification effect in the subdermal model, *in vivo*.

CONCLUSIONS

The anticalcification potency of the novel compounds was ranked as follows: alendronate = pamidronate > **VS-6b** = **VS-5b** = **ISA-225** > tetracycline. The antiresorption activity exhibited by **VS-5b** was similar to that of the clinically employed BP, pamidronate. **VS-6b** was shown to possess higher antiresorption potency than pamidronate. The physicochemical properties of these compounds are slightly different from the BPs in clinical use. In the novel compounds the pKa values are lower, the affinity for hydroxyapatite is lower and the solubilities of the calcium salts are higher. These properties are of potential importance for clinical application. Since there was a good correlation between the different models, it was possible to carry out a facile screening of the novel compounds. The activities of tetracycline and EDTA highlighted the distinct behavior of BPs as "crystal poisons". In addition, tetracycline was found to be a potent anticalcification agent in the BHV subdermal model.

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