Bisacylphosphonates Inhibit Hydroxyapatite Formation and Dissolution *in Vitro* and Dystrophic Calcification *in Vivo*

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Some geminal bisphosphonates are used clinically for a number of important bone/calcium related diseases; however, side effects and lack of selectivity impede their wide use. This work reports the synthesis and evaluation of bisacylphosphonates (e.g., adipoyl- and suberoylbisphosphonate). These compounds were found to inhibit significantly hydroxyapatite formation and dissolution *in vitro* and the calcification of bioprosthetic tissue implanted subdermally in rats. These are the first instances of nongeminal bisphosphonates [P–(C)*n*–P, $n \geq 2$] that have been reported to be active in calcium-related disorders. The reported bisacylphosphonates possess apparent lower toxicity, and their calcium complexes/salts have improved solubility properties. Therefore, they are of potential importance for clinical applications.

KEY WORDS: bisphosphonates; phosphonates; acylphosphonates; ketophosphonates; calcification; calcinosis; hydroxyapatite.

INTRODUCTION

There are several pathological conditions that involve irregularities in calcium metabolism. Such are some bone-related diseases, such as Paget's disease, osteoporosis, and osteolysis in bone cancer, all of which are characterized by excessive destruction of the bone by resorption (1). On the other hand, ectopic calcification is characterized by the deposition of calcium phosphate in a number of clinically important diseases, as, for example, atherosclerosis, kidney and renal calculus, arthritis, and bioprosthetic heart valve calcification (2,3). Bisphosphonates are a relatively new class of drugs (4,5) that have been used in these diseases. Their development followed the recognition that endogenous inorganic pyrophosphate is a physiological inhibitor of calcification and bone resorption. Since inorganic pyrophosphate (characterized by P–O–P-type structure) hydrolyzes rapidly in the body, stable analogues were sought. Such analogues were found about 20 years ago in the long-known class of geminal bisphosphonates [compounds of the general structure P–C–P (4,5)]. These compounds can be considered pyrophosphate analogues in which the oxygen atom linking the two phosphorus atoms has been replaced by a carbon atom. Similarly to pyrophosphate, geminal bisphosphonates have been found to possess the inherent property of inhibiting both mineralization and bone resorption, and there has been a great deal of interest in their clinical application. A number of such geminal bisphosphonates, namely, Pamidronate (ABP), Etidronate (HEBP), and Clodronate (ClMBP), have been approved for clinical use (see Fig. 1).

The biological effects of the geminal bisphosphonates are attributed mainly to their strong affinity to calcium phosphate crystals, although direct interaction with osteoclasts and their influence on a variety of biochemical pathways has also been demonstrated (4). However, side effects and the lack of selectivity impede their wide use (4,6).

Since current research in this field has been confined solely to P–C–P-type compounds (6–8), we considered it of interest to extend the synthetic effort to other types of structures which can reasonably be expected to interact with hydroxyapatite (HAP) and with calcium and yield new biologically active compounds. *In vitro* and *in vivo* testing of new chemical classes of bisphosphonates would provide increased understanding of the relationship between chemical structure and biological activity and might stimulate the development of new generations of such drugs.

In contrast to geminal bisphosphonates, monophosphonates, vicinal bisphosphonates (compounds characterized by the type of structure: P–C–C–P), and compounds in which the distance between the phosphoryl groups is greater [P–(C)*n*–P; $n > 2$] have been reported to be inactive with respect to calcium-related disorders (4,5). On the other hand, it has been recognized that by the addition of potential donor groups adjacent to the phosphonate function in phosphonic acids and esters, these compounds can be endowed with the capability to form bidentate chelates with transition metals (9,10) and calcium (11). Consequently we hypothesized that long-chain bisphosphonates might be made active in mineralization-related disorders, by introducing into them additional donor groups in positions adjacent to the phosphonic functions.

We wish to report here that we have synthesized a series of bisacylphosphonic acids and that representative members of this class, namely, adipoylbisphosphonic acid (AdBP) and suberoylbisphosphonic acid (SuBP), interact with HAP formation and dissolution *in vitro* and inhibit significantly the pathological calcification of bioprosthetic heart valve tissue cusps *in vivo*.

EXPERIMENTAL

General

All reagents used in the characterization of bisphosphonates activity were analytical grade (Sigma, St. Louis, MO). For calcium and phosphorus analyses HCl Ultrex II (BDH, Poole, England) was used. Trimethyl phosphite, bromotrimethylsilane, 1,6-dibromohexane, adipoyl chloride, and suberoyl chloride were obtained from Aldrich Chemical Company (Milwaukee, WI). HAP (fast-flow grade) was obtained from Fluka (Buchs, Switzerland).

Elemental analyses were performed by the Analytical Laboratories of the Hebrew University, Givat-Ram, Jerusa-

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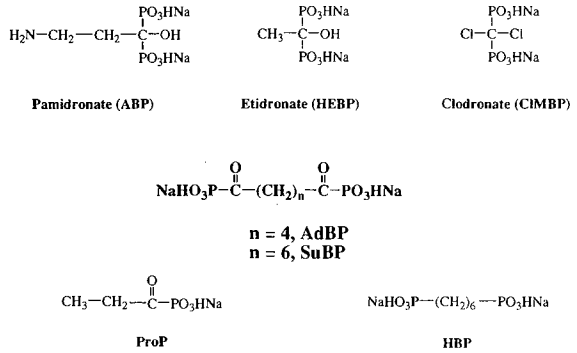

Fig. 1. Structures of known bisphosphonates and of phosphonates examined in this study.

lem. Infrared spectra were determined on an Analect FTIR spectrometer (Analect Instruments FX-6160, Irvine, CA). Nuclear magnetic resonance spectra were obtained on a Varian VXR-300S (Palo Alto, CA) or a Bruker-WH-300 (Brucker, Rheinstetter, Germany) instrument; ^{1}H NMR and ^{31}P NMR recorded in deuteriochloroform or in deuterium oxide solutions. Chemical shifts are reported as parts per million from TMS or TSP as internal standards in ^{1}H 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. Atomic absorption spectroscopy and UV/VIS spectrophotometry analyses were performed on a Perkin-Elmer 403 (Norwalk, CT) and a Spectronic 1001 (Milton Roy, Rochester, NY), respectively.

Syntheses

Tetramethyl Bisacylphosphonates. These were synthesized by a modification of methods previously published (12,13). Dicarboxylic acid dichloride (0.03 mol) was added dropwise, with stirring, to a solution of 7.82 g (0.0636 mol) trimethyl phosphite in dry toluene (60 ml) at −10°C under a nitrogen atmosphere. The reaction mixture was stirred at 25°C for about 24 hr, and then the toluene and the excess trimethyl phosphite were evaporated at reduced pressure to yield an oily product. These compounds decomposed upon attempted distillation, but they were sufficiently pure to be characterized and were used for the next step in the synthesis without further purification.

Tetramethyl Adipoylbisphosphonate. IR (neat): 1697s, 1260s, 1030s, cm^{-1}. NMR ($CDCl_3$) ^{1}H: δ 3.87 (12H, d, J = 10.64 Hz), 2.85 (4H, m), 1.65 (4H, m); ^{31}P: δ 0.57 ppm (sept.).

Tetramethyl Suberoylbisphosphonate. IR (neat); 1697s, 1265s, 1034s cm^{-1}. NMR ($CDCl_3$) ^{1}H: δ 3.87 (12H, d, J = 10.85), 2.82 (4H, t, J = 7.2 Hz), 1.63 (4H, m), 1.32 (4H, m); ^{31}P: δ 0.44 (sept.).

General Method for the Synthesis of Dihydrogen Disodium Bisacylphosphonates

To 0.03 mol of tetramethyl bisacylphosphonate in dry toluene (40 ml), 0.2 mol of bromotrimethylsilane was added dropwise, and the reaction mixture was stirred at ambient temperature for 2 hr. The toluene was evaporated in vacuum (keeping the temperature below 30°C), and the residue was taken up in 40 ml of absolute methanol, followed immediately by the addition of filtered solution of sodium hydroxide (0.06 mol) in methanol (30 ml). The reaction mixture was stirred for 2 hr at ambient temperature and allowed to settle, and the supernatant solution was decanted. Methanol (50 ml) was added to the white precipitate, the mixture was stirred for 30 min, and the product was collected by filtration, washed with methanol (15 ml), and dried in a desiccator containing P_2O_5 under vacuum (1 mm Hg) for 5 hr.

Dihydrogen Disodium Adipoylbisphosphonate (AdBP). Yield, 90%; m.p. >250°C. IR (KBr): 1675, 1191, 1119, 1055 cm^{-1}. NMR (D_2O) ^{1}H: δ 2.88 (4H, m), 1.6 (4H, m); ^{31}P: δ 2.19, s. *Anal.* Calcd. for $C_6H_{10}Na_2P_2O_8$: C, 22.64; H, 3.14. Found: C, 22.41; H, 3.2.

Dihydrogen Disodium Suberoylbisphosphonate (SuBP). Yield, 90%; m.p. ≥250°C. IR (KBr): 1677, 1214, 1110, 1075 cm^{-1}. NMR (D_2O) 1H: δ 2.8 (4H, t, J = 7.2 Hz), 1.58 (4H, m), 1.31 (4H, m); ^{31}P: δ 2.0, s. *Anal.* Calcd. for $C_8H_{14}Na_2P_2O_8$: C, 27.75; H, 4.05. Found: C, 26.93; H, 3.92).

Hydrogen Sodium Propionylphosphonate (ProP). ProP, a monoacylphosphonate, was synthesized as described previously (14).

Tetraethyl 1,6-hexanebisphosphonate. This (devoid of oxo groups) was synthesized by a modification of Kosolapoff's method (15). Triethyl phosphite (20.4 g, 0.123 mol) was added dropwise to stirred 1,6-dibromohexane (10 g, 0.04 mol) at 130°C. The ethylbromide which evolved was distilled off, the reaction mixture was refluxed for 24 hr, and the excess of triethyl phosphite was removed by distillation. The product, tetraethyl 1,6-hexanebisphosphonate, was distilled (178°C, 0.6 mm Hg). Yield, 9.38 g, 64%. NMR ($CDCl_3$) ^{31}P: δ 32.2 (sept., J = 11 Hz).

Dihydrogen Disodium 1,6-Hexanebisphosphonate (HBP). HBP was prepared by the method used for the syntheses of AdBP and SuBP, described above. Yield, 70%. NMR (D_2O) ^{31}P: δ 27.1 (triplet, J = 11 Hz).

In Vitro and *in Vivo* Characterization of Bisphosphonates' Activity

Both *in vitro* and *in vivo* studies were employed in order to characterize the anticalcification and antiresorptive properties of AdBP and SuBP. The inhibitory effect on the formation and dissolution of HAP of the new bisacylphosphonates (AdBP and SuBP) was compared to a monoacylphosphonate (ProP), to 1,6-hexanebisphosphonic acid (devoid of oxo groups, HBP), to the clinically used bisphosphonates (ABP and HEBP), and to the chelating agent EDTA.

Inhibition of Hydroxyapatite Formation

Based on the method of Francis (16) and Golomb (17,18), the inhibition of HAP formation was studied in supersaturated calcium phosphate solution. The concentration product of calcium ($CaCl_2 \cdot 2H_2O$) and phosphate (K_2HPO_4) in the incubation solutions was 9 mM^2, calcium 3.87 mM, and phosphate 2.32 mM, yielding a ratio of Ca/PO_4 = 1.67, as in hydroxyapatite (HAP). Each salt solution was prepared in 0.05 M Tris buffer, pH 7.4, and the drug examined was dissolved in the phosphate solution. Equal volumes of doubled concentrations of 3.87 mM calcium and 2.32 mM phosphate were mixed in borosilicate glass tubes (acid and ace-

tone washed). The vials were placed in a shaker (100 rpm) at 37°C. Calcium (18) and phosphorus (19) concentrations in the filtrate (after 24 hr of incubation) were determined by atomic absorption spectroscopy and UV/vis spectrophotometry, respectively. Specimens for calcium analysis were diluted with lanthanum solution (La^{3+}, 5%; HCl, 3.0 *N*). A standard curve was obtained by using a calcium standard solution containing lanthanum (La^{3+}, 0.5%; HCl, 0.6 *N*).

Inhibition of Hydroxyapatite Dissolution

The inhibition of HAP dissolution by the various compounds was examined by monitoring, over time at pH 5, the calcium and phosphate dissolution from the HAP-adsorbed bisphosphonate [a slight modification of Shinoda's method (20)]. Hydroxyapatite (32.5 mg) was suspended in 100 ml of Tris buffer, pH 7.4, containing 24 μ*M* drug, and the suspension was stirred for 8 hr at 37°C. The suspension was filtered, and the hydroxyapatite with adsorbed drug was suspended in 50 ml of acetate buffer pH 5. At each time point aliquots of 5 ml were withdrawn and were analyzed for calcium and phosphorus concentration, as above. In order to maintain constant slurry concentration, the dissolution medium was replenished with 5 ml of acetate buffer after each sampling.

Inhibition of Ectopic Calcification

The anticalcification effect of the novel bisphosphonates *in vivo* was studied by examining the therapy of pathological calcification of bioprosthetic tissue implanted subdermally in rats (21–25), a representative model for cardiovascular calcific diseases (3,22,26,27). Bioprosthetic heart valve tissue cusps were prepared from bovine pericardium treated with glutaraldehyde (23,25,28,29). Fresh parietal pericardium was obtained at slaughter from 1- to 3-month-old calves and immediately placed in iced sterile saline. After dissection of superficial fat from the external surfaces, pieces of 1 × 1 cm were cut and were incubated in 0.2% glutaraldehyde (25% for electron microscopy, under nitrogen, Merck, Germany) of 0.05 *M* Hepes and 0.1 *M* NaCl, pH 7.4, for cross-linking (for at least a 2-week period) and storage at 4°C.

Osmotic pumps (ALZET 2001, Alza, Stanford, CA) containing 0.14 and 0.014 m*M* drug solution, delivering 16.8 and 1.68 μmol/kg/day, for 14 days, of tested compound, respectively, were placed next to subcutaneous bioprosthetic cuspal (1 × 1 cm) implants in the backs of ether-anesthetized rats. In addition, two similar tissue cusps, were implanted subcutaneously in the abdominal wall of each animal, serving as paired control. An additional group of rats consisted of bioprosthetic tissue implants without treatment. Euthanasia was carried out by placing the rats in a closed chamber containing ether 14 days after implantation. The retrieved tissue was rinsed with copious amounts of DD water and dried to constant weight, and the amount of calcium was determined by atomic absorption spectroscopy on aliquots of 6 *N* HCl hydrolyzates of dried tissue, which was diluted with the lanthanum solution. The amount of calcium was expressed as micrograms calcium per milligram dry tissue weight. The control values represent the average of both control groups' data, tissues implanted in the abdominal wall of treated rats (osmotic pump implanted in the back), and the separate control group. Potential adverse effects on overall somatic growth were assessed by monitoring the weight gain of the treated rats in comparison to the control group.

RESULTS AND DISCUSSION

Following a previous report from our laboratory (14) on the synthesis and characterization of simple acylphosphonic acids, we prepared the two bisacylphosphonate salts reported here, AdBP and SuBP, from the corresponding tetramethyl bisacylphosphonates, described earlier (12,13). Infrared and nmr (^1H and ^{31}P) spectroscopic data obtained for AdBP and SuBP unequivocally confirmed the structures indicated. The results of elemental analyses confirmed the purity of the compounds. To characterize the anticalcification and antiresorptive properties of AdBP and SuBP, three experimental models, which are commonly used for predicting and evaluating the biological activities of bisphosphonates, were utilized: (i) inhibition of calcium phosphate (HAP) formation (16), (ii) inhibition of HAP dissolution (20), and (iii) inhibition of the pathological calcification of bioprosthetic tissue implanted subdermally in rats (21–25).

The effect of the various compounds on calcium precipitation (HAP formation) is presented in Fig. 2. At a 1 m*M* drug concentration, a marked inhibition of calcium precipitation was exhibited by both novel bisphosphonates (AdBP and SuBP), monophosphonate (ProP), EDTA, and to a lesser extent, the known bisphosphonates (ABP and HEBP). However, at a lower drug concentration (0.1 m*M*) SuBP and EDTA were found to be inactive.

The inhibition of HAP dissolution by the various compounds is summarized in Fig. 3. As can be seen, both known bisphosphonates and novel bisacylphosphonates significantly inhibited HAP dissolution. In contrast, EDTA, ProP, and HBP were found to be inactive.

The inactivity of the hexane-1,6-bisphosphonate (devoid of keto groups, HBP) in both models supports our hypothesis that the two oxo groups have an essential role in the interaction with calcium/HAP, presumably by virtue of their donor nature. It is known that bisphosphonates of type P–$(C)_n$–P, in which $n > 2$, are almost inactive (4,5). The reason for the low activity probably is that in such compounds the distance between the two phosphorus atoms allows only separate binding of each phosphoryl group to a different calcium atom, resulting in much weaker binding than in the case of the "P–C–P"-type bisphosphonates, in which the phosphoryl groups can be bound simultaneously to the same calcium atom with the formation of a bidentate, or possibly tridentate, chelate.

ProP, being a monoacylphosphonate, was found to be inactive in inhibiting HAP dissolution, indicating that the presence of one ketophosphonic function is sufficient to confer anticalcification potential, but not the ability to inhibit HAP dissolution. The finding that this compound showed an anticalcification effect is not surprising in view of previous knowledge regarding the contribution of adjacent donor group to the ability of a phosphonate to bind metals (9–11). It has been demonstrated that a related α-hydroxyiminophosphonate (also a monophosphonate containing an additional donor group adjacent to the phosphonic function) forms a multinuclear complex with calcium (11).

The activity of both ABP and HEBP in the anticalcifi-

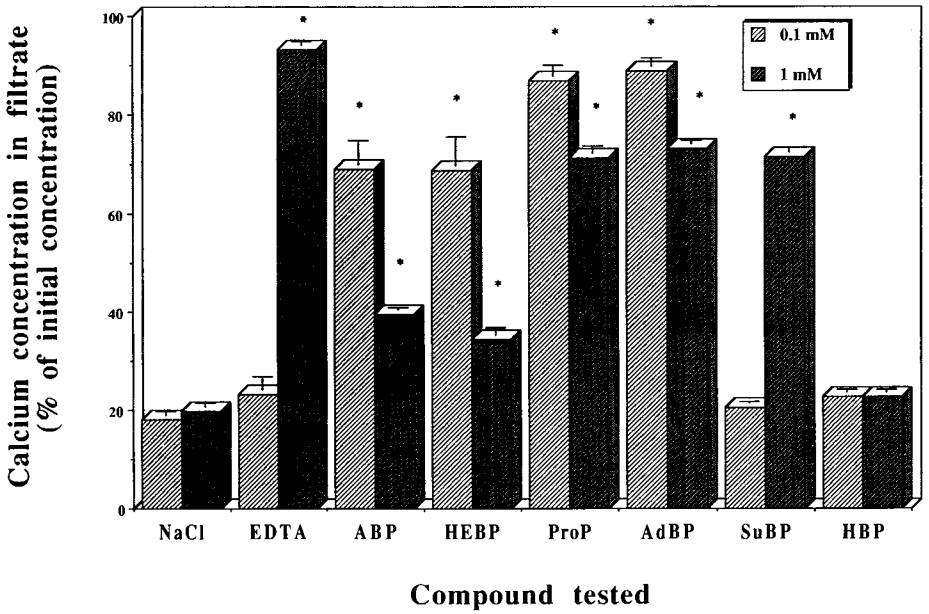

Fig. 2. The inhibition of calcium precipitation (hydroxyapatite formation) in supersaturated calcium phosphate solution ([Ca] × [PO_4] = 9 mM^2) by bisphosphonates (0.1 and 1 mM drug concentration). Novel bisacylphosphonates (AdBP and SuBP) are compared to a monoacylphosphonate (ProP), to known, clinically used, geminal bisphosphonates (ABP and HEBP), to the dideoxo bisphosphonate (HBP), and to EDTA (a chelator). NaCl served as control. (*) Differences were termed statistically significant by the paired t test (n = 10, $P < 0.001$).

cation model was found to decrease at the higher drug concentration of 1 mM (see Fig. 2), and it was lower than those of ProP, AdBP, and SuBP. This apparent inactivity was due to the relatively low solubility of the calcium salts/complexes of the former two drugs. The determination of inorganic phosphate in the precipitate and in the filtrate of the ABP and HEBP experiments (results not shown) revealed that most of the precipitate was composed of calcium-ABP and calcium-HEBP, rather than inorganic calcium phosphate. Opposed to this, the three acylphosphonates (ProP, AdBP,

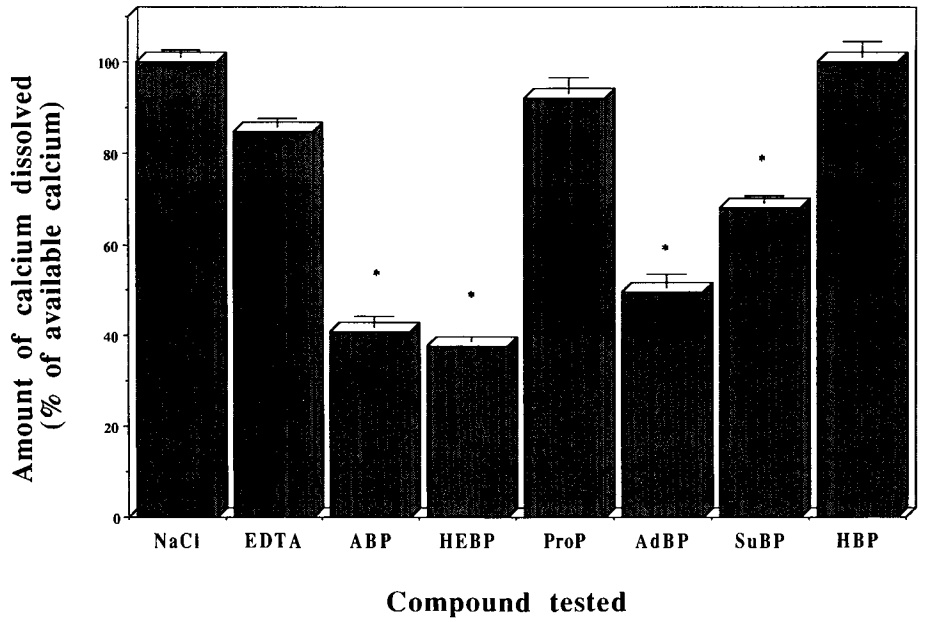

Fig. 3. The inhibition of hydroxyapatite-adsorbed drug dissolution (at steady state) by same compounds as detailed above. The amount of calcium dissolved in the NaCl group (control) was termed as 100%. Novel bisacylphosphonates (AdBP and SuBP) are compared to a monoacylphosphonate (ProP), to known, clinically used, geminal bisphosphonates (ABP and HEBP), to the dideoxo bisphosphonate (HBP), and to EDTA (a chelator). (*) Differences were termed statistically significant by the paired t test (n = 10, $P < 0.001$).

and SuBP) are more effective in preventing calcium precipitation, since even at a higher drug concentration (1 m*M*) their calcium salt/complexes remain soluble.

EDTA (a typical chelating agent) and ProP (a monoacylphosphonate) were active in preventing HAP formation but were found inactive in inhibiting HAP dissolution. The difference in activity between the bisacylphosphonates and these agents in the HAP dissolution model indicates that the bisacylphosphonates do not exert their activity as pure chelators. Their mode of action can best be rationalized by assuming that they act, similarly to gem-bisphosphonates, by strong chemisorption on HAP, known also as the "crystal poisoning" effect (2). From the similarity of the antiresorptive properties of bisacylphosphonates to those of the known drugs *in vitro*, it can be speculated that the novel compounds are likely to show the same kind of activity *in vivo*.

The therapy by bisacylphosphonates of bioprosthetic heart valve (BHV) tissue calcification was studied in the rat subdermal model. As can be seen from Fig. 4, all phosphonates, except for HBP, exhibited a profound inhibition of BHV tissue cusps calcification. These results further support the hypothesis presented earlier, regarding the role of the two oxo groups in the interaction with calcium/HAP. The monoacylphosphonate (ProP) was significantly less active in comparison to both geminal bisphosphonates and bisacylphosphonates. Francis *et al.* (30) showed in 1969 that a simple representative alkylmonophosphonate has a small solubilizing effect on calcium phosphate. Our results show that the presence of one oxo group in a simple alkylphosphonic acid improves its calcium solubilizing property. In contrast, two such functions are needed to impart "crystal poisoning" ability, as well as its potent *in vivo* anticalcification activity.

The novel compounds (AdBP and SuBP) were equally active in inhibiting the pathological calcification of implanted biomaterial, a representative model for cardiovascular calcific diseases, in general (3,22,26,27), and dystrophic calcification, in particular (3,22,27). The correlation found between the ability of a compound to prevent HAP formation *in vitro* (see Fig. 2) and its ability to prevent calcification *in vivo* (see Fig. 4) is consistent with previous reports (31) which imply that the biological activity *in vivo* is at least in part a consequence of the action on crystal growth.

The therapy of BHV tissue calcification was not accompanied by the common adverse effect of overall somatic growth retardation, as evidenced by the normal weight gain of the treated animals (between 96.7 and 102% weight gain of treated and untreated animals). This lack of side effect was due to the site-specific, controlled administration of low drug levels (21,23–25,28). Geminal bisphosphonates have been found highly potent both in inhibiting BHV calcification and in experimental arteriosclerosis (26); however, severe adverse effects on bone development and somatic growth have been noted following the systemic administration of these drugs. In this context it is important to note the results of preliminary acute toxicological studies on bisacylphosphonates. The tolerated dose found (neither deaths nor weight loss) for both AdBP and SuBP (up to 195 mg/kg for 2 days and 211 mg/kg for 3 days, respectively, of i.v. injections in Walker carcinosarcoma-bearing female rats) was markedly higher than that for ABP (50% deaths at a single dose of 47 mg/kg; T. Klenner and H. Stadler, personal communication). The apparent lower toxicity and the better solubility properties of the newly reported bisacylphosphonates clearly indicate their potential for clinical applications.

CONCLUSION

Bisacylphosphonates exhibit activity similar to the currently used gem-bisphosphonates in inhibiting HAP formation and dissolution *in vitro* and anticalcification properties

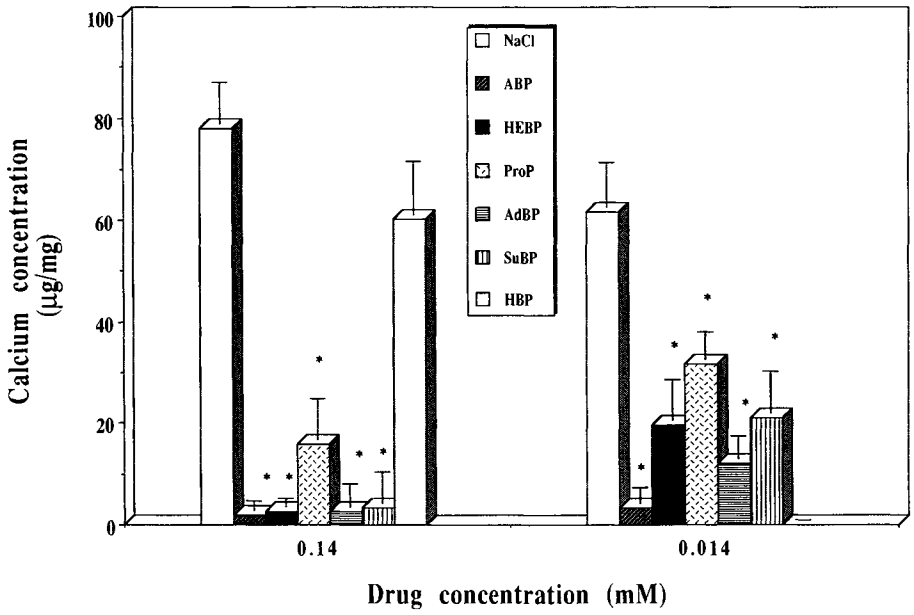

Fig. 4. The effect of bisphosphonates delivered by mini osmotic pump on bioprosthetic heart valve tissue calcification implanted subcutaneously in rats, for 14 days. (*) Differences were termed statistically significant by the paired *t* test ($P < 0.0016$).

in vivo. The introduction of the donor oxo groups in the α positions relative to the phosphonate functions apparently compensates for the long distance between the two phosphonate groups. The better solubility properties and the apparent lower toxicity of the newly reported bisacylphosphonates are of potential importance for clinical applications.

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