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The bisphosphonates HEBP and AHPrBP but not AHBP inhibit mineral mobilization and lysosomal enzyme release from mouse calvarial bones in tissue culture[1]

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Summary. The effect of 3 bisphosphonates, 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP), 3-amino-1-hydroxy-propylidene-1, 1-bisphosphonate (AHPrBP) and azacycloheptylidene-2, 2-bisphosphonate (AHBP), on the release of minerals (^{40}Ca, ^{45}Ca, P_i) and enzymes from cultured mouse calvaria was investigated in an organ culture system. HEBP and AHPrBP reduced PTH-stimulated mobilization of calcium and inorganic phosphate without affecting the release of lactate dehydrogenase. In contrast, no significant effect by AHBP on mineral mobilization and lysosomal enzyme release could be registered. In parallel with inhibited mineral mobilization, HEBP and AHPrBP inhibited the release of the lysosomal enzyme β-glucuronidase. A possible cellular mechanism of action of bisphosphonates is discussed in the light of these data.
Key words. Mouse; calvarial bone; biphosphonates; mineral mobilization; lysosomal enzymes; calcium release; inorganic phosphate release; tissue culture.

Bisphosphonates are compounds which have profound effects on mineralizing tissues[2-4]. They are chemically characterized by a P-C-P bond which is structurally analogous to the P-O-P bond of inorganic pyrophosphate. In contrast to the naturally occurring pyrophosphate the bisphosphonates are resistent to enzymatic hydrolysis. Three different bisphosphonates have been used in man to treat different disorders in bone and cartilage: (HEBP 'EHDP' 1-hydroxyethylene-1, 1-bisphosphonate), Cl_2MBP (dichloromethylene bisphosphonate) and AHPrBP ('APD' 3-amino-1-hydroxypropylidene-1, 1-bisphosphonate). HEBP inhibits bone resorption and bone mineralization at the same concentrations[5] whereas Cl_2MBP is more potent as an inhibitor of bone resorption than as an inhibitor of bone mineralization[6,7]. Recently it has been reported that AHPrBP also has a preferential inhibitory action on bone resorption at doses where no direct effect on bone mineralization could be registered[8-10]. Thus AHPrBP has been used to prevent bone resorption in diseases such as Paget's disease[8], tumor hypercalcemia[11] and rheumatoid arthritis[12]. We here report some data from a study of the effects of AHPrBP on bone cells in tissue culture, as compared to another bisphosphonate, HEBP. We have also examined the effects of a newly synthesized bisphosphonate, AHBP (azacycloheptylidene-2, 2-bisphosphonate), since this compound has been added to a tooth paste recently marketed in Sweden.

Material and methods. Calvarial bones (frontal and parietal) from 5–7-day-old mice (CsA type) were dissected aseptically, washed in Tyrode's solution and divided along the sagittal suture giving 2 half-calvaria. Care was taken during the dissection procedure not to damage the thin periosteum layer. Calvarial halves from 3–4 litters were pooled and randomized in different groups according to the experimental protocol. Subsequently, the individual half-calvaria were transferred to plastic dishes (A/S Nunc, Copenhagen) containing culture media[13] with and without bisphosphonates and incubated for 48 h at 37 °C in a gas phase of 5% CO_2 in air.

In a 1st type of experiment the bones were prelabelled in vivo by a s.c. injection of 1.5 μCi ^{45}Ca (11 μCi/g, New England Nuclear) 4 days prior to sacrifice. The labelled half-calvaria were cultured separately on grids in plastic dishes containing 5.5 ml of CMRL 1066 medium according to the procedures described by Reynolds[14]. The rate of bone resorption was quantified by following the release of ^{45}Ca (% of initial radioactivity) from the bones to the medium.

In a 2nd type of experiments non-labelled half-calvaria were explanted on grids in multi-well dishes (Linbro Scientific Inc., Hamden, Conn.) containing 2 ml phenol-red free BGJ_b medium.

The magnitude of bone resorption was assessed by following the increase in concentrations in the media of calcium (Ca^{2+}) and inorganic phosphate (P_i). Ca^{2+} was analyzed according to Willis[15] and P_i by the method of Chen et al.[16]. The release of lysosomal enzymes from the explants to the media was assessed by analyzing the activities of β-glucuronidase in media and bone extracts. β-glucuronidase was assayed with phenolphthalein-glucuronidate as substrate[17]. The release of cytosolic enzymes was followed by determining the activities of lactate dehydrogenase (LDH) in media and bones after culture. LDH was assayed by determining the oxidation rate of reduced nicotinamide adenine dinucleotide at 25 °C[18]. Statistical analysis was performed with Student's t-test for unpaired samples.

Synthetic, bovine parathyroid hormone (PTH 1–34) with a potency of 6000 IU/mg was obtained from Beckman, Geneva, Switzerland. CMRL 1066 and BGJ_b medium were from Flow

Effect of PTH, AHPrBP, AHBP and HEBP on the release of calcium, inorganic phosphate, lactate dehydrogenase, β-glucuronidase in cultures of mouse calvarial bones stimulated by PTH

Additions	Amount (μM)	Ca^{2+} (μg/half calvarium)	P_i (μg/half calvarium)	LDH (% release)	LDH (U/half calvarium) Total activity	β-Glucuronidase ($U \times 10^{-5}$/half calvarium) Medium	Total activity
–	–	29.2 ± 6.4	15.8 ± 3.3	16.5 ± 1.1	322 ± 18	3.0 ± 0.2	33.6 ± 3.2
PTH	0.01	43.4 ± 2.6[a]	30.8 ± 2.5[b]	17.2 ± 1.9	386 ± 41	7.9 ± 0.5[b]	45.4 ± 3.0[a]
PTH + AHPrBP	0.01 + 30	4.4 ± 2.6[c]	1.8 ± 3.1[c]	12.5 ± 1.2	341 ± 46	5.1 ± 0.3[c]	31.7 ± 1.0[d]
PTH + AHBP	0.01 + 100	48.3 ± 6.0	26.3 ± 4.7	12.8 ± 1.8	303 ± 22	8.0 ± 0.4	46.9 ± 6.2
PTH + HEBP	0.01 + 250	23.8 ± 3.7[c]	19.8 ± 1.8[c]	18.4 ± 3.3	334 ± 25	5.0 ± 0.2[c]	33.7 ± 2.9[e]

Values are means ± SEM for 5–7 unpaired calvarial bones. Culture period 48 h. [a]Significantly different from control, $p < 0.05$; [b]Significantly different from control, $p < 0.01$; [c]Significantly different from PTH alone, $p < 0.001$; [d]Significantly different from PTH alone, $p < 0.01$; [e]Significantly different from PTH alone, $p < 0.05$.

Laboratories. Both media were supplemented with antibiotics, ascorbic acid (150 mg/l), $Fe(NO_3)_3 \times 9\ H_2O$ (100 μg/l) and essentially fatty acid free serum albumin fraction V (1 g/l). AHPrBP (mol. wt 235), HEBP (mol. wt 224) and AHBP (mol. wt 259) were a generous gift from Henkel KGaA, Düsseldorf, West Germany.

Results. When AHPrBP and HEBP were added to bone cultures incubated in the presence of 10 nM PTH, the stimulatory effect of PTH on ^{45}Ca release was significantly reduced by both bisphosphonates at concentrations of 30 and 300 μM (fig.). At a concentration of 3 μM, only AHPrBP produced a statistically significant inhibitory effect. From the figure it also appears that AHBP at 3 and 30 μM was ineffective but at 300 μM produced a moderate inhibition of PTH-stimulated release of ^{45}Ca. Neither AHPrBP nor HEBP influenced the release of ^{45}Ca from dead bones (heated at 70 °C in medium for 5 min, data not shown).

As can be seen in the table, PTH at a concentration of 10 nM stimulated the release of stable calcium (Ca^{2+}) and P_i from the calvaria. When added at optimal and supra-optimal concentrations, based on the effect on ^{45}Ca release, the PTH-stimulated mobilization of Ca^{2+} and P_i was significantly inhibted by AHPrBP (30 μM) and HEBP (250 μM) and again, AHBP (100 μM) was ineffective.

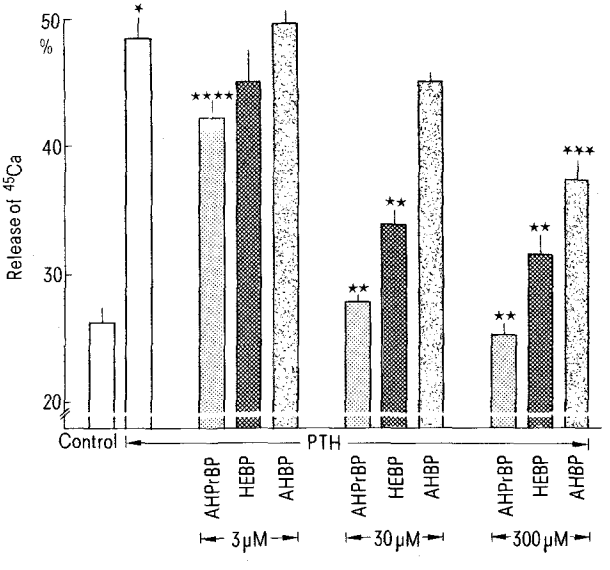
Stimulation by PTH resulted in significantly increased activities in the culture media and in the total culture (medium+bone after culture) of the lysosomal enzyme β-glucuronidase. AHPrBP and HEBP, in addition to inhibiting the release of minerals from the bones, also reduced PTH-stimulated increase in the activity in the culture medium of β-glucuronidase (table). AHPrBP and HEBP also prevented PTH-stimulated increase in the total activity of β-glucuronidase, whereas AHBP did not affect this activity in culture medium or in the total culture (table).

No significant effect of any of the 3 bisphosphonates could be registered on the release of the cytosolic enzyme LDH. Neither PTH nor AHPrBP, HEBP or AHBP affected the total activites of LDH in the bone cultures (table).

Discussion. In the present study, the effects observed by adding AHPrBP or HEBP to the culture media in which mouse calvarial bones were incubated indicate that these compounds exert a direct inhibitory action on cell-mediated bone resorption, with no evidence of a general cell cytotoxic effect. This conclusion is based on our data on mineral mobilization (^{45}Ca, Ca^{2+} and P_i) as well as on enzyme release (β-glucuronidase and LDH) from PTH-stimulated bone. Further support for our conclusion is gained from our observation that the large resorption holes forming in the calvaria after PTH-treatment did not appear when AHPrBP or HEBP were added. The recent findings by Shinoda et al.[19] and by Spiro and Mundy[20], corroborate our finding that AHPrBP seems to be a slightly more potent agent than HEBP. In contrast to the effects of AHPrBP and HEBP, AHBP exerts much smaller or insignificant effects in our in vitro system.

Several different explanations have been presented to explain the mechanism of action of bisphosphonates on bone resorption[2-4]. The present data do not help to elucidate this question, but we demonstrate for the first time that inhibition of mineral release by bisphosphonates (HEBP, AHPrBP) is also paralleled by a reduction of lysosomal enzyme release from the bones to the extracellular milieu. Lysosomal enzyme release has been shown to be intimately associated with bone resorption[21,22]. The inhibitory effect of AHPrBP and HEBP on bone resorption may therefore be related to an inhibitory action on PTH-stimulated exocytotic processes resulting in a reduction of the release of lysosomal enzymes, as indicated by our data on β-glucuronidase inhibition. Interestingly, Morgan et al.[23] reported that Cl_2MBP prevented the enhanced activity in mouse calvarial bones of acid p-nitrophenyl phosphatase and acid pyrophosphatase, caused by PTH-stimulation.

Recently, Reitsma[4] found that AHPrBP is a potent inhibitor of macrophage-mediated dissolution of bone. This observation suggests that AHPrBP has a direct inhibitory action upon macrophages. Whether or not such a direct mechanism is responsible for the inhibition of bone resorption in vitro and in vivo remains to be clarified. Detailed ultrastructural analysis of bone cells exposed to bisphosphonates in vivo and in vitro may help to shed important light upon these questions.



Effect of 3 bisphosphonates AHPrBP, HEBP and AHBP on PTH-stimulated release of ^{45}Ca from mouse calvarial bones cultured for 48 h. Values are means ± SEM for 5 unpaired calvarial halves. * Significantly different from control ($p < 0.001$). ** Significantly different from PTH alone ($p < 0.001$). *** Significantly different from PTH alone ($p < 0.01$). **** Significantly different from PTH alone ($p < 0.05$).

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Genetic and histological aspects of stomach lesions induced by systemic injection of phenylbutazone in the rat

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Summary. Roman high-avoidance (RHA/Verh) rats, food-deprived (F–D) for 5 days, had higher stomach lesion scores than did F-D Roman low-avoidance (RLA/Verh) rats. F–D RLA/Verh rats which were injected i.p. with phenylbutazone (PBZ) 24 h before examination, however, had higher scores than did PBZ-treated, F–D RHA/-Verh rats. Histologically, extensive edema and cellular infiltration (including numerous erythrocytes) were seen below the lesions, in the submucosa, denoting vascular damage. An attenuating influence of food on the ulcerogenic effects of PBZ, which were much more severe in F–D than in fed rats, was also indicated.
Key words. Phenylbutazone; stomach ulcers; food-deprivation; rats, food-deprived; stomach lesion scores; ulcer susceptibility, genetic differences in.

Roman high-avoidance (RHA/Verh) and low-avoidance (RLA/Verh) rats are bred, respectively, for rapid versus non-acquisition of a 2-way, active avoidance response. In addition to showing differences in that, and other, behavioral tests[2], various neurochemical and pharmaco-physiological differences have been found between these psychogenetically-selected lines of rats. For example, RLA/Verh rats were more sensitive to the toxic effects of several drugs, such as pentobarbital[3] and oxotremorine[4], probably due in large part to a less active hepatic microsomal system, leading to a reduced metabolism of these substances[3,5]. Other experiments, concerned with food-deprivation induced gastric lesions, have accentuated potential differences in metabolism between the 2 rat lines. When RHA/Verh and RLA/Verh rats were housed in plastic cages with sawdust bedding, and food-deprived (F–D) for 4–5 days, it was seen that F–D RHA/Verh rats had higher stomach lesion scores that did their unfasted controls and higher lesion scores than did F–D RLA/Verh rats. It was suggested that RHA/Verh rats were more sensitive to this type of stress than were RLA/Verh rats, possibly due to their normally larger appetite and metabolic requirements[6]. The present study sought to investigate these genetically-based differences in ulcer-susceptibility in combination with injections of the anti-inflammatory agent phenylbutazone (PBZ), which is also known to be biotransformed by the hepatic microsomal system. In addition, as PBZ has long been recognized to be extremely ulcerogenic in man[7,8], as well as in the rat[9], its effects were studied in further detail, through histological examination of the lesion area.

Methods. 48 naive, male RHA/Verh rats and 48 naive, male RLA/Verh rats, between 6 and 7 months of age, were used, with each animal being housed individually in a 40 × 25 × 16 cm plastic cage with sawdust bedding during the course of the experiment. The 1st group (N = 16 of each line) was given food and water ad libitum, whereas the 2nd group (N = 32 of each line) was F–D for 5 days with water ad libitum. Half of the rats of each line, in each of the groups, were given an i.p. injection of 100 mg/kg PBZ, 24 h before sacrifice, and the other half were given an i.p. injection of physiological saline (NaCl) solution. At the termination of the 5-day experimental period, each rat was sacrificed with chloroform and its stomach was removed, opened, rinsed and examined under magnification by 2 independent observers (one of which was unaware of the subjects status), for lesions. The following scoring system[9] was used: petechial lesion = 1 point, erosion less than 1 mm = 2 points, erosion 1–2 mm = 3 points, erosion 2-4 mm = 4 points, and erosion greater than 4 mm = 5 points. Due