Effect of a Single Injection of Two New Bisphosphonates on the Hypercalcemia and Hypercalciuria Induced by Walker Carcinosarcoma 256/B in Thyroparathyroidectomized Rats

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ABSTRACT

The effect of one single injection of two new bisphosphonates, 4-amino-1-hydroxybutylidene-1,1-bisphosphonate and 2-(2-pyridyl)ethylidene-1,1-bisphosphonate, on hypercalcemia and hypercalciuria induced by the Walker carcinosarcoma 256/B in the thyroparathyroidectomized rat was evaluated. When given either before or after the development of hypercalcemia and hypercalciuria, 16.1 μmol/kg of 4-amino-1-hydroxybutylidene-1,1-bisphosphonate or 2-(2-pyridyl)ethylidene-1,1-bisphosphonate totally inhibited hypercalcemia, whereas hypercalciuria was only partially reduced over the 14 days of the experiment. At 10 and 100 times lower doses, the effect was strongest in the first days, but still partially present 14 days later. The difference of activity on calcium and calciuria appears to be due to the fact that the tumor increased both bone resorption and renal reabsorption of calcium. Only the former was altered by the bisphosphonates. The two new compounds appeared to be of similar potency and more active than dichloromethylenebisphosphonate. These compounds could be promising for the treatment of malignant hypercalcemia and the other conditions associated with increased bone resorption in humans, even when given only over short periods of time.

INTRODUCTION

Geminial bisphosphonates, compounds characterized by a P—C—P bond, are powerful inhibitors of bone resorption, both in vitro and in various experimental animal models in vivo (for review, see Ref. 1). These compounds have proved useful in the treatment of increased bone resorption in such diseases as Paget’s disease (for review, see Ref. 1), hypercalcemia of malignancy (2–9), or bone metastases (10–12). Three compounds, namely, HEBP, dichloro-MBP, and AHPrBP have been used clinically, but only HEBP is presently on the market.

In this paper, two new more powerful bisphosphonates, AHBuBP (13) and PyEBP (14), were investigated, using as a model the osteolysis induced in rats by W-256. This tumor has been shown to induce bone resorption after s.c. implantation without metastasizing to bone and is therefore a model of humoral hypercalcemia of malignancy (15, 16). It has been found to respond to bisphosphonates (17). In contrast to all previous experiments with bisphosphonates where the drugs were administered daily, in these experiments the compounds were given as a single injection. Indeed, it has been recently suggested that, in humans, a short treatment might be just as effective as a treatment of long duration (18, 19). The compounds were given both preventively before the hypercalcemia had developed and curatively when the increase in blood calcium was already present. To avoid any counterregulation by PTH or calcitonin which may blunt the effect of the drug, the animals were thyroparathyroidectomized. Since the regulation of plasma calcium depends on both bone resorption and renal reabsorption of calcium (20), and since hypercalcemic tumors are known to produce factors changing the renal tubular reabsorption of calcium (21, 22), urinary calcium was also measured. If food intake is kept constant and if intestinal calcium absorption is not changed, then urinary calcium excretion will be a better reflection of bone destruction than calcium.

MATERIALS AND METHODS

Male Fischer 344 rats, weighing 170 to 200 g, were obtained from Iffa Credo, L’Arbresle, France. The animals were housed in individual cages at 23°C and on a 12-h/12-h light cycle. They were fed a maintenance diet for rats (Kliba, Kaeseraugst, Switzerland) containing 1.1% calcium and 0.85% phosphorus.

During the experiment, a daily control of the food consumed was performed. The amount fed was such that, except in rare cases, all the animals ate all the food. The animals had free access to deionized water. Once the rats were accustomed to the pair-feeding, usually after 3 to 5 days, they were TPTX under light Nembutal anesthesia. The success of the operation was verified by measuring plasma calcium after an overnight fast 3 days later. Only animals with a calcium below 1.88 mmol/l were used. From that time all animals received 4 μg of thyroxin 3 times a wk, s.c. At the same time as the TPTX procedure, a subtotal cystectomy was performed in order to minimize urine dead space and to obtain a reliable 2-h urine collection.

Four days after the TPTX operation the animals were given injections under light ether anesthesia with 0.25 ml of W-256 cell suspension in each flank s.c. The W-256 tumor cells, kindly provided by Dr. G. R. Mundy, San Antonio, TX, were maintained by sequential transplantation every 2 wk in male Fischer rats. For transplantations and for the experiments, the tumor tissue was collected under sterile conditions in cold Dulbecco’s modified Eagle’s minimal essential medium. The tissue was then passed through a grid, centrifuged for 5 min at 160 × g, and the pellet diluted 1:1 with the above mentioned medium.

The bisphosphonates were dissolved in 0.15 M NaCl at neutral pH and given at various doses ranging between 0.16 μmol/kg and 161 μmol/kg. One single injection, exceptionally two, was administered s.c. at various times between 3 days before and 10 days after tumor implantation, as indicated in the tables and figures. The control animals were given the solvent.

At various times after tumor implantation, the animals were put into restrictive cages, and urine was collected for 2 h between 9:00 and 11:00 a.m. In the middle of this collection period, blood was taken from the tip of the tail for plasma calcium determination.

Calcium was measured by atomic absorption spectrophotometry, creatinine by the Jaffe method, and phosphorus by the ammonium molybdate complex method of Chen et al. (23), except that 0.5 M HCl was used instead of H₂SO₄. Urinary cAMP was assayed as described by Brown et al. (24). All values are given as the mean ± SE. Grouped data were analyzed using the 2-tailed Student t test.
RESULTS

Fig. 1 shows the influence of 1.61 μmol/kg of AHBuBP, administered s.c. as a single dose at various times before or after tumor implantation, on plasma calcium and the urinary calcium/creatinine ratio. It appears that the compound could reduce but not totally prevent the tumor-induced hypercalcemia. The results were quite similar when the compound was given 2 days before, 1 or 7 days after tumor implantation, although there was a tendency to a smaller effect when the compound was given before tumor implantation. When administered to hypercalcemic animals, the bisphosphonate reduced plasma calcium for the first 2 days, but later on the effect was similar to that obtained when the compound was given earlier.

In contrast, the effect on the urinary calcium/creatinine ratio was dramatic (Fig. 1, bottom). Although calciuria started to increase slightly in the last 2 days, the absolute values were much lower in the treated animals than in the control tumor-bearing rats. Again the results were independent of when the bisphosphonate was given, showing that the compound can act for at least 2 wk after a single injection. When given to hypercalciuric animals, the calcium was reduced to normal values within 2 days, but later the values started again to increase in a similar manner to the other groups.

These results as well as the effect of other doses of AHBuBP given at various times are presented in Table 1. The data are expressed both in absolute values and as the percentage of the values of tumor-bearing animals not receiving bisphosphonates. All doses had a small effect on plasma calcium. Similar reductions were obtained with 0.161 μmol/kg and 1.61 μmol/kg, while 16.1 μmol/kg was more potent. In contrast, urinary calcium excretion was markedly reduced by all doses of

![Graph showing plasma calcium and urinary calcium/creatinine ratio over time](image-url)

Table 1. Influence of one injection of 1.61 μmol/kg s.c. AHBuBP administered -2, +1, or +7 days from tumor implantation on the hypercalcemia and hypercalciuria induced by Walker 256/B tumor in TPTX male Fischer rats. Points: mean; bars, SE (n = 7). O, tumor alone; •, tumor + AHBuBP given on Day -2; △, tumor + AHBuBP given on Day -1; ▲, tumor + AHBuBP given on Day +7. Significance: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Table 2  Influence of two different doses of PyEBP, given on different days (day of administration) with respect to implantation of a Walker tumor 256/B, on the tumor-induced hypercalcemia and hypercalciuria in TPTX male Fischer rats

Data are expressed as absolute values and as percentages of untreated controls.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Day of administration</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td></td>
<td>2.42 ± 0.07* (100)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.07 ± 0.07 (100)</td>
<td>4.1 ± 0.08 (100)</td>
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<tr>
<td>PyEBP, 1.61 μmol/kg (n = 5)</td>
<td>-2</td>
<td>2.03 ± 0.08* (83.85)</td>
<td>2.43 ± 0.1* (79.15)</td>
<td>2.91 ± 0.31* (75.15)</td>
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<tr>
<td></td>
<td>+1</td>
<td>2.02 ± 0.05* (83.38)</td>
<td>2.56 ± 0.16* (83.49)</td>
<td>3.22 ± 0.19* (79.26)</td>
</tr>
</tbody>
</table>

* Mean ± SE.  
<sup>a</sup> Numbers in parentheses, percentage of untreated control.

<table>
<thead>
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<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td></td>
<td>3.67 ± 0.22 (100)</td>
<td>3.54 ± 0.16 (100)</td>
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<tr>
<td>PyEBP, 1.61 μmol/kg (n = 4)</td>
<td>-2</td>
<td>2.92 ± 0.04* (79.64)</td>
<td>2.81 ± 0.08* (79.39)</td>
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<tr>
<td>PyEBP, 1.61 μmol/kg (n = 4)</td>
<td>+8</td>
<td>2.47 ± 0.18* (67.48)</td>
<td>2.37 ± 0.20* (66.8)</td>
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<td>PyEBP, 1.61 μmol/kg (n = 4)</td>
<td>+8 +10</td>
<td>2.47 ± 0.22* (67.45)</td>
<td>2.09 ± 0.28* (58.89)</td>
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</tbody>
</table>

* Mean ± SE.  
<sup>b</sup> Numbers in parentheses, percentage of untreated control.

Table 3  Influence of two different doses of dichloro-MBP, given on different days (day of administration) with respect to implantation of Walker tumor 256/B, on the tumor-induced hypercalcemia and hypercalciuria in TPTX male Fischer rats

Data are expressed as absolute values and as percentages of untreated controls.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Day of administration</th>
<th>Day 9</th>
<th>Day 11</th>
<th>Day 12</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td></td>
<td>2.32 ± 0.17* (100)</td>
<td>3.20 ± 0.12 (100)</td>
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<tr>
<td>Dichloro-MBP, 16.1 μmol/kg (n = 5)</td>
<td>+1</td>
<td>1.95 ± 0.04* (90.27)</td>
<td>2.88 ± 0.05 (83.9)</td>
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<td></td>
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</table>

* Mean ± SE.  
<sup>c</sup> Numbers in parentheses, percentage of untreated control.

<table>
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<tr>
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<th>Day 12</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td></td>
<td>2.16 ± 0.07 (100)</td>
<td>2.67 ± 0.09 (100)</td>
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<tr>
<td>Dichloro-MBP, 161 μmol/kg (n = 6)</td>
<td>-3</td>
<td>2.09 ± 0.08* (96.56)</td>
<td>2.40 ± 0.09 (89.91)</td>
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<tr>
<td>(n = 6)</td>
<td>+1</td>
<td>1.95 ± 0.13* (90.02)</td>
<td>2.21 ± 0.1* (82.70)</td>
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<tr>
<td>(n = 5)</td>
<td>+7</td>
<td>1.75 ± 0.04* (81.03)</td>
<td>2.08 ± 0.08* (77.75)</td>
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* Mean ± SE.  
<sup>d</sup> Numbers in parentheses, percentage of untreated control.

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<th>Day 12</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
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<td>3.07 ± 0.19 (100)</td>
<td>3.60 ± 0.09 (100)</td>
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<tr>
<td>Dichloro-MBP, 16.1 μmol/kg (n = 3)</td>
<td>+10</td>
<td>2.67 ± 0.02* (87.00)</td>
<td>3.51 ± 0.11* (97.48)</td>
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<tr>
<td>Dichloro-MBP, 161 μmol/kg (n = 4)</td>
<td>+10</td>
<td>2.61 ± 0.05 (85.09)</td>
<td>3.18 ± 0.12* (88.31)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE.  
<sup>e</sup> Numbers in parentheses, percentage of untreated control.

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Urinary and plasma calcium during the course of tumor growth was altered. Moreover, the relationship between the activity of the tumor and of the bisphosphonates on the renal handling of calcium. The compounds were active not only when administered preventively before establishment of hypercalcemia and hypercalciuria, but also when given curatively once these variables were already increased under the influence of the tumor.

Similar preventive effects on calcium and calciuria have been previously observed in intact animals with HEBP and dichloro-MBP. The fact that it is present also in TPTX animals rules out any role of endogenous PTH or calcitonin. In analogy to what was found in intact rats (17), the effect was much stronger on calciuria than on calcemia. This is likely to be due to the fact that, in this model, the hypercalcemia is induced not only by increased bone resorption (26), but also by an increase in the renal reabsorption of calcium. Such a renal mechanism has been demonstrated in the Leydig cell tumor rat model (22) and in certain cases of human malignant hypercalcemia (18, 21). Our data in Fig. 2, which show that, for a given urinary calcium excretion, plasma calcium was higher in tumor-bearing rats than in control TPTX rats, support this interpretation.

In the present experiments, where the food intake was kept constant for all animals, calciuria reflects mainly bone resorption. Indeed, it is unlikely that intestinal calcium absorption was altered. Moreover, the relationship between the activity of the three compounds tested on calciuria was similar to that found in other systems used to evaluate antiresorptive agents, such as the metaphyseal density in the growing rat (13) and the inhibition of retinoid-induced hypercalcemia in TPTX rats. Thus, urinary calcium is a better index than plasma calcium for bone destruction in tumoral disease. This also explains the clinical finding that, in humans, bisphosphonates are not always fully active in normalizing calcium, although they cause a drastic decrease in hypercalcemia (18). The relative effect on the two variables will indeed depend upon the relative effect of the tumor on the renal reabsorption of calcium and on bone resorption.

These data suggest the possibility of the use of two new very powerful bisphosphonates in malignant bone disease with increased bone destruction. Furthermore, they suggest that, in

AHBuBP. The effect was most pronounced with the highest dose which completely prevented the development of hypercalciuria up to the last day of the experiment.

Table 2 shows the effect of PyEBP. The results were very similar to those obtained with the same doses of AHBuBP. Again, the effect was much less pronounced on plasma calcium than on calciuria.

Table 3 shows the effect of the reference compound dichloro-MBP. Again, the effect was only small on calciuria, but pronounced on calciuria. However, dichloro-MBP appeared to be less active than AHBuBP or PyEBP.

The discrepancy between the effect of bisphosphonates on urinary and plasma calcium raised the possibility of an effect of these compounds on the renal handling of calcium. The relationship between urinary and plasma calcium measured at various times after tumor implantation in groups of animals treated with different doses of bisphosphonates was therefore plotted and is shown in Fig. 2. For a given urinary calcium excretion, taken as a reflection of bone resorption, plasma calcium increased with increasing time after tumor implantation. Thus, a shift towards the right of the relationship between urinary and plasma calcium during the course of tumor growth was compatible with the hypothesis of a stimulation of the renal tubular reabsorption of calcium induced by the Walker carcinosarcoma.

At the end of several experiments on Day 11 after tumor implantation, plasma creatinine was determined and found to be 54.8 ± 4.2 (n = 7) µmol/liter in control TPTX rats and 57.3 ± 3.1 µmol/liter in TPTX tumor-bearing rats (n = 10, not significant). For values in their respective controls of 52.6 ± 2.0, 64.5 ± 2.4, and 52.4 ± 4.0 µmol/liter, plasma creatinine was 43.5 ± 3.7 in AHBuBP-treated (n = 5, not significant), 58.7 ± 5.8 in PyEBP-treated (n = 10, not significant), and 40.7 ± 1.3 in dichloro-MBP-treated animals (n = 11, P < 0.025). The influence of the tumor and of the bisphosphonates on urinary calcium excretion was not different if the latter were expressed as the amount excreted per 120 min or as the calcium/creatinine ratio (not shown).

To assess whether the bisphosphonates affected the synthesis of a hypercalcemic and hypercalciuric PTH-like factor, urinary cAMP excretion was measured in tumor-bearing rats treated with 1.61 µmol/kg AHBuBP or 161 µmol/kg dichloro-MBP at various days. No significant effect was observed (not shown). Finally, no effect of the bisphosphonate on plasma or urinary phosphate was observed either (not shown).

DISCUSSION

All three bisphosphonates tested, AHBuBP, PyEBP, and dichloro-MBP, showed a similar effect, namely, a small reduction of the tumor-induced hypercalcemia and a dramatic inhibition of tumor-induced hypercalciuria. The two new bisphosphonates, AHBuBP and PyEBP, displayed a similar potency and were more active than dichloro-MBP.

It is interesting that the administration of one dose of the bisphosphonates was effective over a period of at least 2 wk. For dichloro-MBP, this effect was comparable to that obtained with daily injections in either the Walker carcinosarcoma or the Leydig cell tumor models (17, 25). At the intermediate doses the effect was most pronounced for the first 2 days, but nevertheless was also seen at a reduced level later on. At higher doses the effect of AHBuBP and PyEBP was complete up to the end of the experiment. The compounds were active not only when administered preventively before establishment of hypercalcemia and hypercalciuria, but also when given curatively once these variables were already increased under the influence of the tumor.

the future, the bisphosphonates may be administered for short periods only. The drugs are likely to be useful both to prevent the development of bone disease and to treat already established lesions.

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REFERENCES

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