Bisphosphonates Inhibit Angiogenesis in Vitro and Testosterone-stimulated Vascular Regrowth in the Ventral Prostate in Castrated Rats

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ABSTRACT

Bisphosphonates (BPs) are used currently in the treatment of patients with bone metastases because these compounds inhibit bone resorption. We examined here the effects of BPs on inhibition of endothelial cell functions in vitro and in vivo. Treatment of endothelial cells with BPs (clodronate, risedronate, ibandronate, and zoledronic acid) reduced proliferation, induced apoptosis, and decreased capillary-like tube formation in vitro. Quantification of blood vessels in bone biopsy specimens from patients with Paget’s disease before and after clodronate treatment showed a 40% reduction of the vascularization after BP treatment. However, such a decreased vascularity could be secondary to a reduction of bone resorption. Therefore, the tissue distribution of [14C]BPs in male rats was examined to develop an angiogenesis model in a noncalcified tissue where BPs could accumulate. [14C]BPs (zoledronic acid, ibandronate, and clodronate) not only accumulated in bone but also transiently accumulated in the prostate. The effects of BPs on testosterone-induced revascularization of the prostate gland in castrated rats were then studied. Testosterone in combination with ibandronate or zoledronic acid induced a 17% reduction of the prostate weight compared with castrated rats treated with testosterone alone. Blood vessel immunostaining on prostate tissue sections revealed that both ibandronate and zoledronic acid induced a 50% reduction of the revascularization of the prostate gland. Moreover, zoledronic acid did not alter testosterone-induced activity of a luciferase gene reporter construct transfected in androgen-dependent prostatic cells, indicating that this BP did not directly interfere with testosterone. In conclusion, BPs have in vivo antiangiogenic properties, which could be of relevance to improve therapy and prevention of bone metastasis. In addition, our results extend the potential clinical use of BPs to patients with early prostate cancer.

INTRODUCTION

Current trends are to consider that BPs bind avidly to bone mineral and are powerful inhibitors of osteoclast-mediated bone resorption in vivo (1). These findings provided the rationale for using BPs in the treatment of patients with bone metastases because metastatic cells in the bone microenvironment stimulate osteoclast-mediated bone resorption, leading to bone destruction (2, 3). However, we and others have shown previously that BPs inhibit breast and prostate carcinoma cell invasion, proliferation, and adhesion to bone in vitro (4–9). Consistent with these findings (4–9), animal studies have demonstrated that treatment of metastatic nude mice with the BP risedronate, ibandronate, or zoledronic acid produces a marked reduction in the progression of breast cancer-induced osteolytic lesions and a marked decrease in tumor burden in bone (1, 10). BPs also induce the release of factors from osteoblasts that inhibit osteoclast-mediated bone resorption, and they inhibit the proliferation of macrophages and myeloma cells (1). In contrast, they do not affect the proliferation and growth of fibroblasts (5, 7). Therefore, these very important observations (1, 4–10) suggest that BPs not only act on osteoclasts but also on other cell types. In this respect, bone is highly vascularized, and endothelial cells play an essential role during bone remodeling (11). Interestingly, it has been noted previously in Paget’s disease (a bone disorder characterized by increased bone remodeling, bone hypertrophy, and abnormal bone structure) that the decreased bone turnover because of a treatment with the BP clodronate was associated with qualitative histological changes such as a decreased vascularity in the bone marrow and a marked reduction of bone marrow fibrosis (12). It has been suggested that this decreased vascularization was secondary to a reduction of the osteoclastic population (12). However, the possibility remains that clodronate was exerting a direct effect on bone marrow endothelial cells in Paget’s disease. Because angiogenesis is essential for the growth of metastases of solid tumors (13), we examined here the effects of different BPs on inhibition of endothelial cell functions in vitro and in vivo.

MATERIALS AND METHODS

Chemicals. Four BPs were used. Clodronate [dichloromethylene bisphosphonic acid, disodium salt tetrahydrate] was obtained from Leiras Oy (Turku, Finland). Ibandronate [1-hydroxy-3-(methylpentanylamino)-propylidene-bisphosphonic acid monosodium salt, monohydrate] was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Risedronate [2-(3-pyridinyl)-1-hydroxyethylidene-bisphosphonic acid] was obtained from Procter and Gamble Pharmaceuticals (Cincinnati, OH). Zoledronic acid [1-hydroxy-2-(1H-imidazole-1-yl)ethylidene-bisphosphonic acid] was obtained in the form of its hydrated disodium salt from Novartis Pharma AG (Basel, Switzerland). BPs were dissolved in water and stored at 4°C. [14C]Clodronate, [14C]ibandronate, and [14C]zoledronic acid were labeled by Leiras, Roche, and Novartis Pharma, respectively. Radiochemical purity of radiolabelled BPs was >98%, as determined by high-performance liquid chromatography. Specific activity of [14C]clodronate, [14C]ibandronate, and [14C]zoledronic acid was 0.19, 3.55, and 6.1 MBq/mg, respectively. Taxol® (paclitaxel) was purchased from Sigma (Iles d’Abeau, France). The basement membrane Matrigel was from Becton Dickinson (Bedford, MA).

Cell Proliferation Assay. Human umbilical vein endothelial cells (Promo-cell, Heidelberg, Germany) and human prostate PNT1a epithelial cells (a gift from Dr. Thierry Guillaudeux, INSERM U435, Rennes, France) were seeded in flat-bottomed 96-well plates (4 × 103 cells/well and 3 × 104 cells/well, respectively). After a 48-h incubation, growing cells were washed and additionally cultured in complete medium for 3 days in the presence or absence of increasing concentrations of a BP. Cell proliferation was measured using MTT at a concentration of 5 mg/ml (20 µl/well). MTT is reduced by the mitochondrial dehydrogenase of viable cells to a blue formazan product. After a 5-h incubation at 37°C, cells were solubilized in 0.01 N HCl containing 10% SDS for an additional 16-h incubation at 37°C to dissolve the blue formazan product. The absorption was measured spectrophotometrically at 550 nm using

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4 The abbreviations used are: BP, bisphosphonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPF, high power field.
BISPHOSPHONATES INHIBIT ANGIOGENESIS

Effects of BPs on angiogenesis in vitro. Angiogenesis, the sprouting of new blood vessels from preexisting ones, is a multiple-step process including proliferation of endothelial cells and their subsequent realignment to form new capillary tubes (13). The effect of BPs was assessed on each of these two steps. Exposure of endothelial cells to increasing concentrations of zoledronic acid (10⁻¹⁰ to 10⁻⁴ M) for 1–3 days resulted in a significant dose-dependent decrease in cell proliferation (Fig. 1A). Concomitantly to this decreased cell proliferation, zoledronic acid induced endothelial cell apoptosis (Fig. 1B). Apoptosis was assessed by a flow cytometry-based assay using annexin V conjugated to fluorescein (annexin V preferentially binds to phosphatidylserine exposed on the surface of cells undergoing apoptosis). As shown in Fig. 1B, there was a rightward shift in the histograms when endothelial cells were treated with zoledronic acid or Taxol® (a well-known proapoptotic drug). A 3-day exposure of endothelial cells to other BPs (ibandronate, risdronate, and clodronate) at a 10⁻⁴ M concentration also resulted in significant inhibition of cell proliferation (Fig. 1C). Beside the inhibitory effect of BPs on endothelial cell proliferation, zoledronic acid dose-dependently inhibited capillary-like tube formation, reaching 62% inhibition at 10⁻⁴ M (Fig. 2A). Half maximal (IC₅₀) inhibition achieved with zoledronic acid was 5 × 10⁻⁶ M. As exemplified in Fig. 2B, the formation of capillary-like tubes in the presence of zoledronic acid (10⁻⁴ M) was immature, and many endothelial cells remained, forming colonies without elongation. Ibandronate, risdronate, and clodronate used at a 10⁻³ M concentration were as effective as zoledronic acid at inhibiting capillary-like tube formation (Fig. 2C).

Quantification of the Vascularization in Bone Biopsy Specimens from Patients with Paget’s Disease before and after Clodronate Treatment. Qualitative histological changes in pagetic bone after clodronate treatment have been reported previously: a marked reduction in both marrow fibrosis and vascularity (12, 15). Because clodronate inhibited endothelial cell proliferation and capillary-like tube formation, the extent of the vascularization in the bone marrow from pre- and post-treatment pagetic bone biopsies was quantified. As shown in Fig. 3, there was a marked reduction in the number of blood vessels in pagetic bone marrow after clodronate treatment when compared with that observed in bone biopsies before treatment (mean ± SD: 9 ± 4 and 15 ± 7 vessels/HPF, respectively; P = 0.0001 using a paired t test). However, despite the use of different doses (400, 800, 1600, and 2400 mg/day) there was no dose-dependent inhibitory effect of clodronate on the vascularity. Similarly, the

RESULTS

Five-µm sections of the ventral prostate were then subjected to immunohistochemistry after a method described previously (17) using an antibody directed against endothelial cell CD31 (Santa Cruz Biotechnologies, Santa Cruz, CA). The lumen area of 40 immunostained vessels within each ventral prostate section was measured using a semiautomatic analyzer (Biocom, Paris, France), and results were expressed as the average area per vessel (µm²) for each immunostained section.

Statistical Analysis. Data obtained in patients with Paget’s disease were analyzed using paired Student’s t test. Unpaired Student’s t test was used to analyze all of the other experiments, and results were expressed as the mean ± SD. These analyses were performed using the Stat-View v5.0 software. All of the statistical tests were two-sided, and differences were considered to be statistically significant when P < 0.05.

Animals, Treatments, and Immunohistochemistry. All of the procedures were performed on adult male Sprague Dawley rats (weight 330 grams; IFFA CREDO, Saint Germain sur l’Arbresle, France). Studies involving animals, according to the protocol described by these authors (16). Twenty-one days after castration, animals received a s.c. dose of long-acting testosterone (15 mg/pellet; Innovative Research of America, Sarasota, FL) for 6 days. On testosterone treatment, rats were randomized into groups of 5–10 animals, and then received either a BP or the vehicle (PBS; pH 7.0), administrated s.c. at doses ranging from 5 to 100 µg/kg/day for 6 days. At sacrifice, on day 6, the ventral prostate was dissected, formalin-fixed, and paraffin-embedded.
number of osteoclasts per mm$^2$ is significantly decreased in patients treated with clodronate, with no significant difference among the four doses (12, 15).

**Tissue Distribution of the BPs Zoledronic Acid, Ibandronate, and Clodronate in Rats.** Although bone is highly vascularized and could be used as an in vivo angiogenic model, a decreased vascularity in the bone marrow upon BP treatment (as observed here with pagetic bone specimens) could be secondary to a reduction of bone resorption. To overcome this potential confounding effect of BPs, we were therefore interested in developing an in vivo angiogenesis model in a noncalcified tissue where BPs could accumulate. To address this question, we first studied the tissue distribution of different BPs in rats. After s.c. administration of $[^{14}C]$zoledronic acid (60 $\mu$g/kg), $[^{14}C]$ibandronate (100 $\mu$g/kg), or $[^{14}C]$clodronate (2 mg/kg) to male rats, the drug concentration in the bone continuously increased and reached a plateau at 1 h (Fig. 4). As expected, a substantial concentration of $[^{14}C]$BP was also observed in the kidney because it is the only eliminating organ for the drugs (Fig. 4). In addition, the amount of the different drugs in noncalcified tissues like the lung, spleen, and liver was negligible (Fig. 4). In sharp contrast, $[^{14}C]$zoledronic acid, $[^{14}C]$ibandronate, and $[^{14}C]$clodronate accumulated in the prostate, reaching a peak at 30–60 min, then declined rapidly with time (Fig. 4).

**Effect of a 1-h Pulse Treatment with Zoledronic Acid on Proliferation of Endothelial and Prostate Epithelial Cells.** Pharmacokinetic results obtained in the present study showed that the prostate was exposed to zoledronic acid, ibandronate, or clodronate only for a short period of time (i.e., 30–60 min) over a 24-h period. Therefore, we investigated whether a 1-h pulse treatment with zoledronic acid could inhibit cell proliferation to an extent similar to that observed with a continuous treatment. Pulse experiments were performed by incubating cells with zoledronic acid for 1 h, after which the drug was removed and replaced by fresh medium for the next 23 h.
This pulse treatment was repeated each day for 6 days. As observed with endothelial cells (HUVEC) and normal prostate epithelial cells (PNT1a), pulse treatment with zoledronic acid at a 10^{-4} M concentration significantly inhibited cell growth (Fig. 5). Similar results were obtained with ibandronate at a 10^{-4} M concentration, whereas clodronate had no significant inhibitory effect under these experimental conditions (results not shown).

BPs Inhibit Testosterone-stimulated Vascular Regrowth in the Ventral Prostate in Castrated Rats. Previous studies have shown that castration induces an involution of the prostate, which is preceded by a regression of the vasculature in the rat ventral prostate (16, 18). In addition, testosterone treatment rapidly induces a vasodilatation of blood vessels in the ventral prostate of castrated rats (16, 18). Because of the transient accumulation of BPs in the prostate, the effects of zoledronic acid, ibandronate, and clodronate on testosterone-induced revascularization of the prostate gland in castrated rats were studied.

In agreement with previous studies (16, 18), castration induced an involution of the prostate, which was partially normalized by a 6-day testosterone treatment (Fig. 6A). However, as exemplified in Fig. 6A, the size of the prostate gland was consistently smaller in castrated rats treated with testosterone + zoledronic acid. Indeed, ibandronate and zoledronic acid at a dose of 20 μg/kg/day induced a 17% and 35% reduction in the prostate weight, respectively, when testosterone was combined with one of these two different BPs (Fig. 6B). Treatment of castrated rats with testosterone in combination with clodronate (20 mg/kg/day) did not significantly inhibit prostate regrowth (Fig. 6B).

Histological examination of tissue sections showed that castration induced a marked reduction of blood vessel lumina in the stroma of the ventral prostate compared with that observed in intact animals (results not shown). However, a 6-day testosterone treatment induced a revascularization of the prostate as exemplified by the presence of large of blood vessels in the stroma (Fig. 7, top and bottom left panels). Morphometric analysis revealed that the average area per vessel was significantly increased in the ventral prostate of castrated animals treated with testosterone when compared with that observed in the absence of treatment (Table 1). A daily treatment of castrated rats with zoledronic acid for 6 days, at a dose (20 μg/kg) that inhibited prostate weight, resulted in a 47% reduction of the revascularization of the prostate gland under testosterone stimulation (Fig. 7). Similarly, ibandronate at a dose of 20 μg/kg/day for 6 days induced a 51% reduction of the prostate revascularization (Fig. 7). This reduction of the prostate revascularization in the presence of zoledronic acid or ibandronate was highly significant (P = 0.01 and P = 0.007, respectively). In contrast, clodronate (20 mg/kg/day) did not significantly inhibit the revascularization of the prostate gland under testosterone stimulation (30% reduction; P = 0.147). In the light of these findings, additional in vivo experiments were performed with zoledronic acid at doses ranging from 5 to 100 μg/kg/day (Table 1). A daily treatment of castrated rats with zoledronic acid at a dose of 5 μg/kg/day for 6 days did not inhibit testosterone-stimulated vascular regrowth (8% reduction; Table 1). However, a 41–54% reduction of vascular regrowth was observed on treatment with testosterone in combination with zoledronic acid at doses of 10 and 100 μg/kg/day, respectively (Table 1).
Zoledronic Acid Does Not Impair Androgen Receptor Response to Testosterone. A possibility remained that zoledronic acid (or ibandronate) formed a complex with testosterone in the circulation, thereby inhibiting the local effects of testosterone on vascular regrowth in the rat ventral prostate. To exclude this possibility, androgen-dependent LNCaP prostatic cells were transiently transfected with a luciferase gene reporter construct containing or not containing androgen-responsive elements. Zoledronic acid (10^{-8}-10^{-5} M) did not significantly inhibit luciferase activity induced by testosterone (Fig. 8). There was a decreased luciferase activity observed with zoledronic acid at a 10^{-5} M concentration. However, this was because of a direct cytotoxic effect of this BP on LNCaP cells (results not shown).

DISCUSSION

Our study provides evidence that BPs inhibit vascular endothelial functions in vitro and in vivo. This contention is based on a number of findings: (a) BPs reduced endothelial cell proliferation, induced apoptosis, and decreased capillary-like tube formation in vitro; and (b) zoledronic acid, ibandronate, and, to a much lesser extent, clodronate inhibited the revascularization of the prostate under testosterone stimulation, leading to a reduction in prostate regrowth. This observation is in agreement with the fact that the vasculature controls the growth of the prostate gland (16, 18). Pharmacokinetics of zoledronic acid, ibandronate, and clodronate demonstrated a specific accumulation of these BPs in the prostate, explaining therefore why some of these drugs were active in this noncalcified tissue. In addition, there was a correlation between the antiresorptive potency of these BPs and their ability to inhibit testosterone-stimulated prostate regrowth in vivo. For example, clodronate had a modest inhibitory effect on vascular regrowth in the prostate and is much less potent than ibandronate and zoledronic acid at inhibiting bone resorption in vivo (1). We have reported previously a similar structure-activity relationship of BPs on inhibition of cancer cell invasion and adhesion to bone (5, 6).

Mechanisms by which BPs accumulate in the prostate gland to inhibit testosterone-stimulated prostate regrowth are presently unclear. The accumulation of different BPs (alendronate, clodronate, and pamidronate) in liver and spleen has been reported previously in rats when using high i.v. doses of BPs (10-40 mg/kg; Ref. 19). When the drug concentration is too high, BPs form large complexes with metals (iron, calcium, and magnesium), and the phagocytosis of these complexes accounts for the retention of BPs in noncalcified tissues like the liver and spleen (19). However, the retention of BPs in the liver and spleen is observed only after i.v. administration but not after i.p. or s.c. injection at the same dose (19). In the present study, we were using low doses of BPs (ranging from 5 to 100 mg/kg), administrated s.c. in rats. Negligible amounts of these different drugs were present in the lung, spleen, and liver. Thus, it is most unlikely that a phagocytosis process accounted for the specific accumulation of these BPs in the prostate. In contrast, it has long been known that the prostate gland contains the highest level of zinc of all of the organs in the body (20). We have shown previously that zoledronic acid, ibandronate, and clodronate chelate zinc (6). Therefore, it is most conceivable that the
transient retention of BPs in the prostate gland was because of zinc chelation. Similarly, the high affinity of BPs for bone mineral is because of the chelation of calcium phosphate crystals by these compounds (1).

Because of their avidity for bone mineral, high concentrations of BPs (10^{-4}–10^{-3} M) can be achieved locally at sites of active bone resorption in vivo (21). Such high concentrations of BPs cause apoptosis in osteoclasts, breast, and prostate cancer cells in vitro (1, 7, 8), and it has been suggested that these high BP concentrations in metastatic bone lesions could induce apoptosis of cancer cells in vivo (22).

Similarly, in patients with Paget’s disease, it is most likely that high clodronate concentrations achieved in bones account for the marked reduction of the vascularity we observed here in pagetic bone biopsies. However, we could not rule out the possibility that this decreased vascularity in the bone marrow on clodronate treatment was secondary to a reduction of bone resorption. To overcome this potential confounding effect of BPs, we have used an angiogenesis model in the prostate gland (16). We have shown that zoledronic acid and ibandronate inhibited the revascularization of the prostate under testosterone stimulation. These findings are clinically relevant because the lowest effective dose of zoledronic acid used to inhibit vascular regrowth (10^{-6} g/kg/day for 6 days) is equivalent to a single 4-mg dose used currently in the treatment of patients with bone metastases. Mechanisms through which zoledronic acid and ibandronate inhibit the revascularization of the prostate gland are unknown. It is possible that these BPs are acting directly on blood vessels. In our in vitro experiments, maximal inhibitory effects of BPs on inhibition of endothelial cell functions were achieved at a 10^{-4} M concentration. Although it may be considered that such a concentration is high, it is important to note that zoledronic acid and ibandronate amounts in prostate and bone tissues were very similar for at least 30–60 min after their s.c.

**Table 1. Dose-dependent inhibitory effect of the bisphosphonate zoledronic acid on testosterone-stimulated vascular regrowth of the ventral prostate in castrated rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vessel area (μm²)</th>
<th>n⁴</th>
<th>% of control¹</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castration (C)</td>
<td>290 ± 145</td>
<td>10</td>
<td>24</td>
<td>0.001</td>
</tr>
<tr>
<td>C + testosterone (T)</td>
<td>1193 ± 586</td>
<td>20</td>
<td>100</td>
<td>0.76</td>
</tr>
<tr>
<td>C + T + zoledronic acid (5 μg/kg/day)</td>
<td>1102 ± 588</td>
<td>7</td>
<td>92</td>
<td>0.04</td>
</tr>
<tr>
<td>C + T + zoledronic acid (10 μg/kg/day)</td>
<td>705 ± 227</td>
<td>8</td>
<td>59</td>
<td>0.04</td>
</tr>
<tr>
<td>C + T + zoledronic acid (100 μg/kg/day)</td>
<td>552 ± 456</td>
<td>10</td>
<td>46</td>
<td>0.001</td>
</tr>
</tbody>
</table>

⁴ n is the number of animals. Results are a combination of three separate experiments. ¹ Castrated rats treated with testosterone were used as a control group, and data were set to 100%. Results obtained with the other treatments were expressed as a percentage of the control group. ² P when compared with the group treated with testosterone alone.
Results are the mean of two separate experiments; later, cells were solubilized, and the luciferase activity was measured using a luminometer.

in the presence or absence of increasing concentrations of zoledronic acid. Twenty-four h

gene reporter construct containing or not containing androgen-responsive-elements

administration in rats (Fig. 4). For example, 30 min after the s.c.
administration of [14C]zoledronic acid, concentrations in bone, prostate, and lungs were 19 ± 6, 20.7 ± 1, and 2.8 ± 0.6 ng/100 mg protein, respectively. On the basis of the high concentrations of BPs achieved in bone (10−2–10−3 m; Ref. 21), it is therefore possible that blood vessels in the prostate gland are exposed (for 30–60 min) to similar high local concentrations of BPs that substantially inhibited endothelial cell functions in our in vitro experiments. This assumption is supported by the fact that a 1-h pulse treatment with the BPs zoledronic acid and ibandronate (10−4 M) was as efficient as a continuous treatment with these drugs in inhibiting endothelial and epithelial cell proliferation in vitro. In this respect, BPs may also be active on the prostate epithelium in vivo. For example, testosterone treatment increases vascular endothelial growth factor mRNA expression in the prostate epithelium (16). It is possible that BPs indirectly act on blood vessels by inhibiting the angiogenic activity elaborated by the prostate epithelium under testosterone stimulation. This is in agreement with the fact that the BP pamidronate decreases circulating levels of vascular endothelial growth factor in cancer patients (23).

Recent findings strongly support the hypothesis that nitrogen-containing BPs (including ibandronate and zoledronic acid) inhibit enzymes of the mevalonate pathway, thereby preventing the prenylation of GTPases that are essential for osteoclast function (1). Whether ibandronate and zoledronic acid exert their cellular effect on endothelial and epithelial cells via inhibition of protein prenylation warrants additional investigation. Finally, the antiangiogenic activity of BPs was not restricted to our in vivo model of prostate revascularization. Clodronate, pamidronate, and zoledronic acid exert an antiangiogenic activity in the chicken-chorioallantoic membrane assay (24, 25).

In addition, zoledronic acid and, to a much less extent, pamidronate inhibit angiogenesis induced by s.c. implants impregnated with basic fibroblast growth factor (25).

In conclusion, this study has demonstrated that BPs inhibit endothelial cell functions in vitro and inhibit the revascularization of the prostate under testosterone stimulation. Because angiogenesis is essential for the growth of metastases of solid tumors (13), these observations are of potential relevance to improve therapy and prevention of bone metastasis. In addition, our results extend the potential clinical use of BPs to patients with early prostate cancer.

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