Bisphosphonates Inhibit Breast and Prostate Carcinoma Cell Invasion, an Early Event in the Formation of Bone Metastases

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ABSTRACT

The molecular mechanisms by which tumor cells metastasize to bone are likely to involve invasion, cell adhesion to bone, and the release of soluble mediators from tumor cells that stimulate osteoclast-mediated bone resorption. Bisphosphonates (BPs) are powerful inhibitors of the osteoclast activity and are, therefore, used in the treatment of patients with osteolytic metastases. However, an added beneficial effect of BPs may be direct antitumor activity. We previously reported that BPs inhibit breast and prostate carcinoma cell adhesion to bone (Boissier et al., Cancer Res., 57: 3890–3894, 1997). Here, we provide evidence that BP pretreatment of breast and prostate carcinoma cells inhibited tumor cell invasion in a dose-dependent manner. The order of potency for four BPs in inhibiting tumor cell invasion was: zoledronate > ibandronate > NE-10244 (active pyridinium analogue of risedronate) > clodronate. In addition, NE-58051 (the inactive pyridylpropionyl analogue of risedronate) had no inhibitory effect, whereas NE-10790 (a phosphonocarboxylate analogue of risedronate in which one of the phosphate groups is substituted by a carboxyl group) inhibited tumor cell invasion to an extent similar to that observed with NE-10244, indicating that the inhibitory activity of BPs on tumor cells involved the R2 chain of the molecule. BPs did not induce apoptosis in tumor cells, nor did they inhibit tumor-cell migration at concentrations that did inhibit tumor cell invasion. However, although BPs did not interfere with the production of matrix metalloproteinases (MMPs) by tumor cells, they inhibited their proteolytic activity. The inhibitory effect of BPs on MMP activity was completely reversed in the presence of an excess of zinc. In addition, NE-10790 did not inhibit MMP activity, suggesting that phosphonate groups of BPs are responsible for the chelation of zinc and the subsequent inhibition of MMP activity. In conclusion, our results provide evidence for a direct cellular effect of BPs in preventing tumor cell invasion and an inhibitory effect of BPs on the proteolytic activity of MMPs through zinc chelation. These results suggest, therefore, that BPs may be useful agents for the prophylactic treatment of patients with cancers that are known to preferentially metastasize to bone.

INTRODUCTION

A very common metastatic site for breast and prostate carcinomas is bone (1). In bone metastases, metastatic cells stimulate osteoclast-mediated bone resorption, leading to osteolyis (1). This finding provided the rationale for using BPs in the treatment of patients with osteolytic metastases because these compounds bind strongly to bone mineral and are powerful inhibitors of bone resorption (2). However, there may be an added beneficial effect of the BPs that is even more important. Animal studies have demonstrated that pretreatment of nude mice with the BPs risedronate and ibandronate before breast carcinoma cell inoculation produces a marked reduction in osteolytic lesions and a marked decrease in tumor burden in bone (3, 4). Such a beneficial effect of BPs in tumor burden in bone may result from a direct antitumor effect on breast carcinoma cells. For example, BPs inhibit breast carcinoma cell adhesion to bone in vitro (5, 6), and we have shown that BPs act directly on tumor cells to inhibit cell adhesion (6).

Interestingly, BPs also induce apoptosis in human myeloma cells (7) and inhibit MMP production by human PC3 ML prostate carcinoma cells (8, 9). Moreover, adjuvant treatment of breast cancer patients with the BP clodronate in combination with standard hormonal therapy or chemotherapy reduces the incidence and number of bone and non-bone metastases (10). These very important observations (3–10) suggest, therefore, that BPs not only act on osteoclast-mediated bone resorption but may also affect the invasive behavior of metastatic cells in bone. In the present study, we investigated the effect of BPs on tumor cell invasion. Tumor cell invasion in vivo is prerequisite for breast cancer colonization in bone (4). We have found that BPs inhibit breast and prostate carcinoma cell invasion through a specific action on tumor cells. In addition, BPs inhibited the proteolytic activity of MMPs, which is obligatory for successful tumor cell invasion. These results suggest that BPs may be useful agents for the prophylactic treatment of patients with cancers that are known to preferentially metastasize to bone.

MATERIALS AND METHODS

Drugs. Six BPs were used. Clodronate (dichloromethylene bisphosphonic acid) was obtained from Leiras Oy (Turku, Finland); Ibandronate (1-hydroxy-3-(methylpentylamino)-propylidene-bisphosphonic acid) was a gift of Dr. F. Baus (Roche Diagnostics GmbH, Mannheim, Germany); Zoledronate (1-hydroxy-2-(1H-imidazole-1-yl)ethylidene-bisphosphonic acid) was obtained from Novartis (Basle, Switzerland); NE 10244 [methyl 2-(3-pyridyl)-1-hydroxyethylidene-bisphosphonic acid], a potent antiresorptive analogue of risedronate; NE 58051 [3-(3-pyridyl)-1-hydroxypropylidene bisphosphonic acid], an inactive analogue of risedronate; and NE 10790, a phosphonocarboxylate analogue of risedronate were obtained from Procter & Gamble Pharmaceuticals (Cincinnati, OH). Taxol® (paclitaxel) was purchased from Sigma (Isles d’Abeau, France). All BPs were dissolved in water and stored at 4°C. Taxol was dissolved in absolute ethanol and stored at −70°C.

Tumor Cell Lines. Human breast carcinoma cell line MDA-MB-231 was purchased from the American Type Culture Collection (Rockville, MD). Cell line PmPC3 is a highly metastatic variant of human prostate carcinoma cell line PC3 (6). Both the MDA-MB-231 and the PmPC3 cell lines are highly metastatic to bone (3, 4).5 Tumor cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

Drug Treatment of Tumor Cell Lines. Tumor cell monolayers reaching 90% confluency were washed with serum-free RPMI 1640 and treated for 24 h at 37°C with BPs diluted in complete medium. Alternatively, cell monolayers were treated for 1 h at 37°C with Taxol in complete medium. At the end of the incubation, cell monolayers were washed with serum-free RPMI to remove drugs, and cells were harvested with trypsin-EDTA. After a 90-min incubation at 37°C to allow cells to recover from trypsin-EDTA treatment, untreated and treated cells were harvested and counted by trypan blue exclusion.

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3 The abbreviations used are: BP, bisphosphonate; MMP, matrix metalloproteinase.

4 S. Boissier and P. Clézardin, unpublished results.
drug-treated cells in RPMI containing 0.1% (w/v) BSA were used for cell migration and invasion assays.

**Cell Migration Assay.** Cell migration experiments were performed using Bio-Coat cell migration chambers (Becton Dickinson), which consist of a 24-well companion plate with cell culture inserts containing 8 µm pore size filters. The experimental procedure was as described previously (11). Briefly, untreated and drug-treated tumor cells (5 × 10^4/500 µl) were added to each insert (upper chamber), and the chemoattractant (10% FCS) was placed in each well of a 24-well companion plate (lower chamber). After a 6-h incubation at 37°C in a 5% CO2 incubator, the upper surface of the filter was wiped with a cotton-tipped applicator to remove nonmigratory cells. Cells that had migrated through the filter pores and attached on the under surface of the filter were fixed and stained. The membranes were mounted on glass slides, and the cells from 10 random microscopic fields (×400 magnification) were counted. All experiments were run in duplicate, and migration was expressed in terms of cells/mm².

**Matrigel Invasion Assay.** Cell invasion experiments were performed using Bio-Coat cell migration chambers (Becton Dickinson) as described above. Filters (8 µm pore size) were coated with the basement membrane Matrigel (37 µg/filter). The rest of the experimental procedure was as described for the cell migration assay. After a 48-h incubation at 37°C in a 5% CO2 incubator, noninvading cells were removed, and the invading cells on the under surface of the filter were fixed and stained. The membranes were mounted on glass slides, and the cells from 10 random microscopic fields (×400 magnification) were counted. All experiments were run in duplicate, and invasion was expressed in terms of cells/mm².

**Cell Cycle Analysis.** Untreated, BP-treated and Taxol-treated tumor cells harvested with trypsin-EDTA (10^4/500 µl for each experimental condition) were washed in RPMI, and then fixed in 50% (v/v) cold ethanol for 15 min at 4°C. After incubation, fixed cells were stained with propidium iodide (Becton Dickinson) for 10 min at room temperature in the dark. Cell cycle distribution was determined by flow cytometric analysis using the red fluorescence of excited propidium iodide-stained nuclei as a measure of DNA content. Linear displays of fluorescence emissions were used to compare cell cycle phases and quantify the cells with the degraded sub-G1 DNA content characteristic of apoptotic cells. Analysis of the data was performed using software Lysis II (Becton Dickinson).

**Flow Cytometry-based Apoptosis Assay.** Untreated, BP-treated, and Taxol-treated tumor cells were harvested with trypsin-EDTA, and then resuspended in PBS containing 2% (m/v) BSA (10^6 cells/ml for each experimental condition). Detection of apoptosis in tumor cells in suspension was performed using the TACS Annexin V apoptosis detection kit (Genzyme). The staining was analyzed on a FACScan flow cytometer (Becton Dickinson) as described previously (12).

**Analysis of Cell Death in Adherent Tumor Cells.** Tumor cells were plated on chamber slides (3 × 10^5 cells/chamber) and cultured in complete medium for 24 h at 37°C in a 5% CO2 incubator. Tumor cells were treated with BPs or Taxol as described above in the drug treatment section. After incubation, monolayers were washed in RPMI, and adherent cells were fixed in PBS containing 3% paraformaldehyde. After a 30-min incubation, cells were stained with Hoechst 33258 (10 µg/ml; Sigma) for 30–60 min in the dark and examined under fluorescence microscopy.

**Measurement of MMP Activities by Fluorometric Analysis.** Purified recombinant mouse pro-MMP-9 was activated with 1 nM p-aminophenylmercuric acetate for 4 h at 37°C. Human pro-MMP-2 from fibrosarcoma cells (Boehringer Mannheim, Mannheim, Germany) was activated with 1 nM p-aminophenylmercuric acetate for 45 min at 37°C. The active form of rabbit MMP-12 was a gift of Dr. P. Hou (Center for Clinical & Basic Research, Ballerup, Denmark). Enzyme concentrations in the final preparations were estimated by active site titration using the MMP inhibitor BB94 (British Biotech). Purified active MMP-2 (1 nM), MMP-9 (2 nM), and MMP-12 (10 nM) in the presence or absence of increasing concentrations of BPs were preincubated for 3 h at 37°C in 50 mM Tris, 1 mM CaCl₂, 150 mM NaCl, 0.05% (v/v) Brij 35 (pH 7.5) with or without ZnSO₄ (50 µM). MMP activities were then measured by evaluating the proteolytic cleavage of the fluorogenic peptide substrate Mca-Pro-Leu-Gly-Leu-7Np-Ala-Arg-NH₂ (Bachem, Bubenstock, Switzerland) following a previously described method (13). After addition of the fluorogenic peptide substrate (4 µM) to the preincubation mixture, aliquots were taken at time points up to 45 min into 0.1 M sodium acetate (pH 4.0) to stop the reaction before measurement of the increase in fluorescence. This increase in fluorescence corresponding to the release of Mca was measured using a SFM25 Kontron spectrofluorometer with an excitation wavelength of 320 nm and an emission wavelength of 387 nm. The rate of cleavage of the fluorogenic peptide substrate over 45 min obtained with MMs that were not preincubated with BPs was used as a positive control and was set at 100%. Results obtained in the presence of BPs were then expressed as a percentage of each positive control.

**Gelatin Zymography.** The serum-free conditioned media of untreated and BP-treated MDA-MB-231 and PmPC3 cells that had been cultured for 48 h on Matrigel-coated plates were lyophilized. Lyophilized conditioned media were resuspended in 0.5 ml of distilled water, and the protein concentrations were measured with a Bradford protein assay kit (Bio-Rad). Conditioned media (20
were run under non-denaturing conditions on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin in the presence or absence of the BP zoledronate for 4 h at 60 V. After electrophoresis, the gels were incubated in 2.5% (v/v) Triton X-100 for 2 h to remove SDS, washed briefly in distilled water, and then incubated in 50 mM Tris-HCl, 10 mM CaCl2 (pH 7.5) in the presence or absence of the BP zoledronate for overnight at 37°C. The gels were then stained with 0.25% (w/v) Coomassie brilliant blue and destained in methanol-acetic acid-water (20:7:73, v/v/v). White bands indicated gelatinase activity. The conditioned medium from human HT-1080 fibrosarcoma cells treated for 48 h with 12-o-tetradecanoylphorbol-13-acetate (10 ng/ml) was used as a positive control to visualize the gelatinolytic activity of MMP-2 and -9.

RESULTS

**BPs Inhibit Breast and Prostate Carcinoma Cell Invasion.** BPs inhibited MDA-MB-231 breast carcinoma cell invasion in a dose-dependent manner, reaching 60–90% inhibition (Fig. 1). The order of potency for four BPs in inhibiting tumor cell invasion was: zoledronate > ibandronate > NE-10244 (active pyridinium analogue of risedronate) > clodronate. Zoledronate and ibandronate inhibited tumor cell invasion with a half-maximal inhibition (IC50) of 10−12 M, whereas the IC50 for NE-10244 and clodronate were 5 × 10−10 and 5 × 10−5 M, respectively. In addition, NE-58051 (the inactive pyridylpropyldene analogue of risedronate) had no inhibitory effect, whereas NE-10790 (a phosphonocarboxylate analogue of risedronate in which one of the phosphonate groups is substituted by a carboxyl group) inhibited tumor cell invasion to an extent similar to that observed with NE-10244 (Fig. 1). BP pretreatment of PmPC3 prostate carcinoma cells also inhibited tumor cell invasion with the same magnitude (results not shown). The order of potency for zoledronate, ibandronate, NE-10244, NE-10790, and clodronate in inhibiting PmPC3 cell invasion was similar to that observed with MDA-MB-231, and the BP NE-58051 did not exert any inhibitory effect.

**BPs Do Not Induce Apoptosis in Human Breast and Prostate Carcinoma Cells at Concentrations that Inhibit Tumor Cell Invasion.** Human myeloma cells undergo apoptosis after treatment with BPs (7). In the light of this observation (7), we hypothesized that the inhibitory effect of BPs on tumor cell invasion could result from an apoptotic process. To address this question, the effects of zoledronate (the most potent BP used in this study) and Taxol (a well-known mitotic spindle toxin) on MDA-MB-231 and PmPC3 cell cycle progression were investigated. Taxol (100 nM) caused DNA degradation, as judged by the appearance of a sub-G1 peak during MDA-MB-231 cell cycle progression (Fig. 2A). The proportion of Taxol-treated tumor cells within the sub-G1 peak involved 40% of the cell population. Unlike Taxol, zoledronate (1000 nM) did not induce apoptosis in MDA-MB-231 and PmPC3 cells (Fig. 2A, and results not shown). Apoptosis was then assessed by a flow cytometry-based assay using annexin V conjugated to fluorescein as an early indicator of apoptosis (annexin V preferentially binds to phosphatidylserine exposed on the surface of cells undergoing apoptosis). As shown in Fig. 2B, histo-
grams of cell number versus fluorescence intensity showed a rightward shift in the histograms when MDA-MB-231 cells were treated with Taxol then exposed to annexin V, whereas zoledronate did not induce annexin V binding to the tumor cell surface. Similar results were obtained with PmPC3 cells (results not shown). Because both the cell cycle and annexin V binding assays used cells in suspension, analysis of cell death was also performed in adherent tumor cells using Hoechst staining. As shown in Fig. 2C, many of the MDA-MB-231 cells treated with Taxol had nuclei with separate globular structures (apoptotic bodies), whereas nuclei of untreated and zoledronate-treated cells had no such apoptotic bodies. Similar results were obtained with PmPC3 cells (results not shown).

**BPs Inhibit MMP Activity but Not Breast and Prostate Carcinoma Cell Migration.** Tumor cell invasion requires both cell migration and digestion of the basement membrane by secreted or membrane proteases (14). The effect of BPs on tumor cell migration was first investigated. None of the BPs used in this study inhibited MDA-MB-231 or PmPC3 cell migration at concentrations that did inhibit tumor cell invasion (results not shown). We then examined whether BPs inhibited MMP activity. In this respect, kinetic studies were performed using active forms of MMP-2, -9, and -12 (Fig. 3, inset). BPs inhibited the proteolytic activity of MMP-2, -9, and -12 in a dose-dependent manner with IC_{50} of ~40, 160, and 80 μM, respectively (Fig. 3). Surprisingly, despite structural differences in their bioactive moieties, all of the BPs used in this study (including the inactive analogue of risedronate, NE-58051) were equipotent in inhibiting the proteolytic activity of MMP-2, -9, and -12. These BPs have in common a structural motif, the so-called bone hook, which consists of the two phosphate groups that provide binding to bone mineral (15). This bone hook is also responsible for the chelation of divalent cations and the growth inhibition of calcium crystals (15). Because MMPs are zinc-dependent endopeptidases (16), we hypothesized that the phosphate groups of BPs could inhibit the proteolytic activity of MMP-2, -9, and -12 through zinc chelation. To address this question, we first examined the effect of BPs on the proteolytic activity of MMP-2, -9, and -12 in the presence of an excess of zinc (50 μM). The inhibitory effect of BPs on MMP activity was completely reversed in the presence of zinc (results not shown). We also examined the properties of a risedronate analogue (NE-10790), which belongs to the group of phosphonocarboxylates in which one of the phosphate groups is substituted by a carboxyl group (Fig. 1, inset). The phosphonocarboxylate NE-10790 has a markedly reduced affinity for bone mineral compared with that observed with BPs (17). Increasing concentrations of NE-10790 in the absence of an excess of zinc did not inhibit the proteolytic activity of MMP-9 and -12, whereas a modest inhibitory effect was observed on MMP-2 activity (Fig. 3). Thus, the bone hook of BPs is responsible for the inhibition of MMP activity.

We next examined whether BPs may inhibit MMP production by tumor cells. As shown in Fig. 4, pretreatment of MDA-MB-231 cells with zoledronate (10^{-6} M) did not inhibit production of MMP-2 and -9 (Fig. 4, Lane 3) when compared with that observed with untreated cells (Fig. 4, Lane 2). Similarly, the addition of zoledronate (10^{-6} M) in the gels and Ca^{2+}-containing buffers used for gelatin zymography did not significantly inhibit MMP activity (Fig. 4, Lane 4). However, the use of zoledronate at a higher concentration (10^{-4} M) caused a
Fig. 4. The BP zoledronate does not inhibit MMP production by MDA-MB-231 breast carcinoma cells. The serum-free conditioned medium from 12-O-tetradecanoylphorbol-13-acetate-treated HT1080 fibrosarcoma cells was used as a positive control to identify MMP-9 and -2 on gelatin zymograms (Lane 1). Serum-free conditioned medium from untreated (Lane 2) and zoledronate-treated (Lane 3) MDA-MB-231 cells grown on Matrigel were harvested, concentrated, and subjected to gelatin zymography. Alternatively, the serum-free conditioned medium from untreated cells was run on a SDS-polycrylamide gel containing gelatin and zoledronate at a concentration of $10^{-6}$ (Lane 4) or $10^{-4}$ M (Lane 5). White bands indicate gelatinase activity.

DISCUSSION

It has become apparent that BPs not only act on osteoclast-mediated bone resorption but may also affect the metastatic behavior of cells in bone (3–10). In bone metastases, tumor cells reside in the bone marrow and are also found adjacent to the resorbed endosteal bone surface (1). We and others have reported previously that BPs inhibit breast and prostate carcinoma cell adhesion to bone in vitro (5, 6). In the present study, we provided clear evidence that BPs act directly on breast and prostate carcinoma cells to inhibit tumor cell invasion. As shown in Fig. 1 (inset), minor changes in the R2 chain length of NE-10244 (the active analogue of risedronate) drastically affected its inhibitory activity on tumor cell invasion. Indeed, the inactive analogue of risedronate (NE-58051) is 10,000-fold less potent than NE-10244 as an antiresorptive agent in vivo (18), and we previously reported similar large potency variations between NE-10244 and NE-58051 on inhibition of tumor cell adhesion (6). Interestingly, the use of a phosphonocarboxylate analogue of risedronate (NE-10790), in which one of the phosphate groups is substituted by a carboxyl group, inhibited tumor cell invasion to an extent similar to that observed with NE-10244. These data obtained with NE-58051 and NE-10790 strongly suggested that the inhibitory effect of BPs on tumor cells involves the R2 chain (but not the so-called bone hook) of the molecule. Such an inhibitory effect of BPs on tumor cells was independent of apoptosis induction. Zoledronate (the most potent BP used in this study) did not induce apoptosis at concentrations ($10^{-12}$–$10^{-6}$ M) that did inhibit tumor cell invasion (Fig. 2) nor at a higher concentration ($10^{-4}$ M). This is in contrast to the observation that human myeloma cells exposed to high concentrations (up to $5 \times 10^{-4}$ M) of different BPs (including clodronate, pamidronate, and zoledronate) undergo apoptosis (7, 19). However, it must be emphasized that in our study, BP treatment of tumor cells was performed in the presence of serum, which is a survival factor for these cells. It is therefore most conceivable that serum counteracts the pro-apoptotic effect of high concentrations of BPs.

Because tumor cell invasion requires both cell migration and digestion of the basement membrane by MMPs (14), we hypothesized that BPs could affect one or the other of these two mechanisms. In agreement with the observation that the BPs pamidronate and tiludronate do not inhibit the migration of preosteoclasts (20, 21), BPs used in this study failed to inhibit tumor cell migration. In contrast, we observed that BPs inhibited the proteolytic activity of MMP-2, -9, and -12. The BP clodronate also inhibits MMP-1 proteolytic activity (22). These BPs have in common a structural motif, the so-called bone hook, which consists of the two phosphate groups that provide binding to bone mineral (15). Because MMPs are zinc-dependent endopeptidases (16), we suggest that the bone hook of BPs inhibits the proteolytic activity of MMPs through chelation of divalent cations. This contention is based on a number of findings. (a) Despite structural differences in their bioactive moiety (i.e., the R2 chain), all of the BPs used here (including the inactive analogue of risedronate, NE-58051) were equipotent in inhibiting the proteolytic activity of MMP-2, -9, and -12. (b) An excess of zinc reversed the inhibitory effect of BPs on the proteolytic activity of MMP-1, -2, -9, and -12 (Ref. 22, and this study). (c) The phosphonocarboxylate NE-10790, which has a strongly reduced affinity for divalent cations (17), did not inhibit the proteolytic activity of MMP-2, -9, and -12. (d) Zoledronate inhibited the gelatinolytic activity (but not the production) of MMP-2 and -9. The observation that BPs inhibited the proteolytic activity of MMPs is of potential relevance to the treatment of bone metastases. BPs are selectively located on the bone surface in the resorption space where their local concentration can reach several hundred micromolar (15). In bone metastases, it is conceivable that BPs released from resorbed bone inhibit the proteolytic activity of MMPs secreted from tumor cells. However, our results did not give a satisfactory explanation to understand how BPs directly interfere with tumor cell invasion. We have provided clear evidence that the inhibitory activity of BPs on tumor cell invasion required the R2 chain of the molecule (and was effective at low concentrations), whereas inhibition of MMP activity by BPs occurred through the so-called bone hook of the molecule (and was effective only at high concentrations). Thus, other mechanisms are involved. Recently, it has been demonstrated that nitrogen-containing BPs inhibit osteoclastic resorption by affecting enzymes of the cholesterol metabolic pathway involved in the geranylgeranylation of small GTP-binding proteins (23, 24). It is conceivable that BPs also affect the prenylation of some small GTP-binding proteins in breast and prostate carcinoma cells. This hypothesis warrants further investigation.

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