

The Bisphosphonate Ibandronate Promotes Apoptosis in MDA-MB-231 Human Breast Cancer Cells in Bone Metastases¹

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ABSTRACT

Bisphosphonate (BP), a specific inhibitor of osteoclasts, has been widely used as a beneficial agent for the treatment of bone metastases in patients with breast cancer. It is well recognized that BP reduces osteolysis by promoting apoptosis in osteoclasts. However, recent animal and human data suggest that BPs not only reduce osteolysis associated with metastatic breast cancer, but also decrease tumor burden in bone. The mechanisms by which tumor burden is decreased following BP administration are unknown. Here we examined the effects of the BP ibandronate on MDA-231 human breast cancer cells in bone metastases in a well-characterized animal model of bone metastasis. Ibandronate, which was administered (s.c. daily; 4 $\mu\text{g}/\text{mouse}/\text{day}$) after bone metastases were established, inhibited the progression of established osteolytic bone metastases as assessed by radiographic analysis. Histological and histomorphometrical examination revealed that ibandronate reduced osteoclastic bone resorption, with increased apoptosis in osteoclasts. Furthermore, ibandronate also significantly decreased the MDA-231 tumor burden, with increased apoptosis in MDA-231 breast cancer cells in bone metastases. In contrast, ibandronate failed to inhibit MDA-231 tumor formation with no effects on apoptosis in MDA-231 breast cancer cells in the orthotopic mammary fat pads. These data suggest that the effects of ibandronate on apoptosis in MDA-231 breast cancer cells are restricted in bone in which ibandronate selectively deposits. Consistent with these *in vivo* results, a relatively high concentration of ibandronate (100 μM) increased caspase-3 activity and induced DNA fragmentation in MDA-231 breast cancer cells in culture. Moreover, a caspase inhibitor, z-Val-Ala-Asp-fluoromethyl ketone, blocked ibandronate-induced DNA fragmentation in MDA-231 cells, suggesting an involvement of caspase-3 in ibandronate-induced apoptosis. Our results suggest that BP suppresses bone metastases through promotion of apoptosis in metastatic cancer cells as well as in osteoclasts. However, it still remains open whether BP has direct anticancer actions *in vivo*.

INTRODUCTION

BPs⁴ are widely used for the treatment of bone metastases in breast cancer patients. Accumulating clinical data have demonstrated that BPs are beneficial agents in these breast cancer patients (1–4). Because BPs are potent and specific inhibitors of osteoclastic bone resorption (5), these results indicate that osteoclasts play a key role in the pathogenesis of bone metastases and that inhibition of osteoclastic bone resorption is an effective therapeutic intervention for the treatment of bone metastases (6). We recently have shown that BPs inhibit bone resorption by promoting apoptosis in osteoclasts and reduce

metastatic tumor burden in bone, with increased apoptosis in osteoclasts (7, 8). These results suggest that BPs inhibit bone metastases by promoting apoptosis in osteoclasts.

On the other hand, it is unclear why tumor burden in bone is decreased by BPs. Recent *in vitro* data have shown that BPs inhibit the attachment of breast and prostate cancer cells to bone matrix (9, 10) and enhance apoptosis in myeloma cells (11–13) and breast cancer cells (14, 15). Moreover, a clinical study has shown that the BP clodronate suppresses distant metastases in both bone and visceral organs in breast cancer patients (16). These results suggest that BPs may have some anticancer effects, which in turn reduce metastatic tumor burden in bone. However, the effects of BP on cancer cells colonized in bone are yet to be elucidated.

In the present study, to further elucidate the mechanism by which BP inhibits bone metastases, we examined the effects of the BP ibandronate on MDA-231 human breast cancer cells metastasized in bone, using a well-characterized animal model of bone metastasis. Our results show that ibandronate inhibits bone metastases of MDA-231 breast cancer by promoting apoptosis not only in osteoclasts but also metastatic MDA-231 cells.

MATERIALS AND METHODS

Breast Cancer Cells

The estrogen-independent human breast cancer cell line MDA-231 (17) was cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin solution (Life Technologies, Inc.) in a humidified atmosphere of 5% CO₂ in air.

Animal Model

Animals. Four-week-old female BALB/c-*nu/nu* mice (Harlan Industries, Houston, TX) were used for the experiments. Mice were kept in laminar flow isolators in our animal facilities.

Intracardiac Inoculation. Intracardiac injection was performed as described previously (8, 18). Subconfluent MDA-231 cells were refed with fresh medium 24 h before injection. Cells (1×10^5) suspended in 0.1 ml of PBS were injected with a 27-gauge needle into the left cardiac ventricles of mice under anesthesia (pentobarbital, 0.05 mg/g of body weight).

Orthotopic Inoculation. MDA-231 cells (5×10^6) were prepared in a manner similar to the intracardiac injection, suspended in 0.1 ml of PBS, and injected into the mammary fat pads of mice under anesthesia (pentobarbital, 0.05 mg/g of body weight).

BP

The BP ibandronate [1-hydroxy-3-(methylpentylamino)propylidene bisphosphonate; BM 21.0955; Ref. 19], which was kindly provided by Boehringer Mannheim GmbH (Mannheim, Germany), was used after dilution in PBS.

Experimental Protocol

The administration protocol for ibandronate is depicted in Fig. 1.

In bone metastasis experiments, animals were inoculated with MDA-231 cells on day 0 and examined for the development of osteolytic lesions by radiographs at day 21. Animals showing distinct osteolytic metastases were divided into two groups. One group of mice received PBS, and the other group

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⁴ The abbreviations used are: BP, bisphosphonate; TRAP, tartrate-resistant acid phosphatase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Z-VAD-FMK, z-Val-Ala-Asp-fluoromethyl ketone; Ac-DEVD-pNA, N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline.

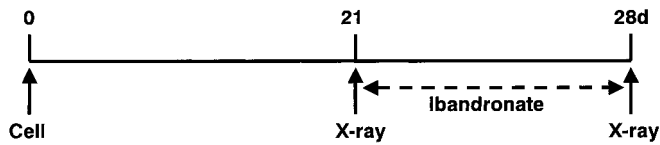
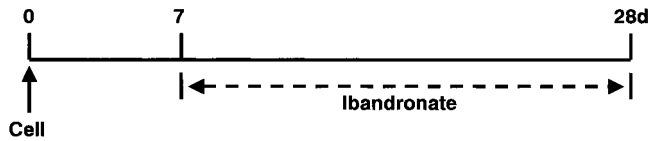
(A) Intracardiac Injection**(B) Intramammary Injection**

Fig. 1. Summary of the experimental protocol. MDA-231 breast cancer cells were inoculated into the left cardiac ventricle (A) or into the mammary fat pad (B) of 4-week-old female nude mice at day 0. Ibandronate ($4 \mu\text{g}/\text{mouse}$) was injected daily s.c. All mice were sacrificed at day 28.

received ibandronate ($4 \mu\text{g}/\text{mouse}$) s.c. daily from day 21 to day 28. At day 28, mice were examined again radiologically for osteolytic bone metastases and sacrificed for histological and histomorphometrical determinations. The experiments were carried out twice.

In orthotopic tumor formation experiments, MDA-231 cells were inoculated in the mammary fat pad on day 0 and ibandronate ($4 \mu\text{g}/\text{mouse}$) was administered s.c. daily from day 7 to day 28. Control mice received PBS. The experiments were carried out twice.

Radiographic Measurement of Osteolytic Lesion Area

Areas of osteolytic lesions were determined on radiographs as described previously (8). Animals were anesthetized deeply with pentobarbital (0.05 mg/g of body weight), laid in prone position against the films ($22 \times 27 \text{ cm}$; X-OMAT AR; Eastman Kodak Co., Rochester, NY), and exposed to an X-ray at 35 kV for 6 s using a Faxitron radiographic inspection unit (43855A; Faxitron X-ray Corporation, Buffalo Grove, IL). Films were developed using a RP X-OMAT processor (M6b; Eastman Kodak Co.). Radiographs were analyzed extensively and carefully by three different individuals who had no prior knowledge of the experimental protocol. The areas of osteolytic bone metastases, which were observed as demarcated radiolucent lesions in the hindlimbs, were quantitatively assessed using a computer-assisted JAVA image analysis system (Jandel Scientific, Corte Madera, CA). Changes in size of osteolytic lesions were analyzed by comparing the radiographs of each individual mouse taken at day 21 with those taken at day 28. Data are shown as the percentage of increase in osteolytic lesion: (size of osteolytic lesion at day 28 – size of osteolytic lesion at day 21)/size of osteolytic lesion at day 21.

Histological and Histochemical Examination

Mice anesthetized with pentobarbital (0.05 mg/g of body weight) were fixed by perfusion first with saline and then with 4% paraformaldehyde buffered with 0.1 M phosphate buffer (pH 7.4) through the left cardiac ventricle as described previously (18). In bone metastasis experiments, the femora and tibiae were dissected, immersed in the same fixative overnight, and decalcified in 4.13% EDTA at room temperature for 1 week. In orthotopic tumor formation experiments, tumors in mammary fat pads were dissected, weighed, and immersed in the same fixative overnight. Paraffin sections were made following conventional methods and stained with H&E. Histochemical examination for TRAP was also performed as described previously (18).

Histomorphometrical Examination

Tumor Burden. We performed histomorphometrical analysis of tumor burden in the metastatic tumors in the distal femoral and proximal tibial metaphyses of both hindlimbs, using longitudinal sections stained with H&E ($\times 200$ magnification) and the OsteoMeasure System (Osteometrics, Atlanta, GA). To determine the tumor area, five fields were randomly selected in each section. Data are shown as tumor area (mm^2)/total area of the field (mm^2).

Osteoclast Number. The number of TRAP-positive multinucleated osteoclasts at the interface between tumor and bone was counted in five fields of each section ($\times 400$ magnification) and expressed per millimeter of this interface distance as described previously (8).

Apoptosis in Osteoclasts. Apoptotic osteoclasts were identified by the presence of chromatin condensation and/or nuclear fragmentation in osteoclasts showing cytoplasmic contraction and loss of adhesion to the underlying bones, using TRAP-stained sections (7). The number of apoptotic osteoclasts at the tumor-bone interface was counted in five fields of each section ($\times 400$ magnification), and data are shown as the number of apoptotic osteoclasts/total number of osteoclasts counted.

Apoptosis and Mitosis of MDA-231 Cells. Apoptosis in MDA-231 cells was determined with TUNEL-stained sections ($\times 400$ magnification). TUNEL staining was performed with the ApopTag Plus *In Situ* Apoptosis Detection Kit (Oncor, Inc., Gaithersburg, MD) according to the manufacturer's protocol. Mitotic cells were quantified using H&E-stained sections at $\times 400$ magnification. To determine the number of apoptotic and mitotic cells, we randomly selected and counted five fields of nonnecrotic areas of metastatic tumors in bone or mammary tumors in each specimen. Data are expressed as number of cells/ mm^2 tumor area.

In Vitro Analysis of Apoptosis

DNA Fragmentation Assay. MDA-231 cells (5×10^5) were cultured in 10-cm plates for 24 h and treated with 1, 10, or $100 \mu\text{M}$ ibandronate for an additional 72 h. Intr nucleosomal DNA fragmentation was detected by agarose gel electrophoresis according to a method described previously (20). In some experiments, DNA fragmentation of MDA-231 cells treated with ibandronate in the presence or absence of a caspase inhibitor, Z-VAD-FMK ($50 \mu\text{M}$; Promega Co., Madison, WI), was also examined.

Caspase Assay. MDA-231 cells (2×10^5) were cultured in 6-well plates for 24 h and treated with $100 \mu\text{M}$ ibandronate for an additional 72 h in the presence or absence of $50 \mu\text{M}$ Z-VAD-FMK. Caspase-3 activity was measured by proteolytic cleavage of the caspase-3 substrate Ac-DEVD-pNA, using the CaspACE Assay System (Promega) according to manufacturer's instruction.

Statistical Analysis

All data were analyzed by Student's *t* test or Welch's *t* test for unpaired samples. All data were presented as the mean \pm SE.

RESULTS

Effects of Ibandronate on the Progression of Established Bone Metastases. Radiographic examination demonstrated the development of small but distinctive radiolucent metastatic osteolytic lesions in the distal femur and proximal tibiae 21 days after cell inoculation (Fig. 2, A-a and A-c). These osteolytic lesions markedly enlarged at 7 days in the untreated group (Fig. 2A-b). In contrast, treatment with ibandronate profoundly impaired the enlargement of these osteolytic lesions (Fig. 2A-d). Quantitative radiological assessment showed that ibandronate significantly inhibited the progression of the osteolytic bone metastases (Fig. 2B).

Effects of Ibandronate on Metastatic MDA-231 Cells. We then examined the effects of ibandronate on metastatic tumor burden in bone. Histological examination of untreated bones revealed that all trabecular bones were destroyed and the bone marrow cavity was almost completely replaced by metastatic MDA-231 breast cancer cells (Fig. 3A-a). In contrast, in the ibandronate-treated bone, colonization of metastatic MDA-231 breast cancer cells in the bone marrow cavity was markedly decreased and the majority of trabecular bones remained undestroyed (Fig. 3A-b). Consistent with these histological observations, histomorphometrical analysis demonstrated that ibandronate significantly decreased the metastatic MDA-231 tumor burden in bone (Fig. 3B).

We subsequently examined whether decreased metastatic MDA-231 tumor burden in bones treated with ibandronate was associated

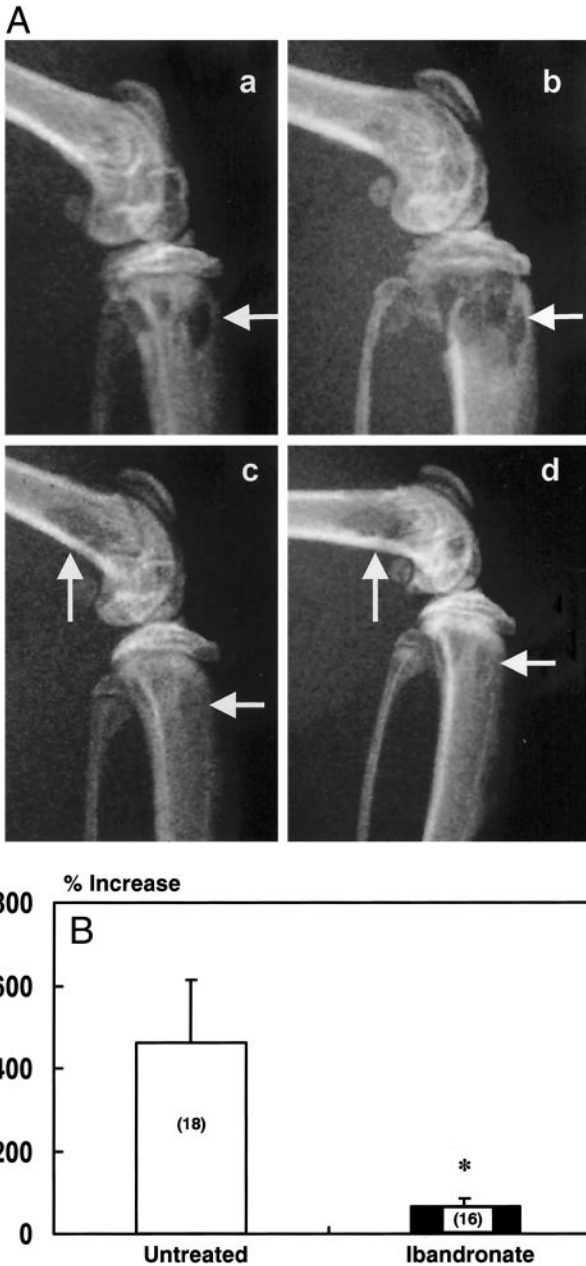


Fig. 2. *A*, representative radiographs of osteolytic lesions in hindlimbs of nude mice treated without (*a* and *b*) or with (*c* and *d*) ibandronate. At day 21 (before treatment), small but distinctive radiolucent osteolytic lesions (arrows) are detected in the distal femur and proximal tibiae (*a* and *c*). The size of these osteolytic lesions in untreated bones is markedly increased 7 days later, at day 28 (*b*). In contrast, in ibandronate-treated bones, enlargement of these osteolytic lesions is profoundly suppressed (*d*). *B*, effects of ibandronate on the progression of established osteolytic bone metastases. Changes in osteolytic lesion size (% increase) were determined as described in "Materials and Methods." Ibandronate markedly inhibited the enlargement of osteolytic bone metastases compared with the untreated group. Data are mean \pm SE (bars). Numbers in parentheses indicate number of animals studied (combination of two separate experiments). *, significantly different from untreated group ($P < 0.01$).

with increased apoptosis in MDA-231 breast cancer cells in bone metastases. We observed that some cancer cells shrank in size and were separated from neighboring cells by a surrounding halo-like clear space (Fig. 4A). Nuclear chromatin condensation and/or fragmentation of the nucleus were also observed in these cancer cells. These histological findings are characteristic features of apoptotic cancer cells (21), which were clearly distinguishable from mitotic cancer cells (Fig. 4A). To quantify the number of apoptotic cancer cells in bone metastases, we used the TUNEL intranucleosomal DNA

fragment end-labeling technique. TUNEL-positive cells were clearly identified as brown-stained cells (Fig. 4B). We found that ibandronate significantly increased the number of apoptotic MDA-231 breast cancer cells in bone metastases (Fig. 4C-a). However, mitosis in metastatic MDA-231 breast cancer cells was not affected by ibandronate (Fig. 4C-b).

To examine whether the apoptosis-promoting effect of ibandronate on MDA-231 breast cancer cells could be observed in sites other than bone, we determined apoptosis and mitosis in MDA-231 cells in the tumors formed in the orthotopic mammary fat pads. There was no difference in MDA-231 tumor burden in the orthotopic site between the untreated and ibandronate-treated groups 4 weeks after cell inoculation [tumor wet weights of untreated and ibandronate-treated were 2.91 ± 0.41 ($n = 10$) and 2.52 ± 0.24 ($n = 9$) g, respectively; not significant]. Moreover, histomorphometrical analysis showed no changes in apoptosis (Fig. 5a) and mitosis (Fig. 5b) in MDA-231 breast cancer cells in these tumors between the untreated and the ibandronate-treated group.

We were not able to determine the effects of ibandronate on the metastases in organs other than bone because heart-inoculated cancer cells rarely spread to visceral organs in this animal model (22).

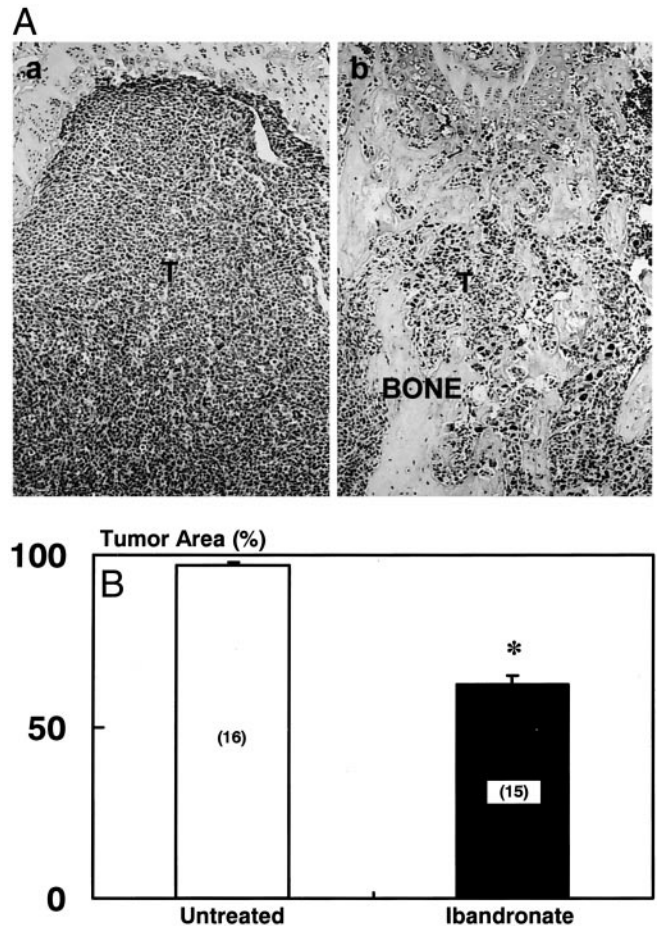


Fig. 3. *A*, histological view of bone metastases treated without (*a*) or with (*b*) ibandronate. In untreated bone (*a*), all trabecular bones are destroyed and the bone marrow cavity is completely replaced by metastatic MDA-231 breast cancer cells (T). In contrast, in ibandronate-treated bone (*b*), colonization of metastatic MDA-231 breast cancer cells is decreased and the majority of trabecular bones remain undestroyed. *B*, histomorphometrical analysis of metastatic tumor burden in bone treated with or without ibandronate. Ibandronate significantly decreased tumor burden in bone. Tumor burden was determined as described in "Materials and Methods." Data are mean \pm SE (bars). Numbers in parentheses indicate number of animals studied (combination of two separate experiments). *, significantly different from untreated group ($P < 0.0001$).

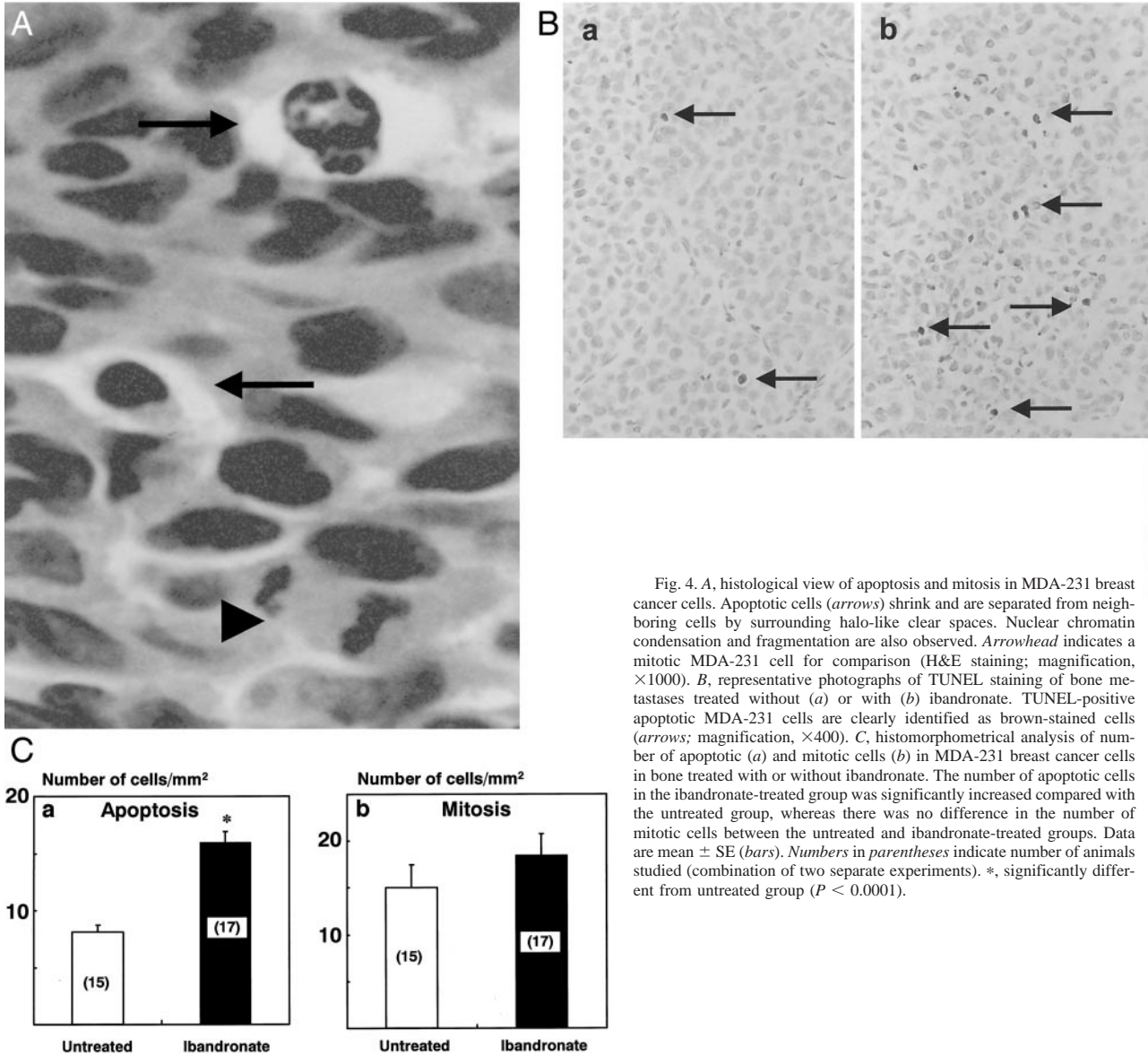


Fig. 4. A, histological view of apoptosis and mitosis in MDA-231 breast cancer cells. Apoptotic cells (arrows) shrink and are separated from neighboring cells by surrounding halo-like clear spaces. Nuclear chromatin condensation and fragmentation are also observed. Arrowhead indicates a mitotic MDA-231 cell for comparison (H&E staining; magnification, $\times 1000$). B, representative photographs of TUNEL staining of bone metastases treated without (a) or with (b) ibandronate. TUNEL-positive apoptotic MDA-231 cells are clearly identified as brown-stained cells (arrows; magnification, $\times 400$). C, histomorphometrical analysis of number of apoptotic (a) and mitotic cells (b) in MDA-231 breast cancer cells in bone treated with or without ibandronate. The number of apoptotic cells in the ibandronate-treated group was significantly increased compared with the untreated group, whereas there was no difference in the number of mitotic cells between the untreated and ibandronate-treated groups. Data are mean \pm SE (bars). Numbers in parentheses indicate number of animals studied (combination of two separate experiments). *, significantly different from untreated group ($P < 0.0001$).

Effects of Ibandronate on Apoptosis in MDA-231 Cells *in Vitro*.

To determine the effects of ibandronate on the apoptosis in MDA-231 cells *in vitro*, we assessed intranucleosomal DNA fragmentation using agarose gel electrophoresis. Ibandronate at 1 and 10 μM had no effects on MDA-231 cell apoptosis, whereas ibandronate at 100 μM induced

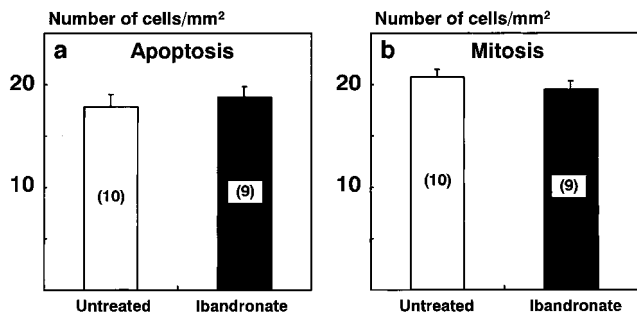


Fig. 5. Histomorphometrical analysis of number of apoptotic (a) and mitotic cells (b) in MDA-231 tumor formed in mammary fat pads in mice treated with or without ibandronate. There were no differences between the untreated and ibandronate-treated group in the numbers of apoptotic and mitotic cells. Data are mean \pm SE (bars). Numbers in parentheses indicate number of animals studied (combination of two separate experiments).

evident apoptosis in MDA-231 cells (Fig. 6A). The DNA fragmentation induced by ibandronate was almost completely inhibited by the caspase inhibitor Z-VAD-FMK (Fig. 6B). To examine the mechanism of apoptosis induced by ibandronate, we measured caspase-3 activity (Fig. 6C). Ibandronate (100 μM) significantly increased caspase-3 activity in MDA-231 cells, and the caspase inhibitor Z-VAD-FMK reduced this increase to a level similar to that of the control (Fig. 6C).

Effects of Ibandronate on Osteoclasts in Bone Metastases. Finally, the effects of ibandronate on osteoclasts were examined. Histochemical examination by TRAP staining revealed that there were numerous osteoclasts facing along the endosteal bone surfaces in untreated bone (Fig. 7A-a). In contrast, the number of osteoclasts was dramatically decreased in ibandronate-treated bone (Fig. 7A-b). Consistent with these histochemical observations, histomorphometrical analysis demonstrated a marked decrease in osteoclast number in ibandronate-treated bone (Fig. 7B). Furthermore, ibandronate also increased the number of apoptotic osteoclasts in bone metastases. Apoptotic TRAP-positive osteoclasts were readily recognizable by their unique morphological appearance. They were detached from bone surfaces and exhibited typical nuclear chromatin condensation (Fig. 8A). Quantitative analysis demonstrated that there was a marked

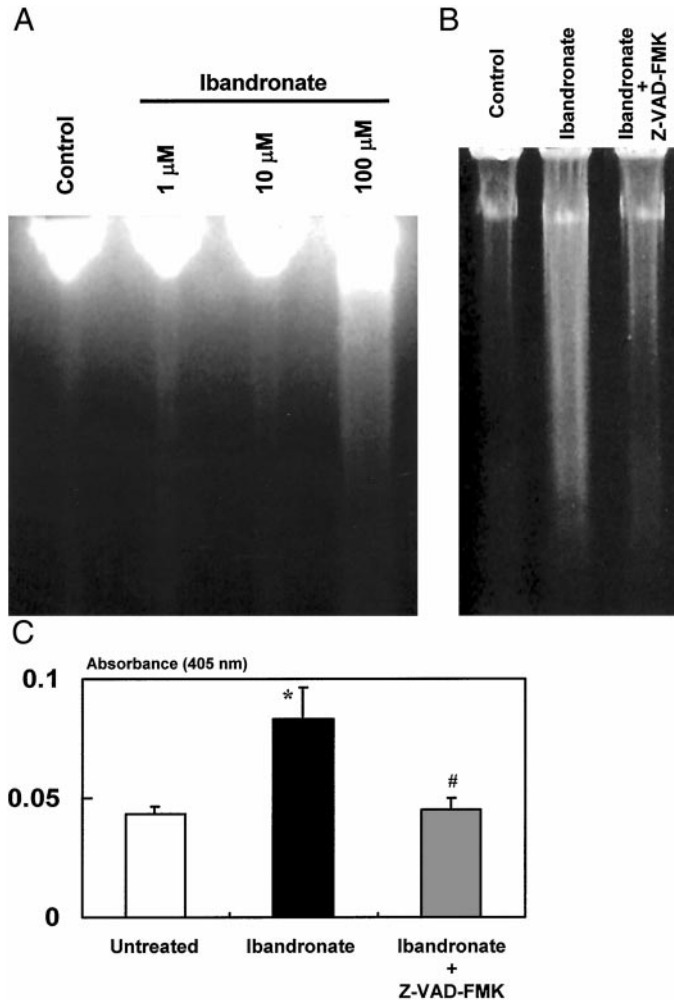


Fig. 6. Effects of ibandronate on apoptosis in MDA-231 breast cancer cells *in vitro*. *A*, apoptosis was examined by electrophoretic analysis of internucleosomal DNA fragmentation following treatment of MDA-231 cells with 1, 10, and 100 μM ibandronate for 72 h. Ibandronate at 100 μM , but not 1 and 10 μM , caused apoptosis in MDA-231 breast cancer cells. *B*, DNA fragmentation in MDA-231 cells treated with 100 μM ibandronate for 72 h in the presence or absence of caspase inhibitor Z-VAD-FMK. DNA fragmentation induced by ibandronate was almost completely inhibited by Z-VAD-FMK. *C*, caspase-3 activity in MDA-231 cells was determined by proteolytic cleavage of the caspase-3 substrate Ac-DEVD-pNA following treatment with 100 μM ibandronate for 72 h in the presence or absence of Z-VAD-FMK. Ibandronate increased caspase-3 activity in MDA-231 cells; the increase was suppressed by Z-VAD-FMK to that of the control. *, significantly different from untreated group ($P < 0.05$). #, significantly different from ibandronate-treated group ($P < 0.05$).

increase in the number of apoptotic osteoclasts in ibandronate-treated bone as measured by the percentage of apoptosis at the tumor bone interface (Fig. 8B). These results are consistent with our previous results (7, 8).

DISCUSSION

BPs have been widely used for the treatment of bone metastases in breast cancer patients (1–4). Nevertheless, the effects of BPs on breast cancer cells in bone metastases remain unclear. Our data show that ibandronate increased apoptosis in MDA-231 breast cancer cells in bone metastases and decreased metastatic MDA-231 tumor burden, whereas mitosis of these MDA-231 breast cancer cells was not changed. These results suggest that ibandronate has apoptosis-promoting effects on metastatic MDA-231 breast cancer cells in bone and that the suppressive effect of ibandronate on the MDA-231 breast tumor burden in bone is attributable to enhancement of apoptosis

rather than inhibition of cell proliferation in metastatic MDA-231 breast cancer cells. On the other hand, ibandronate showed no effects on apoptosis and mitosis in MDA-231 breast cancer cells in the tumors formed in the orthotopic sites. Thus, ibandronate exerts its apoptosis-promoting effects selectively in bone. Because BPs are shown to preferentially accumulate in bone after systemic administration (5), this differing effect of ibandronate on apoptosis between bone and the orthotopic site could be a result of differences in the local concentrations of ibandronate deposited in each site.

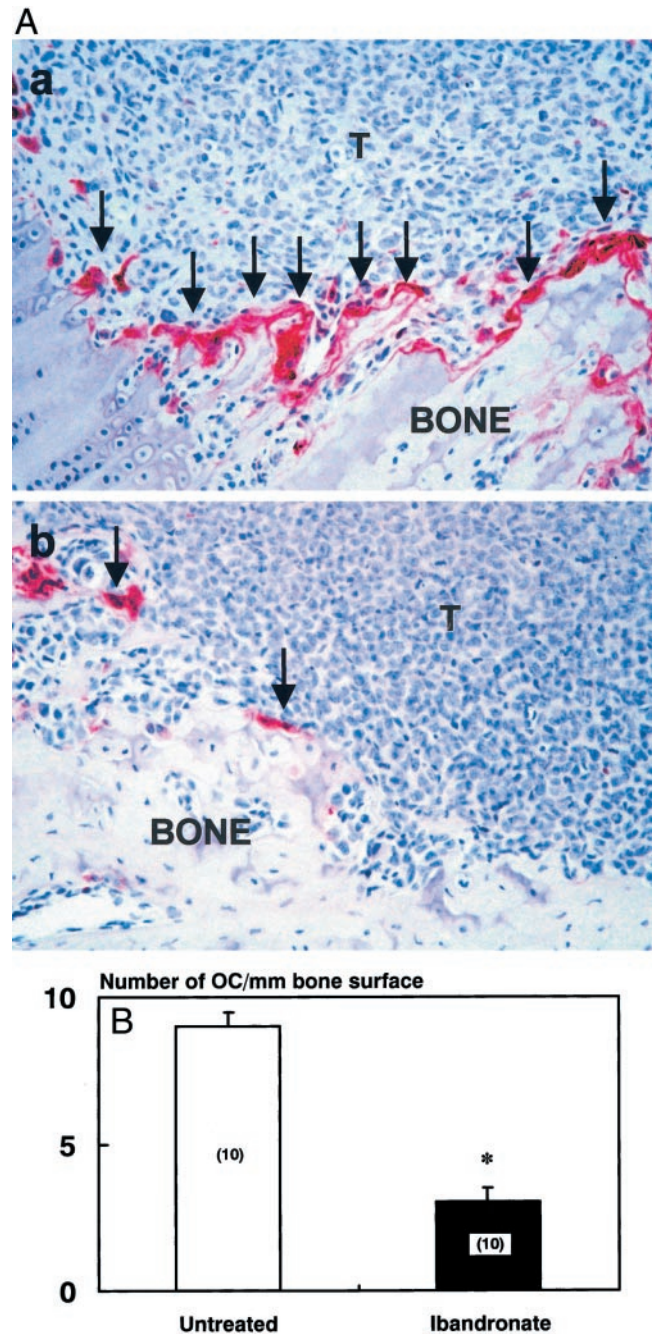


Fig. 7. *A*, histological view of bone metastases in untreated (*a*) or ibandronate-treated (*b*) mice. In untreated bone (*a*), there are numerous TRAP-positive osteoclasts facing along the bone surfaces (arrows). In contrast, number of osteoclasts is dramatically decreased in ibandronate-treated bone (*b*). TRAP staining: T, tumor. Magnification, $\times 200$. *B*, histomorphometrical analysis of osteoclast number in bone metastases of mice treated with or without ibandronate. Number of osteoclasts (OC) is expressed per mm of tumor-bone interface. Number of osteoclasts is significantly decreased in ibandronate-treated group compared with untreated group. Data are mean \pm SE (bars). Numbers in parentheses indicate number of animals studied (combination of two separate experiments). *, significantly different from untreated group ($P < 0.0001$).

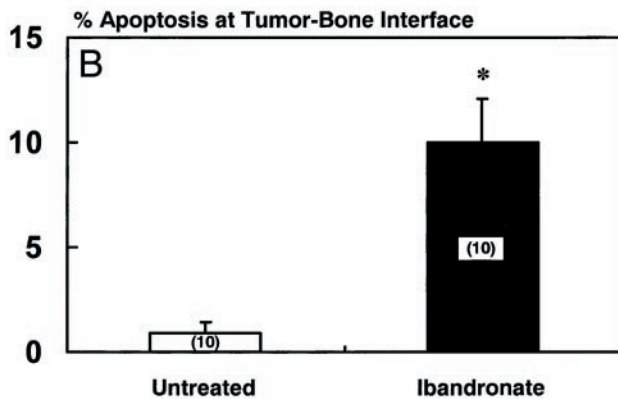
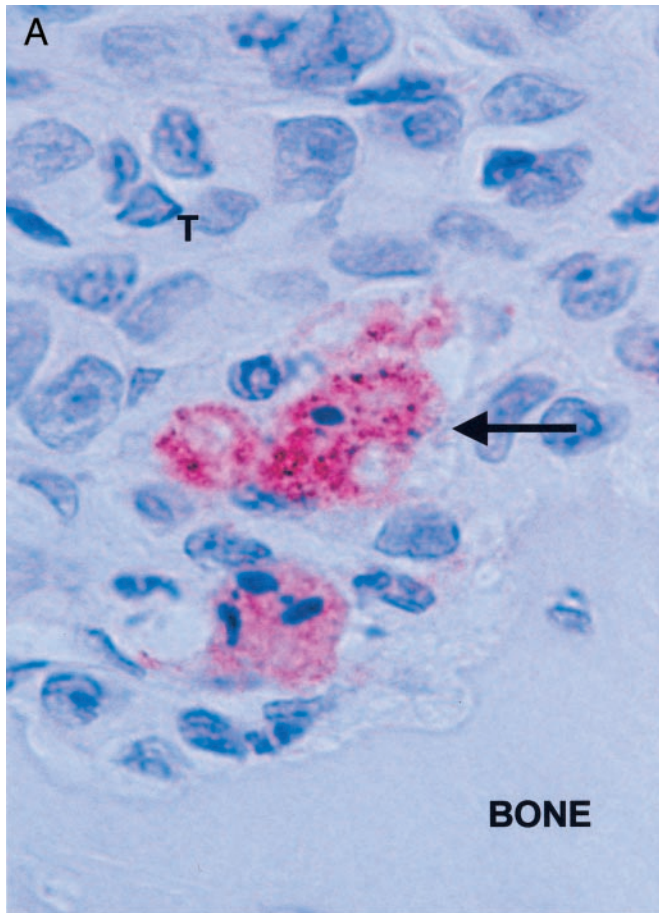


Fig. 8. A, histological view of apoptotic osteoclasts in bone metastases in ibandronate-treated mice. Apoptotic osteoclasts (arrows) are detached from bone surfaces and exhibit typical nuclear chromatin condensation. TRAP staining: T, tumor. Magnification, $\times 200$. B, histomorphometrical analysis of apoptosis in osteoclasts in bone metastases of mice treated with or without ibandronate. Osteoclast apoptosis is expressed as the percentage of apoptosis at tumor-bone interface. Ibandronate markedly enhanced apoptosis in osteoclasts in bone metastases compared with the untreated group. Data are mean \pm SE (bars). Numbers in parentheses indicate number of animals studied (combination of two separate experiments). *, significantly different from untreated group ($P < 0.0001$).

Whether the enhancement of MDA-231 cell apoptosis in bone following ibandronate treatment in bone is caused by direct anticancer actions on MDA-231 breast cancer cells or indirect mechanisms associated with the uniqueness of the bone microenvironment needs to be elucidated. Bone is a storehouse of varieties of growth factors, such as insulin-like growth factor and transforming growth factor β (23). These growth factors are released into the bone microenvironment in active forms as a consequence of osteoclastic bone resorption (24). BP probably limits the supply of these bone-derived growth factors that

facilitate the proliferation and survival of breast cancer cells colonizing bone through inhibition of osteoclastic bone resorption. This growth factor-deficient condition may, in turn, cause increased apoptosis in these bone-colonizing breast cancer cells. In support of this notion, Kostenuik *et al.* (25) have demonstrated that stimulation of bone resorption increases the growth of metastatic Walker 256 tumor in bone. Moreover, we recently observed that the conditioned medium of resorbed bone promoted the proliferation of MDA-231 breast cancer cells in culture in a preliminary experiment. Of note, the conditioned medium of ibandronate-treated bones in which bone resorption was inhibited failed to stimulate the proliferation of MDA-231 breast cancer cells in the same experiment.⁵ When these results are taken together, it seems likely that ibandronate, at least in part, promotes apoptosis in metastatic MDA-231 breast cancer cells in bone through inhibition of osteoclastic bone resorption.

Several previous reports have shown that BPs induce apoptosis in cancer cells through inhibition of the mevalonate pathway (13), reduction of bcl-2 expression (14), and activation of caspase (14, 15). Our *in vitro* data showed that ibandronate (100 μM) elevated caspase-3 activity and induced apoptosis in MDA-231 breast cancer cells as determined by DNA fragmentation. These effects of ibandronate were reversed in the presence of the caspase inhibitor Z-VAD-FMK. Thus, ibandronate likely enhances apoptosis in MDA-231 breast cancer cells, at least in part, through an activation of caspase-3. Our results are consistent with those obtained in previous studies (9–15) and support the notion that BPs have direct anticancer actions *in vitro*. However, our *in vivo* data demonstrated that ibandronate reduced MDA-231 tumor burden in bone but failed to inhibit MDA-231 tumor formation in the mammary fat pad, suggesting that the anticancer effects of ibandronate may be bone specific. Furthermore, contrary to the results reported by Diel *et al.* (16), more recent studies showed that the bisphosphonate clodronate had no effects on visceral organ metastases in breast cancer patients (26, 27). Thus, whether BPs possess direct anticancer effects *in vivo* is still an open question, and further study is needed.

Recent studies have shown that BPs promote apoptosis in osteoclasts (28, 29) and cancer cells (13) through the same mechanism that involves mevalonate pathway. However, it should be noted that the concentrations required to promote apoptosis in MDA-231 breast cancer cells *in vitro* are much higher than those required in osteoclasts. We previously have shown that BPs, including pamidronate, clodronate, and risedronate, at 1 μM markedly increased apoptosis in osteoclasts (7). On the other hand, we show here that ibandronate at 1 and 10 μM fails to induce apoptosis in MDA-231 breast cancer cells and that apoptosis is observed only when MDA-231 cells are treated with 100 μM ibandronate. Consistent with our results, other groups have also reported that relatively high concentrations of BPs ($>10 \mu\text{M}$) are required to promote apoptosis in tumor cells (11–15). Thus, it is likely that higher concentrations of BP are necessary to promote apoptosis in cancer cells than in osteoclasts in bone metastases. Regarding the concentration of BP in bone, the authors of one study suggested that the concentration of the BP alendronate reaches as high as 800 μM at the osteoclast-bone interface (30), whereas the authors of another study reported that an estimated concentration of the BP incadronate is as low as 0.7 $\mu\text{g/g}$ ($<1 \mu\text{M}$) in the metastatic tumor nests in bone (31). If the former is the case, it is possible that BPs directly promote apoptosis in tumor cells in bone. On the other hand, if the latter is the case, BPs seem likely to promote apoptosis in tumor cells in bone indirectly through inhibition of osteoclastic bone resorption. Whichever is the case, it is suggested that BP concentrations in

⁵ T. Yoneda, A. Myoui, A. Sasaki, P. J. Williams, N. Hashimoto, G. R. Mundy, manuscript in preparation.

bone need to be maintained at sufficiently high levels to enhance apoptosis in metastatic cancer cells.

In conclusion, our results suggest that BPs inhibit bone metastasis of breast cancer by promoting apoptosis in both osteoclasts and metastatic breast cancer cells. These two cellular mechanisms may be cumulatively responsible for the suppression of osteolytic bone metastases by BPs in breast cancer.

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