

Zoledronic Acid Inhibits Visceral Metastases in the 4T1/luc Mouse Breast Cancer Model

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ABSTRACT

Purpose: It is established that bisphosphonates (BPs), specific inhibitors of osteoclasts, have beneficial effects on bone metastases of breast cancer. In addition, recent studies have reported that BPs have anticancer effects and suppress visceral metastases, too. However, the results of clinical studies are still conflicting. In the present study, we examined the effects of the BP zoledronic acid (ZOL), one of the most potent BPs currently available, on visceral metastases of breast cancer using an animal model in which mouse breast cancer cells 4T1/luc implanted at the orthotopic mammary fat pad spontaneously metastasize to multiple organs including bone, lung, and liver in female BALB/c mice.

Experimental Design and Results: The 4T1/luc-bearing mice received single or four i.v. injections of ZOL (0.5 or 5 µg/mouse) during the whole experimental period. Bone metastases were reduced by the ZOL treatment. More importantly, ZOL significantly suppressed lung and liver metastases. Furthermore, ZOL prolonged overall survival of the tumor-bearing mice. Of interest, apoptosis in 4T1/luc cells colonized in bone was increased by ZOL; however, those in lung were not changed. *In vitro* studies demonstrated that ZOL inhibited cell migration and invasion and promoted apoptosis of 4T1/luc cells.

Conclusions: These results are consistent with the notion that ZOL affects breast cancer metastasis to visceral organs as well as bone. These effects of ZOL may be attributable to inhibition of migration and invasion of breast cancer cells. Clinical relevance of our experimental results

needs to be determined in breast cancer patients with visceral metastases.

INTRODUCTION

Bisphosphonates (BPs) have been widely and successfully used for the treatment of bone metastases in breast cancer patients (1–3). In addition to their potent antiosteoclast effects, recent preclinical studies have shown that BPs induce apoptosis of breast cancer cells (4, 5). It has also been demonstrated that BPs inhibit cancer cell invasion (6, 7) and angiogenesis (8, 9). These data suggest that BPs have anticancer effects and inhibit cancer metastases. In support of this notion, Diel *et al.* (10) reported that the BP clodronate combined with anticancer therapies exhibits adjuvant inhibitory effects on the metastases to visceral organs as well as to bone and prolongs survival in breast cancer patients. However, subsequent clinical studies failed to reproduce their results (11, 12), although the inclusion criteria of patients studied were slightly different. Because of these conflicting results of the clinical studies, the guideline from American Society of Clinical Oncology does not recommend the use of BPs for breast cancer patients without osteolytic bone metastases (13). Thus, it is an important issue to be resolved whether BPs have any effects on nonbone organ metastases.

Zoledronic acid [2-(imidazol-1-yl)-1-hydroxyethylidene-1,1-bisphosphonate; ZOL] is the third generation BP, which has the most potent inhibitory effect on osteoclast-mediated bone resorption among currently available BPs (14–16). Phase III clinical trials in cancer patients have been completed recently, and the compound is now registered in the United States and Europe for the treatment of bone metastases associated with a wide variety of tumors (17, 18). However, the effects of ZOL on visceral metastases of breast cancer have not been extensively examined yet.

We have developed recently an animal model in which mouse breast cancer cells 4T1/luc implanted at the orthotopic site spontaneously metastasize to bone, lung, and liver in immunocompetent syngeneic female BALB/c mice (19–21). Using this model, the effects of ZOL were examined in this study. Our data show that ZOL reduced the metastases of 4T1/luc cells to visceral organs as well as to bone and prolonged survival of the 4T1/luc-bearing mice.

MATERIALS AND METHODS

Chemicals and Antibodies

ZOL and pamidronate (3-amino-1-hydroxypropylidene-bisphosphonate) were provided by Novartis Pharma AG (Basel, Switzerland). Clodronate (dichloromethylene-bisphosphonate; CLO) was a gift from Dr. Ross I. Garrett (OsteoScreen, Inc., San Antonio, TX). Type IV collagen, fibronectin, geranylgeraniol (GGOH), and farnesol (FOH) were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibodies to phospho-Akt (Thr308), Akt, and cleaved caspase-3 were purchased from Cell

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Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal antibody to Rap1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), which recognizes both prenylated and unprenylated Rap1 (22). All of the other chemicals used in this study were purchased from Sigma or Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise described.

4T1/luc Mouse Breast Cancer Cells

The mouse breast cancer cell line 4T1 was generously provided by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI; Ref. 23). This cell line was isolated from a single spontaneously arising mammary tumor from a BALB/cfC3H mouse. Inoculation of 4T1 cells into the orthotopic mammary fat pad in mice causes distant metastases to multiple organs including lung, liver, and bone (19). 4T1/luc was established by the stable transfection of the firefly luciferase gene (pGL3-control; Promega, Madison, WI) to 4T1 cells using LipofectAMINE PLUS Reagent (Life Technologies, Inc., Grand Island, NY) for quantitative assessment of tumor burden in visceral organs. pcDNA3 vector (Invitrogen Co., Carlsbad, CA) was cotransfected for the selection by G418 (Sigma). The 4T1/luc cells showed an equivalent metastatic potential with the parental 4T1 cells (20, 21). The cells were cultured in DMEM supplemented with 10% FCS (Asahi Glass Techno Corp., Tokyo, Japan) and 1% penicillin-streptomycin solution (Life Technologies, Inc.) in a humidified atmosphere of 5% CO₂ in air.

Animal Model

Six-week-old female BALB/c mice were purchased from Harlan Industries (Houston, TX) or Japan SLC, Inc. (Shizuoka, Japan). Subconfluent 4T1/luc cells were refed with fresh medium 24 h before injection. Cells (1×10^6) were suspended in 0.1 ml PBS and injected into the mammary fat pad in mice under the anesthesia with pentobarbital (0.05 mg/g body weight; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).

All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry before the experiments were started.

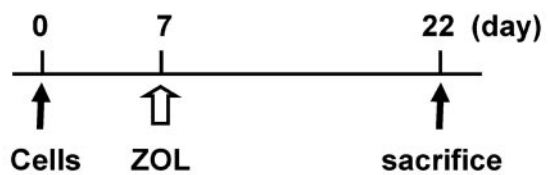
Protocols of Animal Experiments

The summary of the experiments is depicted in Fig. 1. The number of mice studied in each experiment is described in each figure. In all of the experiments, 4T1/luc cells were inoculated at day 0. The mice were divided into each group at day 7, when discernible orthotopic tumor formed. The dose and the treatment schedule of ZOL were designed according to the clinical studies (17, 18, 24, 25).

Mice received either single (protocol 1) or four (protocol 2) i.v. injections of ZOL (0.5 or 5 μ g/mouse) from tail vein during the whole experimental period and were sacrificed at day 22. The primary mammary tumors were dissected and weighed at the sacrifice. The tumor burden in lung, liver, and bone was determined as described below.

In other experiments in which the effects of ZOL on survival of tumor-bearing mice were assessed, the mice were treated with ZOL (5 μ g/mouse) similarly to the protocol 2, and were sacrificed when they began to show profound loss of body weight.

Protocol 1



Protocol 2

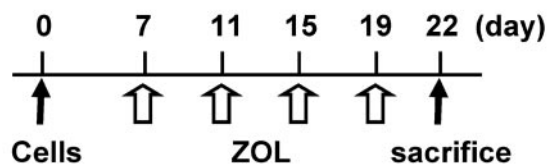


Fig. 1 Summary of the experimental protocols. 4T1/luc cells (*Cells*) were inoculated into the mammary fat pad of 6-week-old female BALB/c mice at day 0. Zoledronic acid (0.5 or 5 μ g/mouse) was i.v. given once (*Protocol 1*, at day 7) or 4 times (*Protocol 2*, at days 7, 11, 15, and 19) during the whole experimental period. The mice were sacrificed at day 22.

Histomorphometrical Examination

Tumor Burden of 4T1/luc in Bone. The femora and tibiae dissected were immersed in 4% paraformaldehyde buffered with 0.1 M phosphate buffer (pH 7.4) overnight, decalcified in 4.13% EDTA at room temperature for 1 week, and embedded in paraffin. Sagittal sections were made following conventional methods and stained with H&E. Using representative sections chosen from center part of each femur and tibia, tumor burden of 4T1/luc was measured under microscope at $\times 100$ magnification using the image analysis software (Image Pro Plus; Media Cybernetics, Silver Spring, MD) as described previously (21, 26). Then, total tumor area in both femora and tibiae in each mouse was calculated. Data are shown as tumor area (mm²/mouse).

Tumor Burden of 4T1/luc in Lung. The lungs were dissected and fixed in 4% paraformaldehyde buffered with 0.1 M phosphate buffer (pH 7.4) overnight. Paraffin sections were made following conventional methods and stained with H&E. Metastasis was quantified by measuring the total tissue area per lung section (D1) and metastasis present in the same area (D2) using Image Pro Plus. Data are shown as tumor area (%) calculated by the ratio D2:D1 as described previously (27).

Apoptosis in Metastatic 4T1/luc Cells in Lung and Bone. Paraffin sections of lungs, femora, and tibiae were made as described above. Apoptosis in 4T1/luc cells was determined with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. TUNEL staining was performed with DeadEnd Colorimetric TUNEL System (Promega Corp.) according to the manufacturer's instruction. Five fields of metastatic tumors at $\times 400$ magnification were randomly selected in each specimen, and the TUNEL-positive cells were counted. Data are expressed as number of apoptosis/mm² tumor area as described (26).

All of the histomorphometrical analyses were performed extensively and carefully by two different individuals, both of whom were without knowledge of the experimental protocol.

Determination of Luciferase Activity

The tumor burden of 4T1/luc cells in lung and liver was evaluated by measuring luciferase activity (20). The lung and liver were dissected, homogenized in Reporter Lysis Buffer (Promega Corp.), centrifuged, and the supernatants were used for the assay. The luciferase activity was determined by a luminometer (Promega Corp.) using Luciferase Assay System (Promega Corp.) according to the manufacturer's protocol. The luciferase activity in each organ was corrected by protein concentration. Data are shown as luciferase activity (percentage of control).

Osteoclast-Like Cell Formation

Effects of ZOL on osteoclast formation were examined by mouse bone marrow cultures as described before (21). Bone marrow cells were harvested from femora and tibiae of ddY mice (male, 4-week-old; Japan SLC, Inc.), plated in a 48-well plate at a final density of 1×10^6 cells/well and cultured in α MEM supplemented with 10% FCS for 6 days. Fifty ng/ml of parathyroid hormone-related protein (PTHrP) and varying concentrations of ZOL were added to the culture medium during whole period of the culture. The cells were fed every 2 days with fresh α MEM containing 10% FCS, PTHrP, and ZOL. At the end of the culture, the cells were stained with tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts using a commercially available kit (Sigma). TRAP-positive multinucleated (three or more nuclei) cells in each well were counted under light microscope. Data are shown as number of TRAP-positive multinucleated cells/well.

Apoptosis *in Vitro*

Apoptosis in 4T1/luc cells *in vitro* was determined using a fluorescence-activated cell sorter (FACS) technique as described (21). Subconfluent 4T1/luc cells in six-well plates were treated with ZOL in the presence or absence of GGOH and FOH

for 48 h. After the treatment, the cells were harvested, fixed in 70% ethanol, and incubated in PBS containing propidium iodide (10 μ g/ml) and RNase A (10 μ g/ml) for 20 min. DNA content of 4T1/luc cells was determined by FACS (FACScan; Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). The percentages of sub-G₁ nuclei in the population were determined as percentage of apoptosis.

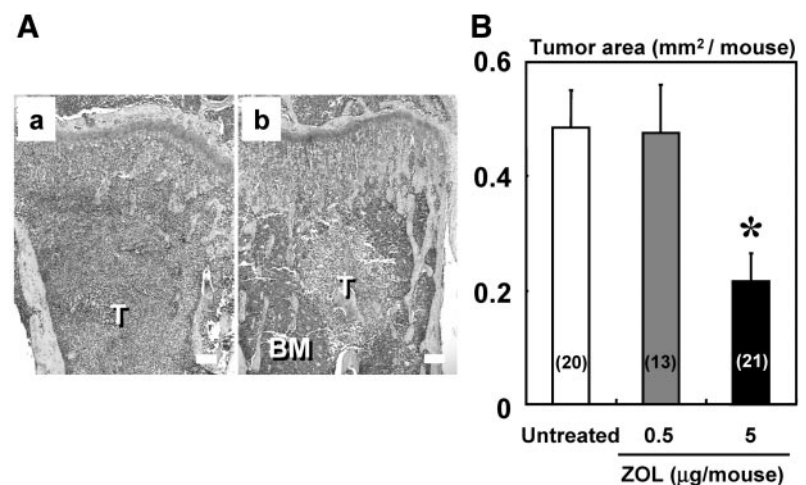
Western Blotting

Subconfluent 4T1/luc cells in 10-cm dishes were treated with 100 μ M ZOL or 100 μ M CLO in the presence or absence of 100 μ M GGOH and 100 μ M FOH for 36 h. Cells were then washed three times with ice-cold PBS and solubilized in lysis buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM sodium orthovanadate]. The cells lysates obtained were boiled in SDS sample buffer containing 0.5M β -mercaptoethanol. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with primary antibodies to Rap1, phospho-Akt, Akt, and cleaved caspase-3. Separated proteins are visualized with horseradish peroxidase coupled with protein A (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) with enhancement by chemiluminescence using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products Inc., Boston, MA).

Cell Migration and Invasion

Cell migration and invasion analyses were performed as described previously (28). Transwell polycarbonate membranes (8- μ m pore size; Corning Costar Co., Cambridge, MA) coated with type IV collagen (25 μ g/ml) or Matrigel-coated invasion chambers (Biocoat Matrigel Invasion Chamber, 8- μ m pore size; Becton Dickinson Labware, Bedford, MA) were used for migration and invasion assay, respectively. As a chemoattractant, fibronectin (25 μ g/ml) was added in the lower chamber. 4T1/luc cells (5×10^4) suspended in DMEM containing 0.1% BSA in the presence or absence of ZOL were added to the upper

Fig. 2 Effects of the single injection of zoledronic acid (protocol 1) on bone metastases of 4T1/luc. **A**, representative histological view of bone metastases treated without (*a*) or with (*b*) ZOL (5 μ g/mouse). In the untreated bone (*a*), the bone marrow cavity was almost completely replaced by cancer cells, and the trabecular bones were destroyed. In contrast, in the ZOL-treated bone (*b*), the tumor burden was markedly smaller and bone marrow and trabecular bones still remained undestroyed. (H&E staining; T, tumor; BM, bone marrow; original magnification, $\times 50$; bar = 200 μ m). **B**, histomorphometrical analysis of tumor burden of 4T1/luc in bone. Numbers in parentheses indicate the number of mice studied. Data are mean; bars, \pm SE. *, significantly different from untreated group ($P < 0.05$)



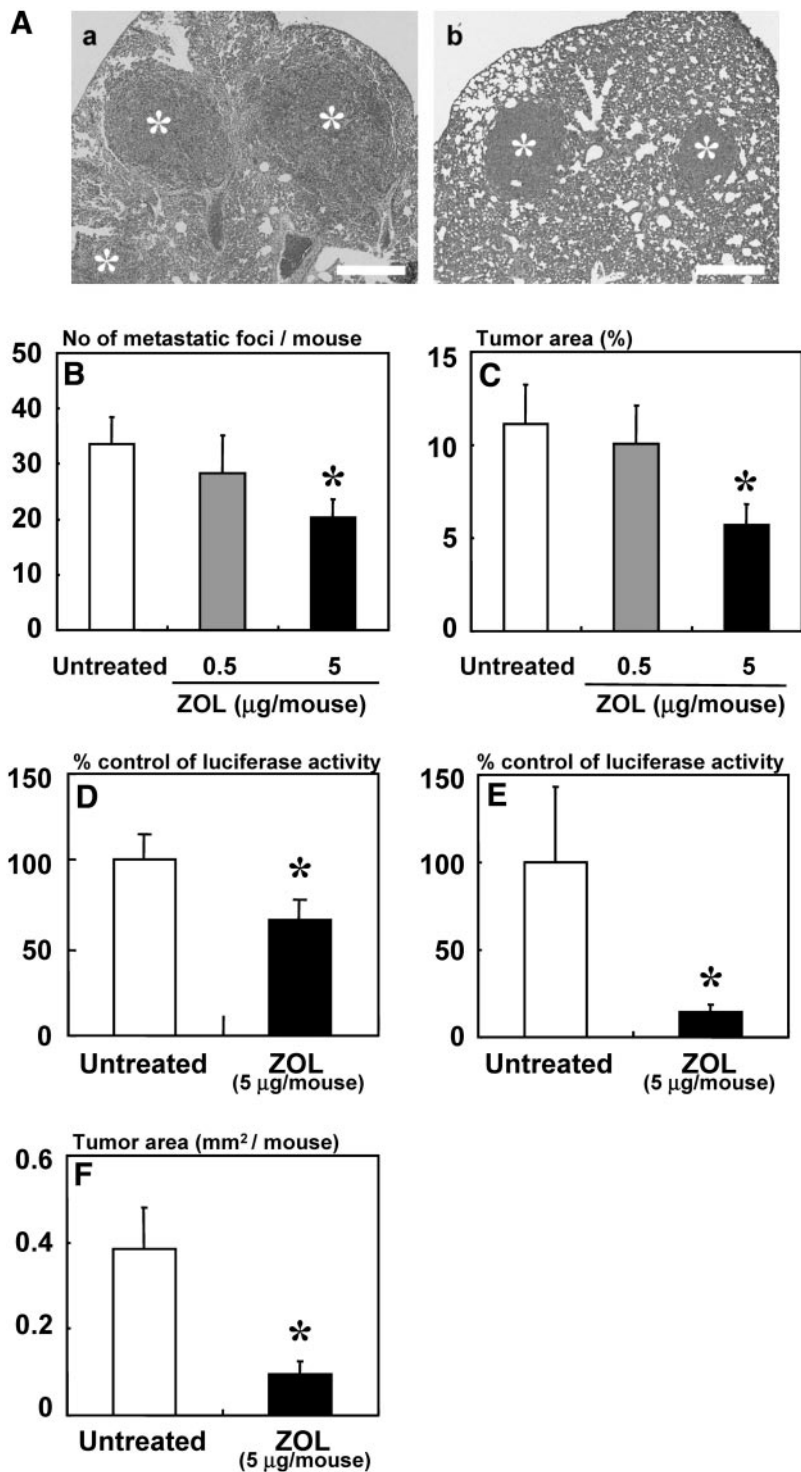


Fig. 3 Effects of the repeated injections of zoledronic acid (protocol 2) on lung, liver, and bone metastases of 4T1/luc. **A**, representative histological view of lung metastases treated without (**a**) or with (**b**) ZOL (5 $\mu\text{g}/\text{mouse}$). H&E staining; *, tumor; original magnification, $\times 50$; bar = 500 μm . **B**, number of metastatic foci of 4T1/luc in lung. Data are mean; bars, \pm SE ($n = 10$ animals for each group). *, significantly different from untreated group ($P < 0.05$). **C**, histomorphometrical analysis of tumor burden of 4T1/luc in lung. Data are mean; bars, \pm SE ($n = 10$ animals for each group). *, significantly different from untreated group ($P < 0.05$). **D** and **E**, metastatic tumor burden of 4T1/luc in lung (**D**) and liver (**E**) determined by luciferase activity. Data are mean; bars, \pm SE ($n = 10$ animals for each group). *, significantly different from untreated group ($P < 0.05$). **F**, histomorphometrical analysis of tumor burden of 4T1/luc in bone. Data are mean; bars, \pm SE ($n = 10$ animals for each group). *, significantly different from untreated group ($P < 0.05$).

chamber and incubated for 6 h in the migration assay and for 24 h in the invasion assay. After the incubation period, cells that passed through the membrane were stained with 0.1% crystal violet and counted under the microscope. Data are shown as number of cells/membrane.

Statistical Analysis

The data were analyzed by one-way ANOVA followed by Fisher's PLSD post-hoc test (StatView; SAS Institute Inc., Cary, NC) for determination of differences between more than two groups. Student's *t* test or Welch's *t* test was conducted when

two groups were compared. The survival experiments were analyzed by Wilcoxon's test. P values of <0.05 were considered statistically significant. All of the data were presented as mean \pm SE.

RESULTS

Effects of ZOL on Bone and Visceral Metastases in 4T1/luc-Bearing Mice. Histological and histomorphometrical examinations demonstrated that single i.v. injection of ZOL (5 $\mu\text{g}/\text{mouse}$; protocol 1) significantly decreased 4T1/luc tumor burden in bone (Fig. 2), whereas lung and liver metastases determined by luciferase activity were not changed (data not shown). On the other hand, repeated injections of ZOL (5 $\mu\text{g}/\text{mouse}$; protocol 2) significantly reduced the number of metastatic foci and tumor burden in lung determined by histomorphometry and luciferase activity (Fig. 3). In contrast, other bisphosphonates, CLO and pamidronate, showed no effects on the lung metastases (Fig. 4). The tumor burden in liver and bone was also decreased in ZOL (5 $\mu\text{g}/\text{mouse}$)-treated mice (Fig. 3). Furthermore, ZOL significantly prolonged overall survival of 4T1/luc-bearing mice in the protocol 2 (Fig. 5). ZOL did not affect the development of orthotopic 4T1/luc tumors in the mammary fat pad in the both protocols (data not shown).

Effects of ZOL on Apoptosis in Metastatic Cancer Cell Colonizing in Bone and Lung. We next examined the apoptotic effects of ZOL on metastatic 4T1/luc cells colonizing in bone and lung in mice. As shown in Fig. 6A, the number of apoptosis in 4T1/luc cells in bone was significantly increased in ZOL-treated mice compared with that in untreated mice. In contrast, ZOL did not change the apoptosis in metastatic 4T1/luc cells in the lung (Fig. 6B).

Effects of ZOL on Osteoclast-Like Cell Formation. It has been suggested that osteoclastic bone resorption is a critical process in the development of bone metastases. Therefore, to examine the mechanisms by which ZOL inhibited bone metastases, effects of ZOL on osteoclast-like cell formation were

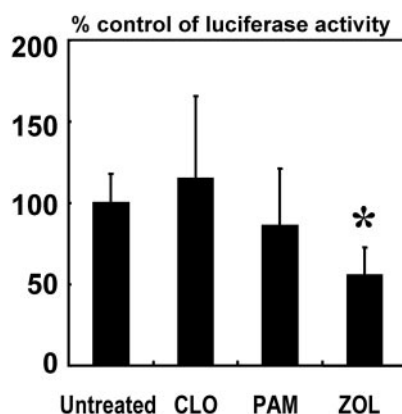


Fig. 4 Effects of clodronate, pamidronate, and zoledronic acid on lung metastases of 4T1/luc. CLO (6 mg/mouse), PAM (360 $\mu\text{g}/\text{mouse}$), or ZOL (32 $\mu\text{g}/\text{mouse}$) was i.v. injected twice at days 7 and 14. Mice were sacrificed at day 22. Metastatic tumor burden of 4T1/luc in lung was determined by luciferase activity. Data are mean; bars, \pm SE ($n = 10$ animals for each group). *, significantly different from untreated group ($P < 0.05$).

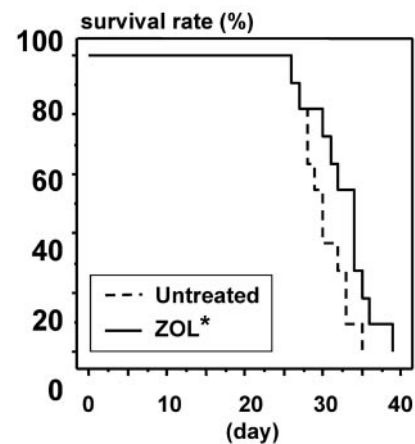


Fig. 5 Effects of zoledronic acid (ZOL; 5 $\mu\text{g}/\text{mouse}$; protocol 2) on overall survival of the 4T1/luc-bearing mice ($n = 10$ animals for each group). *, significantly different from untreated group ($P < 0.05$).

examined in mouse bone marrow cultures. ZOL significantly inhibited PTHrP-induced osteoclast-like cell formation in a dose-dependent manner (Fig. 7).

Effects of ZOL on Apoptosis in 4T1/luc Cells. Because ZOL treatment increased apoptosis in metastatic 4T1/luc cells in bone (Fig. 6A), we examined apoptotic effects of ZOL on 4T1/luc cells *in vitro*. FACS analysis demonstrated that ZOL significantly increased apoptosis in 4T1/luc in a dose-dependent manner (Fig. 8). We then examined whether the ZOL-induced apoptosis is rescued by the mevalonate pathway intermediates, GGOH and FOH, because nitrogen-containing BPs including ZOL have been shown to induce apoptosis by suppressing prenylation of small G proteins through inhibition of mevalonate pathway (5, 16, 29). FACS analysis demonstrated that both GGOH and FOH significantly decreased ZOL-induced apoptosis in 4T1/luc cells (Fig. 9A). GGOH showed greater effect than FOH. Consistently, the ZOL-decreased Rap1 prenylation and Akt phosphorylation were reversed by GGOH but not by FOH (Fig. 9B). In addition, GGOH suppressed ZOL-induced caspase-3 activation, whereas FOH showed little effect (Fig. 9B). CLO, which belongs to another class of BPs (16), did not show these effects (Fig. 9B).

Effects of ZOL on Migration and Invasion of 4T1/luc *in Vitro*. Our data showed that ZOL decreased the tumor burden not only in bone but also in lung (Fig. 3). Nevertheless, ZOL increased apoptosis in metastatic 4T1/luc cells in bone but not in lung (Fig. 6). Therefore, to determine how ZOL suppresses the visceral metastases of 4T1/luc, we examined effects of ZOL on migration and invasion of 4T1/luc cells *in vitro*. One hundred μM of ZOL markedly suppressed migration of 4T1/luc cells (Fig. 10A). More importantly, all of the concentrations of ZOL tested (1, 10, and 100 μM) dose-dependently inhibited cell invasion (Fig. 10B).

DISCUSSION

Large bodies of clinical studies demonstrated that BPs have beneficial effects on bone metastases in breast cancer patients (1–3). On the other hand, the effects of BPs on nonbone metas-

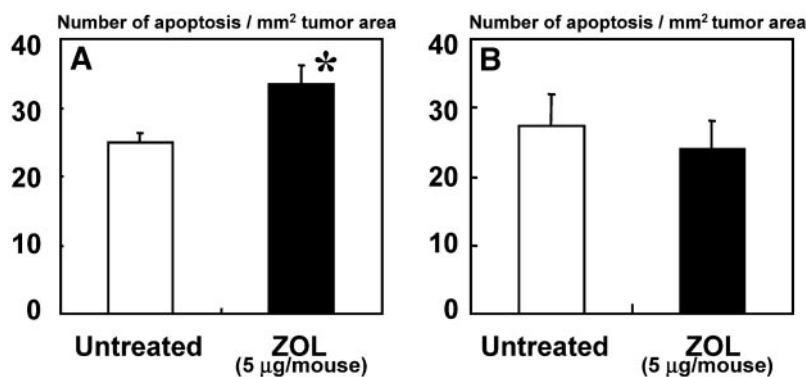


Fig. 6 Effects of zoledronic acid (5 µg/mouse; protocol 2) on apoptosis in metastatic 4T1/luc cells colonized in bone (A) and lung (B). Apoptotic cancer cells were determined by terminal deoxynucleotidyl transferase-mediated nick end labeling staining as described in "Materials and Methods." Data are mean; bars, \pm SE ($n = 5$ animals for each group). *, significantly different from untreated group ($P < 0.05$).

tases are still controversial (10–12). In the present study, we demonstrated that the BP ZOL, one of the most potent BPs currently available, at doses that are equivalent to clinically administered dosages significantly inhibits metastases of the 4T1/luc mouse breast cancer cells to lung and liver, as well as to bone. Of importance, ZOL consequently showed prolonged overall survival of 4T1/luc tumor-bearing mice. These results suggest that ZOL has inhibitory effects on visceral metastases of breast cancer.

We reported previously that the BP ibandronate increased apoptosis in MDA-MB-231 human breast cancer cells in bone metastases and decreased metastatic MDA-MB-231 tumor burden in bone in nude mice (26). Consistent with these findings, our present study showed that ZOL decreased bone metastases of 4T1/luc with an increased number of apoptosis in 4T1/luc cells colonized in bone. In contrast, ZOL did not increase apoptosis of 4T1/luc cells metastasized in lung, suggesting that the apoptotic effects of BPs on cancer cells seem to be bone-specific. In this context, it has been suggested that there exist two possible mechanisms by which BPs increase apoptosis in cancer cells in bone. One is that BPs limit the supply of bone-stored growth factors such as insulin-like growth factors and transforming growth factor β from bone by inhibiting osteoclastic bone resorption, which results in increased apoptosis in the bone-colonizing cancer cells, because those growth factors are released into the bone microenvironment in active forms as a consequence of osteoclastic bone resorption and facilitate the proliferation and survival of breast cancer cells (26, 30–34). Another possibility is that BPs directly induce apoptosis in cancer cells. BPs including ZOL have been shown to induce apoptosis in breast cancer cells *in vitro* (4, 5). Our study also demonstrated that ZOL suppressed Rap1 prenylation and Akt phosphorylation, activated caspase-3, and increased apoptosis in 4T1/luc cells. Furthermore, the mevalonate pathway intermediate GGOH reversed all of these ZOL-induced changes and those effects were markedly more potent than FOH, suggesting that ZOL induced apoptosis in 4T1/luc cells by impairing protein prenylation, especially geranylgeranylation, through the inhibition of mevalonate pathway. In contrast, these phenomena were not observed in the cells treated with CLO. Consistent with our result, it has been suggested that non-nitrogen-containing BPs including CLO induce cell apoptosis

through the mechanism that is independent of the inhibition of mevalonate pathway (16). Although the concentrations of BPs required for inducing apoptosis are relatively high, BPs are known to be highly accumulated in bone, and a report by Sato *et al.* (35) suggests that those concentrations are attainable at the osteoclast-bone interface. These data suggest that BPs may directly induce apoptosis in cancer cells specifically in bone. However, of note, our previous study revealed that the concentrations of ZOL that induce apoptosis in 4T1/luc cells also increase apoptosis in bone marrow stromal cells (21), suggesting that apoptotic effects of ZOL are nonselective at such high concentrations. Nevertheless, we have not observed an increased number of apoptosis in bone marrow stromal cells in mice (21). The observation suggests that ZOL concentration in bone metastases is not as high as that tested *in vitro*. Taken together, it seems likely that ZOL increases apoptosis in bone primarily by inhibiting bone resorption, which limits the supply of bone-stored growth factors to cancer cells colonized in bone.

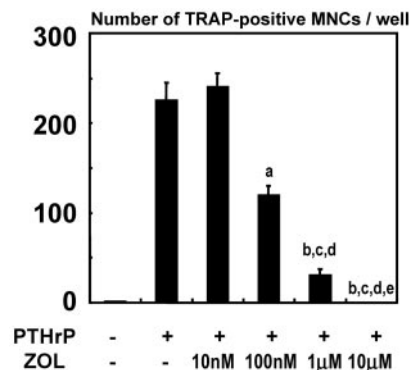


Fig. 7 Effects of zoledronic acid on osteoclast-like cell formation. Mouse bone marrow cells were cultured with 50 ng/ml PTHrP in the presence or absence of varying concentrations of ZOL for 6 days. At the end of culture, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) were counted. Data are mean; bars, \pm SE ($n = 4$ /group). *a*, significantly different from PTHrP alone ($P < 0.01$). *b*, significantly different from PTHrP + 10 nM ZOL ($P < 0.01$). *c*, significantly different from PTHrP + 100 nM ZOL ($P < 0.001$). *d*, significantly different from PTHrP + 1 µM ZOL ($P < 0.05$). *e*, significantly different from PTHrP + 10 µM ZOL ($P < 0.05$).

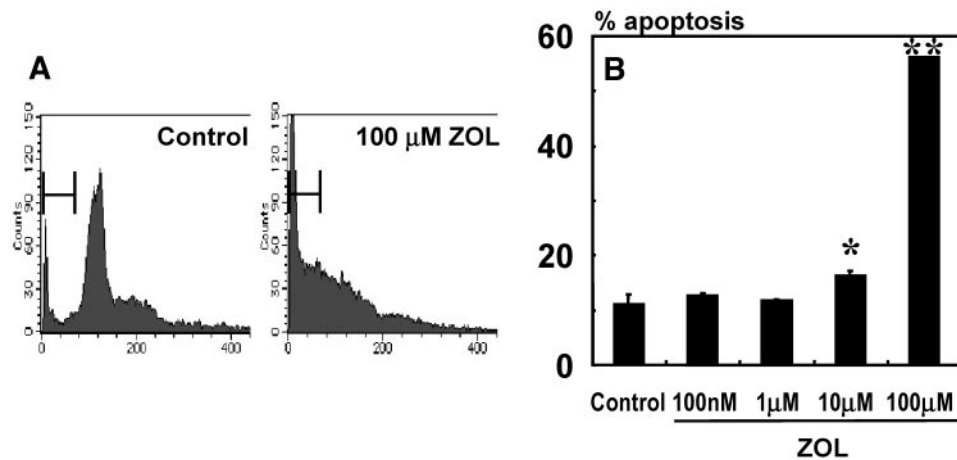


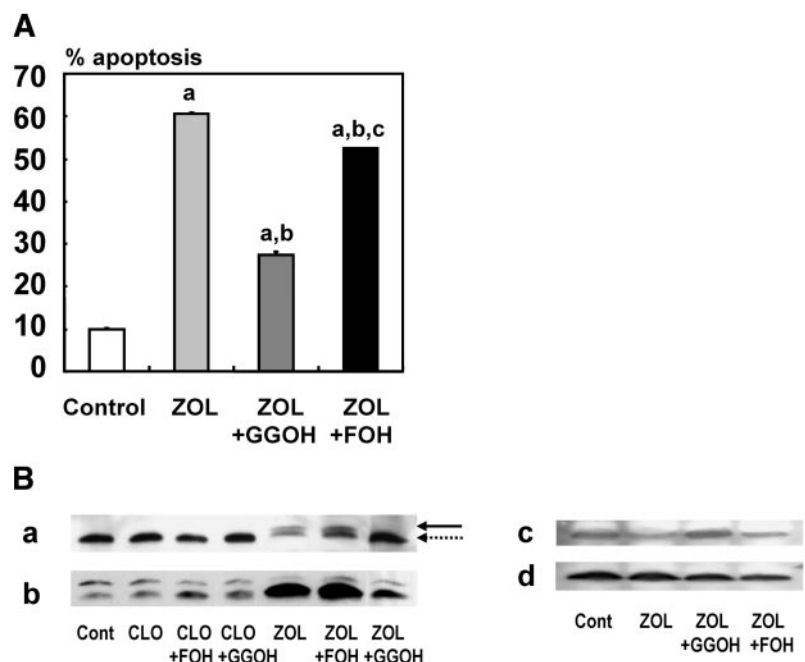
Fig. 8 Effects of zoledronic acid on apoptosis in 4T1/luc cells *in vitro*. 4T1/luc cells were treated with varying concentrations of ZOL for 48 h. Apoptosis was assessed by fluorescence-activated cell sorter as described in "Materials and Methods." **A**, representative histograms of propidium iodide staining of 4T1/luc cells treated without or with ZOL (100 μM) examined by fluorescence-activated cell sorter. The percentages of sub-G₁ nuclei (indicated by bars) in the population were determined as percentage of apoptosis. **B**, quantitative data of percentage of apoptosis. Data are mean; bars, \pm SE ($n = 4/\text{group}$). *, significantly different from control group ($P < 0.05$). **, significantly different from control group ($P < 0.01$).

In contrast to the effects of ZOL on bone metastases, ZOL did not affect apoptosis in metastatic 4T1/luc cells in lung. However, ZOL reduced the metastatic tumor burden of 4T1/luc in lung as well as in bone. To explain this discrepancy, we examined the effects of ZOL on the migration and invasion of 4T1/luc cells using the Boyden chamber method. Consistent with the previous reports (6, 36), our data showed that ZOL inhibited cell migration and invasion of 4T1/luc cells in a dose-dependent fashion. Moreover, it is of particular note that 1 μM of ZOL, which did not increase apoptosis

in 4T1/luc cells, significantly inhibited the cell invasion. Boissier *et al.* (6) have shown that ZOL inhibited invasion of MDA-MB-231 cells even at 1 pM. Because cell migration and invasion are essential steps for cancer cells to spread (37, 38), our data suggest that inhibition of migration and invasion of 4T1/luc cells by ZOL contribute to the suppression of the visceral and bone metastases.

BPs are synthetic analogs of PP_i in which the oxygen bridge is replaced by a carbon that allows the development of several kinds of BPs with various side chains (15, 16). It is

Fig. 9 **A**, effects of geranylgeraniol (GGOH) and farnesol (FOH) on zoledronic acid-induced apoptosis in 4T1/luc cells. 4T1/luc cells were treated with 100 μM ZOL in the presence or absence of 100 μM GGOH or 100 μM FOH for 48 h. Apoptosis was assessed by fluorescence-activated cell sorter. The percentages of sub-G₁ nuclei in the population were determined as percentage of apoptosis. Data are mean; bars, \pm SE ($n = 4/\text{group}$). *a*, significantly different from control group ($P < 0.001$). *b*, significantly different from ZOL group ($P < 0.001$). *c*, significantly different from ZOL + GGOH group ($P < 0.001$). **B**, effects of clodronate (CLO) or ZOL on Rap1 prenylation (*a*: arrow, unprenylated Rap1; broken arrow, prenylated Rap1), cleaved caspase-3 expression (*b*), Akt phosphorylation (*c*), and Akt expression (*d*) in the presence or absence of GGOH and FOH in 4T1/luc cells were examined by Western blot. 4T1/luc cells were treated with 100 μM CLO or 100 μM ZOL in the presence or absence of 100 μM GGOH or 100 μM FOH for 36 h.



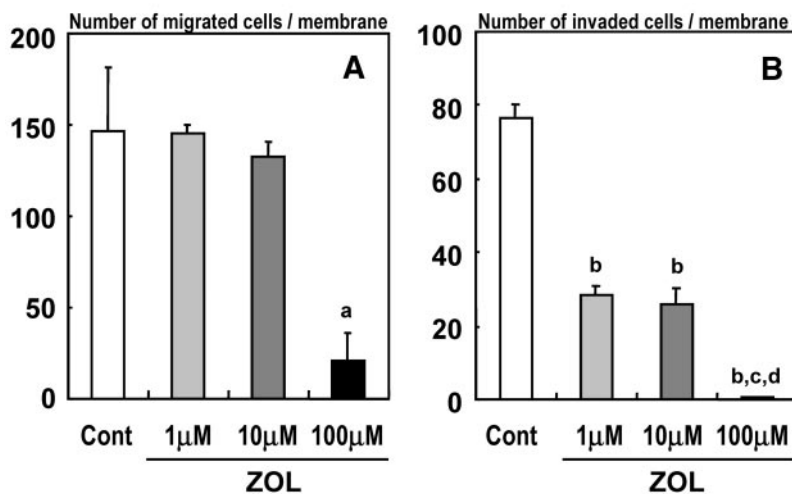


Fig. 10 Effects of zoledronic acid on migration (A) and invasion (B) of 4T1/luc cells *in vitro*. Data are mean; bars, \pm SE ($n = 4/\text{group}$). a, significantly different from control group ($P < 0.05$). b, significantly different from control group ($P < 0.01$). c, significantly different from 1 μM ZOL group ($P < 0.01$). d, significantly different from 10 μM ZOL group ($P < 0.01$).

well described that these BPs possess various inhibitory potencies on osteoclastic bone resorption; however, it is still unclear whether these structurally different BPs show similar effects on other types of cells. We showed that ZOL decreased the visceral metastases of 4T1/luc in this study. In contrast, CLO and pamidronate showed no effects. In addition, our previous study using the same animal model has shown that ibandronate (4 $\mu\text{g}/\text{mouse}$, s.c., daily from day 0 to the end of experiments) did not inhibit lung metastases of 4T1/luc (20). Fromigue *et al.* (5) have shown that ZOL and ibandronate have a similar potency to induce apoptosis in breast cancer cells *in vitro*. It has also been demonstrated that ZOL and ibandronate equivalently inhibit angiogenesis (9), one of the critical factors in cancer metastasis (37). On the other hand, Boissier *et al.* (6) demonstrated that ZOL inhibits invasion of MDA-MB-231 cells more potently than ibandronate. CLO and pamidronate were less potent than ZOL and ibandronate in all of these experiments (5, 6, 9). These data collectively support a possibility that the inhibition of 4T1/luc cell invasion is a primary mechanism underlying the decreased visceral metastases of 4T1/luc by ZOL. The results also suggest that structurally different BPs may have various effects on cancer metastases to visceral organs.

Preclinical studies have shown that the BPs risedronate and alendronate significantly increased survival rate of the breast or prostate cancer-bearing mice (39, 40). The present study also demonstrated that the repeated injections of ZOL significantly prolonged the survival of 4T1/luc-bearing mice accompanied with the reduced tumor burden in the visceral organs. However, the results of the clinical studies are still conflicting (10–12). It is of note that the BP CLO, which is used in all of these clinical studies (10–12), showed no effects on lung metastases of 4T1/luc in this study. Additional extensive clinical studies are required to conclude whether ZOL inhibits visceral metastases and show a survival benefit in cancer patients.

In conclusion, our results are in good agreement with the

notion that the BP ZOL influences distant metastases not only to bone but also to visceral organs. These effects of ZOL may be attributable to inhibition of migration and invasion of cancer cells. The effects of ZOL on visceral metastases in cancer patients need to be determined.

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