INTRODUCTION

Advanced breast cancer is frequently associated with destructive osteolytic bone metastases that are accompanied by serious complications, inside and outside the skeleton, including severe bone pain, pathological fractures, hypercalcemia, neural compression syndrome, and bone marrow suppression (1-3). In turn, there is an increase in morbidity and mortality among breast cancer patients. Arguello et al. (4) established a bone metastasis model in which injection of cancer cells into the left cardiac ventricle of nude mice causes the development of osteolytic lesions. Recently, Sasaki et al. (5) have used this approach to develop a model of human breast cancer, the MDA-MB-231 human breast cancer cell line, that selectively produces osteolytic lesions similar to those observed in breast cancer patients. This model was then used to demonstrate a beneficial effect of bisphosphonates in the treatment and prevention of metastatic cancer to bone (5-8). Because bisphosphonates work primarily by inhibiting osteoclastic bone resorption but have probably no direct effect on metastatic tumor cells, it would be advantageous to devise alternative therapy that could directly target tumors within bone, alone or in combination with bisphosphonates.

The biologically active metabolite of vitamin D_3, 1,25(OH)_2D_3, has functions and therapeutic potential that extend beyond those of regulating bone mineralization and calcium homeostasis. At present, it is well-documented that 1,25(OH)_2D_3 is involved in essential cell regulatory processes, such as proliferation, differentiation, and apoptosis (9, 10). It has been shown that this hormone promotes cellular differentiation and inhibits the proliferation and the invasive potential of a number of different cancer cells in vitro (11-14). Recently, 1,25(OH)_2D_3 has been shown to induce apoptosis in human breast cancer cell lines (11, 15) and can also inhibit tumor-induced angiogenesis (16). In vivo studies demonstrated that 1,25(OH)_2D_3 slows the progression of breast, prostate, and other carcinomas (17-19). These properties suggest the possible clinical use of 1,25(OH)_2D_3 in the treatment of benign or malignant hyperproliferative disorders such as psoriasis and prostate and breast cancer. However, the potent hypercalcemic activity of 1,25(OH)_2D_3 has precluded its application as a pharmacological agent. For this reason, various synthetic vitamin D_3 compounds with reduced calcemic activity that retain the antiproliferative effects of 1,25(OH)_2D_3 have been developed (12, 20-22). Among these analogues, EB 1089 (Leo Pharmaceutical Ltd., Ballerup, Denmark) has been studied extensively. The effect of EB 1089 on calcium metabolism in vivo is ~50% lower than that of 1,25(OH)_2D_3 (23, 24). Moreover, this compound has a half-life similar to 1,25(OH)_2D_3 in vivo (25). In this analogue, the side chain is elongated with introduction of terminal ethyl groups, and double bonds have been introduced at positions C22 and C24 (Fig. 1), resulting in an increased metabolic stability (26). Previous studies have clearly demonstrated the efficacy of EB 1089 in reducing the growth of breast cancer cells in vitro (22, 23, 27). EB 1089 has also been tested in vivo and exhibited the best profile for regression of tumor growth without affecting serum calcium levels (15, 18, 27-29). On the basis of these findings and the fact that the vitamin D_3 receptor is present in a wide variety of human breast cancer cells (30-32) and in >80% of breast tumors (30, 31), we examined the capacity of EB 1089 to inhibit human breast cancer cell growth in vitro and the development of bone metastases in vivo.

We report here that in the MDA-MB-231 bone metastasis model, EB 1089 significantly decreases the development of osteolytic bone metastases, as demonstrated by radiological and histomorphometric examinations.

MATERIALS AND METHODS

Animals. Female, athymic nude mice (BALB/c-nu/nu; Charles River, Quebec, Canada), 4 weeks of age, were used for all experiments. Animals were maintained in a specific pathogen-free environment under controlled conditions of light and humidity for several weeks. These studies were approved by the Institutional Review Board of the Royal Victoria Hospital (Montreal, Canada).

Culture Conditions. The MDA-MB-231 (MDA-231) human breast cancer cell line was initially isolated from a pleural effusion of a 51-year-old woman and found to be estrogen receptor negative (33). This cell line was obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Wisent, Montreal, Quebec, Canada)
Intracardiac Injection of MDA-231 Cells in Nude Mice and Administration of EB 1089. Intracardiac injection of MDA-231 cells was performed according to the procedure described previously by Sasaki et al. (5). Subconfluent MDA-231 cells were fed with a fresh medium 24 h before intracardiac injection into nude mice. Cells (1 × 10⁶) were suspended in 0.1 ml of PBS and then injected into the left cardiac ventricle of female nude mice, 4 weeks of age, using a 27-gauge needle under anesthesia.

A preventative protocol was designed in which EB 1089 was administered continuously using an osmotic minipump (model 2 ML4 Alzet; Alza Corp., Palo Alto, CA) implanted s.c. the same day as the inoculation of MDA-231 breast cancer cells (Fig. 1). In preliminary experiments, we used increasing concentrations of EB 1089 (10, 14, 16, and 18 µM/24 h) to determine the minimal effective dosage that will not cause hypercalcemia in non-tumor-bearing mice. An infusion rate of 14 µM/24 h was chosen, and each minipump contained EB 1089 dissolved in 50% propylene glycol, 10% ethanol, and 40% saline to deliver a continuous dose of EB 1089 for up to 4 weeks at a delivery rate of 2.5 µl/h. Untreated animals were implanted with a minipump containing vehicle alone. Radiographs were taken 35 days after cell inoculation and prior to sacrifice to assess the number of osteolytic bone metastases. Histomorphometric analysis of tumor burden within bone was then performed in this group. In a separate protocol, survival, development of bone metastases, and hind limb paralysis were determined from the day of cell inoculation to the animal’s death using Kaplan-Meier analysis.

Assessment of the Number and Area of Bone Metastases. The number and area of osteolytic bone metastases were determined on radiographs. Animals were anesthetized, placed in a prone position against the films (18 × 24-cm; Mamoray Screens, AGFA, Mortsel, Belgium), and exposed to an X-ray at 25 kV for 5 s using a Mammo Diagnost UC (Philips, Hamburg, Germany). Films were developed using a Curix Compact processor (AGFA). The radiographs were extensively evaluated by three investigators including and 1× antibiotic-antimycotic solution (Life Technologies, Inc.) in a humidified atmosphere of 5% CO₂ in air. The medium was changed twice weekly.

Assessment of Cell Growth in Vitro. The effects of EB 1089 and 1,25(OH)₂D₃ on the proliferation of breast cancer MDA-231 cells in vitro were assessed by cell count and [³H]thymidine incorporation. Cells were seeded at a density of 4 × 10⁴ cells/well in 24-well cluster plates in DMEM containing 10% fetal bovine serum for 24 h. After 24 h in serum-deprived DMEM, fresh medium containing 2.5% charcoal-stripped FCS with or without increasing concentrations of EB 1089 or 1,25(OH)₂D₃ (10⁻¹⁰ to 10⁻⁷ M) was added to cultured cells, and incubations continued for 3–5 days. Media was changed every 2 days thereafter. EB 1089 and 1,25(OH)₂D₃ were dissolved in ethanol, and the final concentration of ethanol in all cultures did not exceed 0.1%. Cells were trypsinized at timed intervals, and an aliquot was counted (Coulter Electronics, Beds, United Kingdom).

DNA synthesis was assessed by measuring [³H]thymidine incorporation into cellular DNA. [³H]Thymidine (1 Ci/ml; DuPont New England Nuclear) was added to the cells during the last 2 h of incubation. The medium was aspirated, and cells were then washed twice with cold HBSS and incubated in 5% cold trichloroacetic acid for 15 min. After aspiration of the trichloroacetic acid, the cells were dissolved in 0.5 ml of 0.6 N NaOH, and an aliquot was counted by liquid scintillation. Results were expressed as a percentage of [³H]thymidine incorporation measured in the absence of 1,25(OH)₂D₃ or EB 1089.

Fig. 2. Effects of EB 1089 and 1,25(OH)₂D₃ on MDA-231 cell growth in vitro. Cells were treated without or with increasing concentrations of 1,25(OH)₂D₃ or EB 1089 (10⁻¹⁰ to 10⁻⁷ M) for 3–5 days in DMEM supplemented with 2.5% charcoal-stripped FCS. A, cell number was determined at 3 and 5 days with 10⁻² M of each compound. B, [³H]thymidine incorporation expressed as a percent of control (100%). Each value represents the mean of three different experiments done in quadruplicate; bars, SE. *, a significant difference from control values (vehicle treated cells); ∗, a significant difference between 1,25(OH)₂D₃ and EB 1089 (p < 0.05). □, control; □, 1,25(OH)₂D₃; □, EB 1089.
one radiologist, who had no knowledge of the experimental protocol. The area of osteolytic metastases was determined in both fore and hind limbs using an image analysis system in which prints of radiographs were captured and measured using a digitizing tablet attached to an IBM-compatible computer. Bone specimens were cut completely through. Levels of longitudinal sections were spaced by 50 μm if tumor was identified or by 125 μm if no tumor was seen on sections stained by methylene blue. Sections of 5 μm were obtained using a polycut-E microtome (Reichert-Jung, Leica, Heerbrugg, Switzerland), placed on gelatin-coated glass slides, and stained with hematoxylin, eosin, and Goldner.

Histomorphometrical determination of total tumor depth and area of metastatic cancer infiltrations were measured in the femora of each treatment group on Goldner-trichrome-stained longitudinal sections. The metastatic tumors in bone were recognized, and their areas were measured on an osteoMeasure system (Osteometrics, Inc., Atlanta, GA) using an IBM-compatible computer.

### Table 1

<table>
<thead>
<tr>
<th>Animals with bone metastases (%)</th>
<th>No. of lesions</th>
<th>Animals with limb paralysis (%)</th>
<th>Bone metastases/paralysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB 1089 (n = 9)</td>
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<td></td>
<td></td>
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<tr>
<td>Untreated (n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>33</td>
<td>8</td>
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<td>2</td>
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<tr>
<td>77</td>
<td>10</td>
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<tr>
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<td>72</td>
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<td>100</td>
<td>8</td>
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</table>

*Ps were derived with the log-rank test.*
Analytical Methods. Animals were bled once a week for measurement of total plasma calcium and albumin. Plasma calcium and albumin levels were determined by microchemistry (Kodak Ektachrome, Mississauga, Ontario, Canada). Corrected plasma calcium was calculated using the formula: plasma total calcium \( \times \frac{1}{[(40 - \text{plasma albumin}) \times 0.02].}

Statistical Analysis. All results are expressed as mean ± SE. Statistical comparisons for in vitro study were made using the unpaired Student’s t test (a probability value of \( P < 0.05 \) was considered significant). Statistical significance of the difference in numbers of osteolytic metastases and tumor volume between EB 1089-treated groups and untreated groups was analyzed by Mann-Whitney test for nonparametric samples. The statistical difference of survival rate of the animals was determined by Kaplan-Meier analysis.

RESULTS

Effects of EB 1089 and 1,25(OH)\(_2\)D\(_3\) on MDA-231 Cell Growth in Vitro. We examined the effects of 1,25(OH)\(_2\)D\(_3\) and its low calcemic analogue EB 1089 on the proliferation of breast cancer MDA-231 cells in vitro. Cells were grown as described in “Materials and Methods” and treated with increasing concentrations of EB 1089 or

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Table 2 Area of osteolytic lesions measured on radiographs of long bones at the death of the animals

<table>
<thead>
<tr>
<th></th>
<th>Untreated (( n = 11 ))</th>
<th>EB 1089 (( n = 9 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.654</td>
<td>0.00</td>
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<tr>
<td>0.654</td>
<td>1.428</td>
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<tr>
<td>0.00</td>
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<tr>
<td>2.580</td>
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<tr>
<td>3.391</td>
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<tr>
<td>1.309</td>
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<td>3.807</td>
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<td>1.011</td>
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<td>0.00</td>
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<tr>
<td>1.130</td>
<td>0.00</td>
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<tr>
<td>1.606</td>
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Mean ± SE 1.449 ± 0.381 0.511 ± 0.229

\(^a\) P < 0.05.

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Fig. 6. Bone histology in EB 1089-treated and untreated mice. This slide is representative of typical lesions observed in femora of animals. A. section from a control animal given EB 1089 alone. B and D, osteolytic lesions in an animal injected with MDA-231 cells and treated with vehicle alone. The primary and secondary spongiosa were replaced by metastatic breast cancer cells (T) as compared with the bone of non-tumor-bearing control mice (A). Goldner-trichrome staining. ×20 and ×80. C and E, show osteolytic metastases in an animal treated with EB 1089. Colonization of metastatic MDA-231 cells (T) was more localized compared with vehicle-treated animals (B), and the marrow cavity remained intact with an appearance similar to that of non-tumor-bearing mice (A). Goldner-trichrome staining. ×20 and ×80. BM, bone marrow; Ct. B, cortical bone; SM, skeletal muscle.
1.25(OH)\(_2\)D\(_3\). As shown in Fig. 2, treatment of cells with 10\(^{-7}\) M of each compound for 3 and 5 days resulted in a time-dependent decrease of cell number (Fig. 2A). Moreover, addition of increasing concentrations of EB 1089 or 1.25(OH)\(_2\)D\(_3\) to the culture medium caused a significant dose-dependent inhibition of \(^{3}H\)thymidine incorporation (Fig. 2B). The minimal dosage producing a significant inhibition of cell growth was 10\(^{-3}\) M for EB 1089 and 10\(^{-5}\) M for 1.25(OH)\(_2\)D\(_3\). In addition, the degree of inhibition observed with EB 1089 at any one dose appeared greater than 1.25(OH)\(_2\)D\(_3\).

**Osteolytic Bone Metastases Caused by MDA-231 Breast Cancer Cells in Nude Mice.** Nude mice injected with breast cancer MDA-231 cells into the left cardiac ventricle showed multiple and well-defined osteolytic bone metastases in lower and upper extremities between 4 and 5 weeks after cell inoculation. Furthermore, mice developed severe cachexia with a marked decrease in muscle and adipose tissue, leading to body weight loss (data not shown). These observations are consistent with those reported previously (5, 36).

**Effects of Continuous Treatment with EB 1089 on the Development of Osteolytic Bone Metastases.** The number of mice that developed osteolytic bone metastases was analyzed longitudinally by Kaplan-Meier analysis and found to be significantly lower in the EB 1089-treated group as compared with the untreated group (Fig. 3A). At the time of death, the percentage of mice that developed osteolytic bone metastases was only 66% in the EB 1089-treated group compared with 100% in the untreated group (\(P < 0.002\); Table 1). In addition, the total number of bone lesions at each site analyzed (femur, tibia, and humerus) was significantly reduced in animals treated with EB 1089 (\(P < 0.01\)). Furthermore, EB 1089-treated mice developed less hind limb paralysis as compared with untreated mice (\(P < 0.013\); Fig. 3B; Table 1).

Radiographs taken 35 days after tumor cell inoculation in the untreated group showed multiple and obvious osteolytic lesions in the distal femora and proximal tibia. In contrast, nude mice treated with EB 1089 (14 pm/day/mouse) from the time of MDA-231 cell inoculation developed fewer radiographically detectable osteolytic bone lesions (Fig. 4). Only 28% of mice treated with EB 1089 developed osteolytic bone metastases as compared with 85% of untreated mice (\(P < 0.03\)).

The number of bone lesions per animal in the EB 1089-treated group was markedly lower than that in the untreated group at 5 weeks (Fig. 5A) and at the death of the animals (Fig. 5B). Moreover, the mean lesion area was significantly reduced in the EB 1089-treated group compared with the group receiving vehicle alone (Table 2).

**Histological Examination of Bones with Metastatic Lesions.** Representative histological sections through the femora of both untreated and EB 1089-treated groups 35 days after inoculation of tumor cells are illustrated in Fig. 6. Bone sections from untreated mice revealed that in most cases metastatic tumor cells filled a substantial amount of bone marrow space. In contrast, tumor cells present in the bone marrow space in EB 1089-treated mice were small and associated with little or no bone destruction. Furthermore, most of the EB 1089-treated mice had intact cortical and trabecular bone, and many bones had no evidence of tumor involvement.

**Histomorphometric Analysis of Metastatic Cancer Burden in Bone.** Histomorphometrical analysis of bone from mice in both untreated and EB 1089-treated groups 35 days after inoculation of tumor cells confirmed radiographic observations. The number and area of osteolytic lesions were significantly decreased in mice treated with EB 1089 compared with vehicle-treated mice (Fig. 7, A and B). Moreover, tumor depth was significantly less in EB 1089-treated mice compared with untreated mice (Fig. 7C).

**Effect of EB 1089 on Plasma Calcium.** Tumor-bearing animals receiving EB 1089 did not show any significant change in plasma calcium when compared with the vehicle-treated control group. In both groups, plasma calcium concentrations remained normal for the duration of the experiment (Fig. 8).

**Effect of EB 1089 on Animal Survival.** We assessed the effect of EB 1089 on survival time of tumor-bearing animals. Mice receiving EB 1089 IN OSTEOLYTIC BONE METASTASES
vehicle alone (untreated) died within 6 weeks after MDA-231 cell inoculation (Fig. 9). In contrast, animals treated with EB 1089 displayed a marked and statistical increase in survival at 35 days from 54.6 ± 15.0% in untreated animals to 88.9 ± 10.5%. (P < 0.007).

DISCUSSION

Metastatic breast cancer to bone is a particularly challenging problem in the clinical setting. Once tumors have invaded bone, response to classical chemotherapeutic agents is low, and the prognosis for these patients is poor. Recently, bisphosphonates have been used in this setting and shown to reduce the number of events in metastatic breast cancer without influencing survival (37–42). Bisphosphonates are directed primarily at inhibiting osteoclastic bone resorption. However, they neither prevent the spread of tumor cells to bone or directly affect tumor growth within bone. Consequently, other approaches aimed at preventing either attachment or growth of breast cancer cells at the bone site are needed and may be used alone or in combination with bisphosphonates.

In this study, we used an animal model of human breast cancer to demonstrate that EB 1089, a low calcemic analogue of 1,25(OH)2 D3, inhibits the formation of osteolytic bone lesions. Previous studies had shown that synthetic vitamin D3 analogues with low calcemic activities relative to the native hormone 1,25(OH)2 D3, are of potential value as anticancer agents (18, 21, 43–46). EB 1089, has been extensively studied and shown to inhibit both estrogen dependent and independent human breast cancer cell growth in vitro and in vivo (22, 23, 27, 28). In a Phase I clinical trial, designed to evaluate the calcemic effect of EB 1089 in patients with advanced breast and colorectal cancers, this compound was well tolerated, and 16% of patients on treatment for >90 days showed stabilization of disease (47). In view of the fact that vitamin D receptors are expressed in breast cancer without influencing survival (37–42), Bisphosphonates in the present protocol, examined the effect of EB 1089 as a prophylactic treatment of bone metastases. In the clinical setting, this would represent a situation similar to tamoxifen prevention for recurrence of breast cancer (53) in patients without evidence of tumor spread. Our data clearly indicate radiological, histological, and histomorphometric suppression of bone metastases by continuous administration of EB 1089. This effect occurs without significant calcium elevation, indicating that this analogue could be administered safely without undesirable side effects. Our study also indicates that inhibition of the bone metastatic process prolongs survival. Our results are in keeping with previous studies using bisphosphonates in a preventive manner in both animal protocols (6–8) or in clinical trials (37–42), showing a good correlation between reduction of metastatic bone lesions and survival.

In conclusion, EB 1089 is highly effective in reducing metastatic bone lesions associated with human breast cancer and warrants further study as a therapeutic agent in this condition.

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The Vitamin D Analogue EB 1089 Prevents Skeletal Metastasis and Prolongs Survival Time in Nude Mice Transplanted with Human Breast Cancer Cells

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