ARTICLE

Antitumor Effects of Clinical Dosing Regimens of Bisphosphonates in Experimental Breast Cancer Bone Metastasis

Florence Daubiné, Céline Le Gall, Juerg Gasser, Jonathan Green, Philippe Clézardin

Background   Bisphosphonates exhibit direct antitumor activity in animal models, but only at high doses that are incompatible with the clinical dosing regimens approved for the treatment of cancer patients with skeletal metastases. We compared the antitumor effects of clinical dosing regimens of the bisphosphonates zoledronic acid and clodronate in a mouse model of bone metastasis.

Methods   Mice (n = 6–10 per group) were treated with zoledronic acid, clodronate, or vehicle starting before (preventive protocols) or after (treatment protocols) intravenous injection with human B02/GFP.2 breast cancer cells, which express green fluorescent protein (GFP) and luciferase and metastasize to bone. Zoledronic acid was given as daily, weekly, or single doses at a cumulative dose of 98–100 µg/kg body weight, equivalent to the 4-mg intravenous dose given to patients. Clodronate was given as a daily dose (530 µg/kg/day), equivalent to the daily 1600-mg oral clinical dose given to patients. Bone destruction was measured by radiography, x-ray absorptiometry or tomography, and histomorphometry (as the ratio of bone volume to tissue volume). Skeletal tumor burden was measured by histomorphometry (as the ratio of tumor burden to soft tissue volume [TB/STV]) and luciferase activity. All statistical tests were two-sided.

Results   In treatment protocols, daily clodronate was less effective at decreasing the TB/STV ratio than daily (53% versus 87%, difference = 34%, 95% confidence interval [CI] = 16% to 44%, *P*<.001) or weekly (53% versus 90%, difference = 37%, 95% CI = 19% to 46%, *P*<.001) zoledronic acid–dosing regimens. Compared with vehicle, a single dose of zoledronic acid decreased tumor burden by only 16% (95% CI = 9% to 22%, *P*<.001). In preventive protocols, daily clodronate and daily or weekly zoledronic acid decreased the TB/STV ratio by 49% (95% CI = 40% to 57%, *P* = .006), 83% (95% CI = 68% to 98%, *P*<.001), and 66% (95% CI = 47% to 84%, *P*<.001), respectively, compared with vehicle, whereas a single dose of zoledronic acid decreased tumor burden by only 13% (95% CI = −2% to 28%, *P* = .84). Mice treated with a daily preventive regimen of clodronate or with a daily or weekly preventive regimen of zoledronic acid showed a decreased B02/GFP.2 cell tumor burden compared with vehicle, whereas a single preventive dose of zoledronic acid had no effect.

Conclusion   Daily or repeated intermittent therapy with clinical doses of bisphosphonates inhibits skeletal tumor growth in a mouse model.

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Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption and have demonstrated clinical utility in the palliative treatment of patients with bone metastases (1). There is now extensive in vivo preclinical evidence that bisphosphonates can reduce skeletal tumor burden and inhibit the formation of bone metastases in animal models (2). Several mechanisms have been proposed to explain these observations. For example, bisphosphonates may render the bone a less favorable microenvironment for tumor cell colonization by reducing osteoclast-mediated bone resorption, which, in turn, would deprive tumor cells of bone-derived growth factors released from the bone matrix (3,4). In addition, bisphosphonates appear to have direct antitumor effects (2). Bisphosphonates have been shown to inhibit tumor cell adhesion, invasion, and proliferation, and they induce apoptosis of a variety of human tumor cell

lines in vitro (2). Bisphosphonates also inhibit tumor growth in vivo through antiangiogenic, anti-invasive, and immunomodulatory activities (2). However, the experimental conditions that have been

used to study the efficacy of bisphosphonates in tumor-bearing animals are far removed from the conditions that have been used to treat patients with bone metastases (2). For example, the high doses of bisphosphonates that have been used in most animal studies are incompatible with the clinical dosing regimens that have been approved for the treatment of cancer patients with skeletal metastases. Moreover, the bisphosphonate-dosing regimens that have been used in clinical trials to date have shown no convincing antitumor effects (1,3). Thus, it is important to determine if a clinically relevant dosing regimen of bisphosphonate can achieve meaningful antitumor effects in animal models of bone metastasis.

We used a mouse model of human breast cancer bone metastasis to examine the effects of different dosing regimens with two bisphosphonates, zoledronic acid and clodronate, on osteolysis and skeletal tumor growth.

Materials and Methods

Bisphosphonates

Clodronate (dichloromethylene bisphosphonic acid) and zoledronic acid [1-hydroxy-2-(1H-imidazole-1-yl)ethylidene-bisphosphonic acid], as the disodium salts, were provided by Novartis Pharma AG (Basle, Switzerland). These compounds were dissolved in water and stored at 4 °C.

Mouse Model of Bone Metastasis

All procedures involving mice including their housing and care, the method by which they were killed, and all experimental protocols were conducted in accordance with a code of practice established by the Experimentation Review Board from the Laennec School of Medicine. This study was monitored on a routine basis by the attending veterinarian to ensure continued compliance with the proposed protocols. Four-week-old female BALB/c athymic (nu/nu) mice were purchased from Charles River (St Germain sur l'Arbresle, France). The bone metastasis experiments in mice were conducted as previously described (5,6) using B02 cells, a subpopulation of the human MDA-MB-231 breast cancer cell line that was selected for the high efficiency with which it metastasizes to bone after intravenous inoculation (5). We specifically used B02 cells that had been stably transfected with the genes encoding green fluorescent protein (GFP) and luciferase (B02/GFP.2 cells); the characteristics of this cell line were described elsewhere (6). On day 0, B02/GFP.2 cells (5×10^5 cells in 100 μL of phosphate-buffered saline [PBS]) were injected into the tail vein of mice anesthetized with 130 mg/kg ketamin and 8.8 mg/kg xylazin. In this model, mice usually develop bone metastases 18 days after tumor cell injection, as judged by radiography (6). For the treatment protocols (see "Dosing Regimens and Experimental Protocols"), mice were analyzed by radiography on day 18, and the area of osteolytic lesions on the skeleton of each animal was measured. Tumor-bearing animals were then distributed among the different treatment groups (n = 6–10 mice per group) to balance these groups for the extent of bone destruction at baseline. For the preventive protocols (see "Dosing Regimens and Experimental Protocols"), mice were randomly assigned to treatment groups (n = 6–10 mice per group) 1 day before B02/GFP.2 tumor cell injection. For both protocols, on day 32 after tumor cell inoculation, radiographs of anesthetized

CONTEXT AND CAVEATS

Prior knowledge
At high doses, bisphosphonates exhibit antitumor properties in animal models. However, the high doses of bisphosphonates used in animals are incompatible with approved treatment regimens for patients, and the bisphosphonate doses that are approved for patients do not have convincing antitumor effects.

Study design
In vivo study of clinical dosing regimens of bisphosphonates in a mouse model of breast cancer cell metastasis to bone.

Contribution
Bisphosphonates administered at low dosages on a daily or weekly dosing schedule were shown to inhibit skeletal tumor growth in a mouse model of bone metastasis.

Implications
Continuous or frequent intermittent low-dose therapy with bisphosphonates may facilitate the prolonged exposure of bone marrow to these drugs, thus enabling a direct effect on tumor cells that reside in bone.

Limitations
The mouse model does not recapitulate all steps required for spontaneous metastasis of breast cancer cells to bone and does not take into account the role of the immune system.

mice were taken with the use of MIN-R2000 film (Kodak, Rochester, NY) in an MX-20 cabinet X-ray system (Faxitron X-ray Corp, Wheeling, IL). Osteolytic lesions were identified on radiographs as demarcated radiolucent lesions in the bone. The area of the osteolytic lesions was measured using a Visiolab 2000 computerized image analysis system (Explora Nova, La Rochelle, France), and the extent of bone destruction per animal was expressed in square millimeters, as described previously (5,6). Anesthetized mice were killed by cervical dislocation after radiography on day 32.

Dosing Regimens and Experimental Protocols
The bisphosphonate doses we used were calculated on the basis of the current clinical doses that have been approved for the treatment of cancer patients with skeletal metastases (i.e., for zoledronic acid, 4 mg via intravenous injection every 3–4 weeks; for clodronate, 1600 mg taken orally daily), using an average body weight of 60 kg for patients and an oral bioavailability of 2% for clodronate (7). Based on an average body weight of 20 g for 4-week-old mice, clodronate was administered to mice at a dosage of 530 μg/kg body weight/day for both the treatment and preventive protocols (Fig. 1). For zoledronic acid, we used three dosing regimens (daily, weekly, and single) for both the treatment and preventive protocols (Fig. 1). Zoledronic acid was administered to mice at a daily dose of 3 and 7 μg/kg body weight for the preventive and treatment protocols, respectively, and a weekly dose of 20 and 50 μg/kg body weight in the preventive and treatment protocols, respectively. However, the same total cumulative dose of zoledronic acid was given to each mouse (i.e., 98–100 μg/kg body weight), regardless of the protocol or dosing regimen, which enabled us to directly compare the efficacy of zoledronic acid among the different dosing regimens and the two different protocols. This dose (100 μg/kg) is theoretically
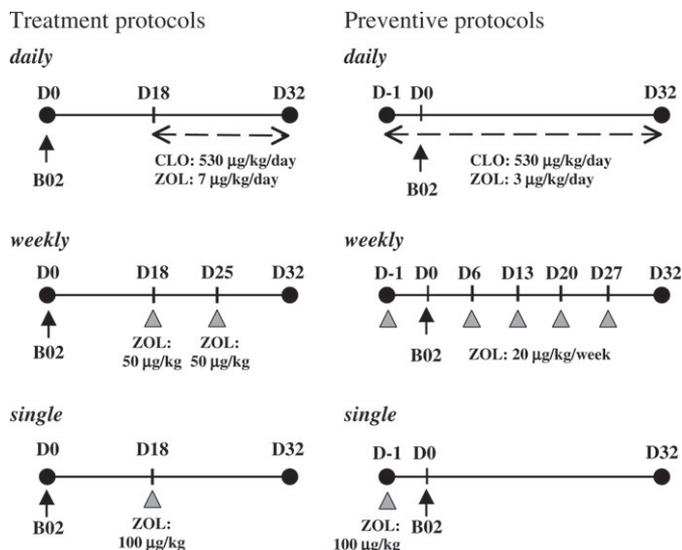
Fig. 1. Summary of treatment and preventive protocols for bisphosphonates in mouse model of human B02 breast cancer cell metastasis to bone. B02 cells were inoculated intravenously into 4-week-old female BALB/c nude mice on day 0 (D0). In the treatment protocols, bisphosphonates were administered by subcutaneous injection beginning on day 18 (D18) after tumor cell inoculation, at which time the mice had developed bone metastases. In the preventive protocols, bisphosphonates were administered by subcutaneous injection beginning 1 day before tumor cell inoculation (D-1). In both protocols, zoledronic acid (ZOL) was administered daily, weekly, or once (on day 18 in the treatment protocol and on day 1 in the preventive protocol); the total cumulative dose of ZOL was 98–100 µg/kg. For the daily dosage, ZOL was administered at a dose of 3 and 7 µg/kg in the preventive and treatment protocols, respectively. A weekly dose of 20 and 50 µg/kg of ZOL was used for the preventive and treatment protocols, respectively. Clodronate (CLO) was administered daily at a dose of 530 µg/kg in both the treatment and preventive protocols. The mice were killed 32 days after tumor cell inoculation.

equivalent to the clinical dose of 6 mg every 3–4 weeks; the actual dose given to patients is 4 mg. However, zoledronic acid in the commercial clinical vial (free acid monohydrate) has a lower molecular weight than the pure research-grade substance (disodium salt, 4.75 hydrate) used in this study. Because of the difference in molecular weights between the free acid monohydrate and the disodium salt, a 4-mg clinical dose of zoledronic acid is equivalent to 5.9 mg of pure research-grade substance. A total cumulative dose of 100 µg/kg was therefore chosen to adjust for the higher molecular weight of the pure research-grade substance. All doses of each bisphosphonate were administered by subcutaneous injection in 100 µL PBS (vehicle). Control mice received a daily treatment with vehicle only.

Detection of Luciferase-Expressing Tumor Cells in Bone Lysates

B02/GFP.2 cells were inoculated intravenously into mice (5 × 10^5 cells in 100 µL of PBS). Mice injected intravenously with B02/GFP.2 cells and treated on the three preventive protocols with zoledronic acid, clodronate, or vehicle were killed 14 days after tumor cell inoculation (n = 4–6 mice per group) and analyzed by radiography. None of these mice had radiographic evidence of bone destruction on the day they were killed. To produce a bone extract for each mouse, we harvested both tibiae, snap froze the limbs in liquid nitrogen, and crushed them together in 2 mL of lysate buffer (25 mM Tris-phosphate, pH 7.8, 2mM dithiothreitol,

2mM EDTA, 1% [vol/vol] Triton X-100, and 5 mg/mL lysozyme [Promega, Madison, WI]). We mixed 30 µL of bone extract of each mouse at room temperature with 100 µL of beetle luciferin (Promega) and then measured the luciferase activity of the mixture with the use of an automated luminometer (Lumat LB 9507; Berthold Technologies, Thoiry, France). Results are expressed as relative light units (RLUs).

Bone Mineral Density Measurement

Vehicle- and bisphosphonate-treated mice bearing radiographically confirmed tumors (n = 6–10 mice per group) were killed on day 32 after tumor cell inoculation for both protocols, and both hind limbs from each animal were dissected and fixed in 70% (vol/vol) alcohol. We measured the bone mineral density of tibiae with radiographic evidence of osteolytic lesions by dual-energy x-ray absorptiometry scanning with the use of a PIXI-mouse densitometer (Lunar Corp, Copenhagen, Denmark) or by peripheral quantitative computed tomography with the use of an XCT Research SA+ scanner (Stratec Medizintechnik, Pforzheim, Germany) fitted with a 0.5-mm collimator, as previously described (8,9). For the peripheral quantitative computed tomography, the measurement of bone mineral density in tibiae was performed on four different locations within the proximal tibial metaphysis (S1–S4, with S1 being the closest to the growth plate). Only the data obtained at the S1 location are shown in this study because this region is highly vascularized and is the site where osteolytic lesions start to develop. Results obtained using dual-energy x-ray absorptiometry and peripheral quantitative computed tomography are expressed as milligram per square centimeter and milligram per cubic centimeter, respectively.

Bone Histology and Histomorphometry

Bone histology and histomorphometric analysis of bone tissue sections were performed as previously described (5,6). Briefly, vehicle- and bisphosphonate-treated tumor-bearing mice (n = 6–10 mice per group) were killed on day 32 after tumor cell inoculation for both protocols, and both hind limbs from each animal were dissected, fixed in 80% (vol/vol) alcohol, dehydrated, and embedded in methylmethacrylate. A microtome (Polycut E; Reichert-Jung, Heidelberg, Germany) was used to cut 7-µm-thick sections of undecalcified long bones, and the sections were stained with Goldner's trichrome (5,6). Histologic and histomorphometric analyses were performed on Goldner's trichrome–stained longitudinal medial sections of tibial metaphysis with the use of a computerized image analysis system (Visiolab 2000), as previously described (5, 6). Histomorphometric measurements (i.e., bone volume to tissue volume [BV/TV] and tumor burden to soft tissue volume [TB/STV] ratios) were performed in a standard zone of the tibial metaphysis, situated at 0.5 mm from the growth plate, including cortical and trabecular bones. The BV/TV ratio represents the percentage of bone tissue. The TV/STV ratio represents the percentage of tumor tissue.

Statistical Analysis

All data were analyzed with the use of StatView software (version 5.0; SAS Institute Inc, Cary, NC). Pairwise comparisons were carried out by performing a nonparametric Mann–Whitney U test. P values less than .05 were considered statistically significant. All statistical tests were two-sided.

Results

Effects of Zoledronic Acid and Clodronate on the Progression of Established Breast Cancer Bone Metastases (Treatment Protocols)

We used a mouse model of human breast cancer bone metastasis in which animals display radiographic evidence of osteolytic lesions in hind limbs 18 days after tumor cell injection (5,6). We first compared the effects of different dosing regimens of zoledronic acid and a daily dosing regimen of clodronate on the progression of established bone metastases by using a treatment protocol in which drug administration to tumor-bearing mice was initiated on day 18 after tumor cell injection (Fig. 1). For all three regimens involving zoledronic acid (i.e., daily, weekly, and single), the total cumulative dose (administered by subcutaneous injection) was 98–100 µg/kg body weight, which we calculated was equivalent to the 4-mg clinical intravenous dose given to breast cancer patients on the basis of body weight. For the daily regimen involving clodronate, the dosage of 530 µg/kg/day (also administered by subcutaneous injection) was similarly calculated to be equivalent to a clinical oral dose of 1600 mg/day (assuming an oral bioavailability of 2%) (7).

Radiographic analysis on day 32 after tumor cell injection revealed that tumor-bearing mice treated with a single dose of zoledronic acid had osteolytic lesions that were 52% (95% confidence interval [CI] = 37% to 67%, $P<.001$) smaller than those of tumor-bearing mice treated with the vehicle (Table 1 and Fig. 2). Similarly, tumor-bearing mice treated with daily or weekly dosing regimens of zoledronic acid had osteolytic lesions that were 66% (95% CI = 56% to 76%, $P<.001$) and 50% (95% CI = 13% to 83%, $P = .04$) smaller, respectively, than those of vehicle-treated animals (Table 1 and Fig. 2). The inhibitory effect of zoledronic acid on the progression of osteolytic lesions did not differ statistically significantly among the different dosing regimens (Table 1). In addition, hind legs bearing metastases from mice treated with each of the three zoledronic acid–dosing regimens had statistically significantly higher bone mineral density than those from mice treated with vehicle (Table 1). Compared with vehicle, the daily, weekly, and single regimens of zoledronic acid increased the bone mineral density by 24% (95% CI = 18% to 31%, $P<.001$), 16% (95% CI = 10% to 23%, $P = .004$), and 14% (95% CI = 7% to 20%, $P = .01$), respectively (Table 1). Moreover, the daily dosing regimen with zoledronic acid was statistically significantly more effective than a

single dosing of zoledronic acid at increasing the bone mineral density (633 mg/cm³ versus 579 mg/cm³, difference = 54 mg/cm³, 95% CI = 9 to 99 mg/cm³, $P = .02$). By contrast, the bone mineral density did not differ statistically significantly among the weekly and single dosing regimens. Daily clodronate was less effective than daily zoledronic acid (580 mg/cm³ versus 633 mg/cm³, difference = 53 mg/cm³, 95% CI = 3 to 103 mg/cm³, $P = .04$) but similar to the weekly and single zoledronic acid–dosing regimens at increasing the bone mineral density.

Histomorphometric analysis of hind limbs with metastases showed that mice treated with each of the three regimens of zoledronic acid had statistically significantly higher BV/TV ratios (indicating a prevention of bone loss) than vehicle-treated mice. For example, the daily, weekly, and single regimens of zoledronic acid increased the BV/TV by 138% (95% CI = 126% to 150%), 79% (95% CI = 67% to 89%), and 90% (95% CI = 82% to 98%), respectively, compared with vehicle ($P<.001$ for all three regimens) (Table 1 and Fig. 2). Moreover, mice treated with a daily zoledronic acid regimen had a statistically significantly higher BV/TV ratio than mice receiving a weekly or single dosing regimen of zoledronic acid (mean BV/TV for daily zoledronic acid: 41%, mean BV/TV for weekly zoledronic acid: 30%, mean BV/TV for single zoledronic acid: 32%; difference for daily versus weekly zoledronic acid = 11%, 95% CI = 9% to 14%, $P<.001$; difference for daily versus single zoledronic acid = 9%, 95% CI = 7% to 11%, $P<.001$). Mice treated with daily clodronate also had statistically significantly higher BV/TV ratios than vehicle-treated mice (Table 1). However, daily clodronate was less effective than daily zoledronic acid at increasing the BV/TV ratio (28% versus 41%, difference = 13%, 95% CI = 10% to 16%, $P<.001$).

Compared with the vehicle, the daily and weekly regimens of zoledronic acid also substantially decreased the TB/STV ratio (a measure of the skeletal tumor burden) by 87% (95% CI = 75% to 93%, $P<.001$) and 90% (95% CI = 80% to 94%, $P<.001$), respectively, whereas a single dose of zoledronic acid decreased the TB/STV ratio by only 16% (95% CI = 9% to 22%, $P<.001$) (Table 1 and Fig. 2). Daily clodronate decreased the TB/STV ratio by 53% (95% CI = 39% to 64%, $P<.001$) compared with vehicle (Table 1 and Fig. 2). However, daily clodronate was statistically significantly less effective at decreasing skeletal tumor burden than the daily (53% versus 87%, difference = 34%, 95% CI = 16% to

Table 1. Effect of different dosing regimens of zoledronic acid and clodronate on the progression of established breast cancer bone metastases*

Bisphosphonate or vehicle (dosing schedule)	Radiography			Bone mineral density			Histomorphometry				
	No. of mice	mm²/mouse (95% CI)	P	No. of legs with bone metastasis	mg/cm³ (95% CI)	P	No. of legs with bone metastasis	BV/TV, % (95% CI)	P	TB/STV, % (95% CI)	P
Vehicle (daily)	27	9.7 (7.9 to 11.5)	–	11	510 (482 to 538)	–	22	17 (15 to 20)	–	96 (94 to 98)	–
ZOL (daily)	6	3.3 (2.4 to 4.3)	<.001	9	633 (599 to 667)	<.001	12	41 (40 to 43)	<.001	12 (6 to 19)	<.001
ZOL (weekly)	5	4.8 (1 to 8.6)	.04	9	592 (552 to 631)	.006	10	30 (29 to 32)	<.001	10 (5 to 16)	<.001
ZOL (single)	16	4.7 (3.2 to 6.1)	<.001	8	579 (544 to 614)	.001	16	32 (31 to 34)	<.001	81 (76 to 87)	<.001
CLO (daily)	6	3.5 (2.2 to 4.7)	<.001	10	580 (539 to 621)	.009	11	28 (25 to 32)	<.001	45 (31 to 60)	<.001

* Data are mean values from two to three independent experiments. All measurements were made 32 days after tumor cell injection. *P* values (two-sided) are for pairwise comparisons with vehicle-treated control group using the Mann–Whitney *U* test. ZOL = zoledronic acid; CLO = clodronate; CI = confidence interval; BV/TV = bone volume–to–tissue volume ratio; TB/STV = tumor burden–to–soft tissue volume ratio; – = not applicable (referent).

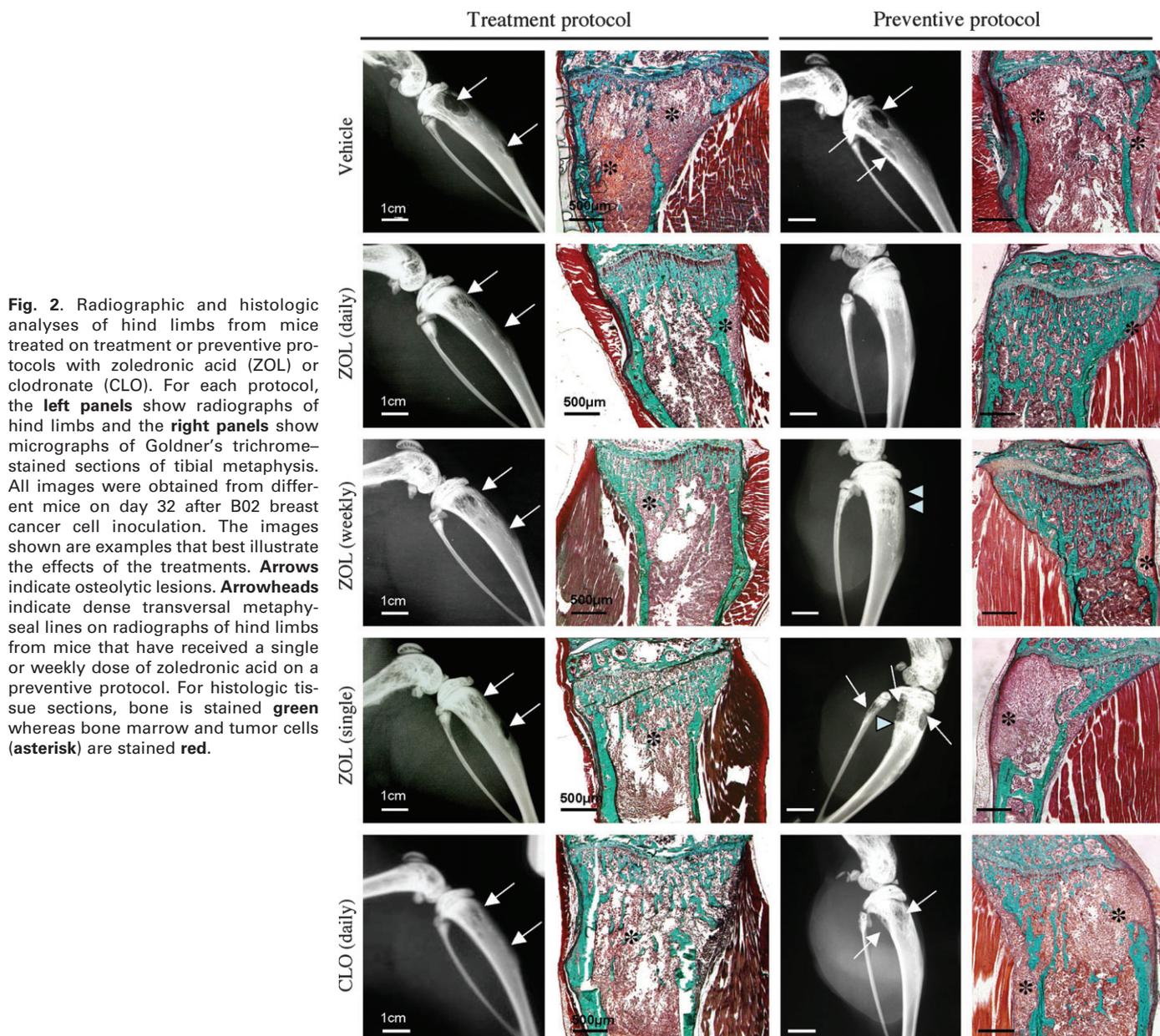Treatment protocol   Preventive protocol

Fig. 2. Radiographic and histologic analyses of hind limbs from mice treated on treatment or preventive protocols with zoledronic acid (ZOL) or clodronate (CLO). For each protocol, the **left panels** show radiographs of hind limbs and the **right panels** show micrographs of Goldner's trichrome–stained sections of tibial metaphysis. All images were obtained from different mice on day 32 after B02 breast cancer cell inoculation. The images shown are examples that best illustrate the effects of the treatments. **Arrows** indicate osteolytic lesions. **Arrowheads** indicate dense transversal metaphyseal lines on radiographs of hind limbs from mice that have received a single or weekly dose of zoledronic acid on a preventive protocol. For histologic tissue sections, bone is stained **green** whereas bone marrow and tumor cells (**asterisk**) are stained **red**.

44%, P<.001) or weekly zoledronic acid–dosing regimens (53% versus 90%, difference = 37%, 95% CI = 19% to 46%, P<.001).

Effects of Zoledronic Acid and Clodronate on the Formation of Breast Cancer Bone Metastases (Preventive Protocols)

We next compared the effects of the different dosing regimens of zoledronic acid and the daily dosing regimen of clodronate on the formation of breast cancer bone metastases by using a preventive protocol in which drug administration was initiated 1 day before tumor cell inoculation (Fig. 1). We found that mice treated 1 day before tumor cell inoculation with a single dose of 100 μg zoledronic acid/kg body weight had osteolytic lesions that were the same size as those of mice treated with vehicle (Table 2 and Fig. 2). The average area of osteolytic lesions was decreased by only 13% (95% CI = −2% to 28%, P = .84) compared with the vehicle. By contrast, mice treated with the daily or weekly regimens of zole-

dronic acid beginning 1 day before tumor cell inoculation had osteolytic lesions that were 88% (95% CI = 81% to 95%) and 80% (95% CI = 71% to 90%), respectively, smaller than those of mice treated with vehicle (P<.001 for both regimens). Moreover, mice treated with the daily or weekly regimens of zoledronic acid had statistically significantly smaller osteolytic lesions than mice treated with a daily regimen of clodronate (mean area of osteolytic lesion for daily zoledronic acid: 1.4 mm²; weekly zoledronic acid: 2.1 mm²; daily clodronate: 5.5 mm²; difference for daily zoledronic acid versus daily clodronate = 4.1 mm², 95% CI = 2.3 to 6 mm², P<.001; difference for weekly zoledronic acid versus daily clodronate = 3.4 mm², 95% CI = 1.2 to 5.6 mm², P = .006) (Table 2 and Fig. 2). Radiographic analysis revealed the presence of dense transverse lines on the tibial metaphysis of mice treated with the weekly and single regimens of zoledronic acid (Fig. 2, arrowheads).

The bone mineral density of hind legs from mice treated with the daily or weekly regimens of zoledronic acid was statistically

Table 2. Effect of different dosing regimens of zoledronic acid and clodronate on the formation of breast cancer bone metastases*

Bisphosphonate or vehicle (dosing schedule)	Radiography			Bone mineral density			Histomorphometry				
	No. of mice	mm²/mouse (95% CI)	P	No. of legs with bone metastasis	mg/cm² (95% CI)	P	No. of legs with bone metastasis	BV/TV, % (95% CI)	P	TB/STV, % (95% CI)	P
Vehicle (daily)	17	11 (9.2 to 13)	–	6	77 (67 to 86)	–	11	12 (8 to 16)	–	59 (46 to 73)	–
ZOL (daily)	8	1.4 (0.6 to 2.1)	.002	8	94 (85 to 104)	.01	11	34 (30 to 37)	.001	10 (1 to 19)	.001
ZOL (weekly)	7	2.1 (1.1 to 3.2)	.004	5	104 (91 to 117)	.008	9	25 (20 to 29)	.004	20 (8 to 31)	.002
ZOL (single)	10	11 (9.7 to 12.4)	.86	3	87 (83 to 91)	.07	6	13 (10 to 16)	.7	60 (41 to 78)	.85
CLO (daily)	9	5.5 (3.7 to 7.3)	.004	7	72 (68 to 76)	.31	12	20 (17 to 22)	.005	30 (24 to 35)	.002

* Data are mean values from two to three independent experiments. All measurements were made 32 days after tumor cell injection. *P* values (two-sided) are for pairwise comparisons with vehicle-treated control group using the Mann–Whitney *U* test. ZOL = zoledronic acid; CLO = clodronate; CI = confidence interval; BV/TV = bone volume–to–tissue volume ratio; TB/STV = tumor burden–to–soft tissue volume ratio; – = not applicable (referent).

significantly greater than that of mice treated with vehicle, with the single dose of zoledronic acid before tumor cell inoculation or with the daily regimen of clodronate (Table 2). Histomorphometric analysis of metastasis-bearing hind limbs revealed that mice treated with the daily or weekly regimens of zoledronic acid had statistically significantly higher BV/TV ratios than mice treated with vehicle, whereas mice treated with the single dosing regimen of zoledronic acid did not (Table 2 and Fig. 2). For example, the daily and weekly regimens of zoledronic acid increased the BV/TV ratio by 180% (95% CI = 152% to 208%) and 103% (95% CI = 65% to 142%), respectively, compared with vehicle (*P*<.001 for both regimens). The BV/TV ratio for mice treated with daily clodronate was 63% higher (95% CI = 43% to 83%, *P* = .006) than that of vehicle-treated mice (Table 2). However, mice treated with the daily or weekly regimens of zoledronic acid had statistically significantly higher BV/TV ratios than mice treated with the daily regimen of clodronate (34% versus 20%, difference = 14%, 95% CI = 10% to 18%, *P*<.001, and 25% versus 20%, difference = 5%, 95% CI = 0.5% to 9%, *P* = .03, respectively). The daily and weekly regimens of zoledronic acid also reduced the TB/STV ratio by 83% (95% CI = 68% to 98%) and 66% (95% CI = 47% to 84%), respectively, compared with vehicle (*P*<.001 for both regimens). Daily clodronate also reduced the skeletal tumor burden by 49% (95% CI = 40% to 57%, *P* = .006) compared with vehicle (Table 2 and Fig. 2). However, mice treated with a daily clodronate regimen had a statistically significantly higher skeletal tumor burden than mice treated with a daily zoledronic acid regimen (30% versus 10%, difference = 20%, 95% CI = 10% to 29%, *P*<.001).

Our assessment of the histology of Goldner's trichrome–stained bones bearing metastatic lesions revealed that the reduction of skeletal tumor growth we observed with either bisphosphonate treatment was not associated with the concomitant invasion of soft tissues (muscle, tendon, or connective tissue) adjacent to bones (Fig. 2).

Effects of Zoledronic Acid and Clodronate on Tumor Cell Homing to Bone Marrow

The measurement of luciferase activity expressed by tumor cells is a highly sensitive and quantitative method to detect the early development of bone metastases (10). Our use of B02 breast cancer cells that expressed a stably transfected gene encoding luciferase (6) allowed us to examine whether mice treated on the preventive protocols with bisphosphonates displayed evidence of luciferase-expressing B02/GFP.2 cells in bone extracts on day 14 after tumor cell inoculation, at which time there was no radiographic evidence of osteolytic lesions (data not shown). We assumed that detection of luciferase activity (expressed in RLUs) was an indication that B02 breast cancer cells were present in the bone marrow. Bone extracts of hind limbs from vehicle-treated mice (n = 6) that had been inoculated with tumor cells had a mean luciferase activity of 147 894 RLUs (95% CI = 85 525 to 210 263 RLUs). By contrast, bone extracts of hind limbs from age-matched control mice (n = 5) that had not been inoculated with tumor cells had a very low (i.e., background) level of luciferase activity (443 RLUs, 95% CI = 238 to 648 RLUs), further indicating that the detection of luciferase activity was inherent to the presence of inoculated tumor cells.

The mean luciferase activities of bone extracts from mice treated with the daily, weekly, or single dosing regimens of zoledronic acid were 86 911 RLUs (95% CI = 71 838 to 101 983 RLUs; n = 4 mice), 55 236 RLUs (95% CI = 41 474 to 68 998 RLUs; n = 4 mice), and 152 692 RLUs (95% CI = 58 714 to 246 669 RLUs; n = 4 mice), respectively. Mice treated with the daily or weekly regimens of zoledronic acid had a statistically significantly lower B02 tumor burden than mice treated with the vehicle (mean luciferase activity for daily zoledronic acid: 86 911 RLUs; weekly zoledronic acid: 55 236 RLUs; vehicle: 147 894 RLUs; difference for daily zoledronic acid versus vehicle = 60 983 RLUs, 95% CI = −9484 to 131 451 RLUs, *P* = .01; difference for weekly zoledronic acid versus vehicle = 92 658 RLUs, 95% CI = 22 278 to 163 038 RLUs, *P* = .01). The mean luciferase activity measured in bone extracts from clodronate-treated mice was 46 693 RLUs (95% CI = 16 124 to 71 261 RLUs; n = 3 mice), which was also statistically significantly lower than that of vehicle-treated mice (difference for clodronate versus vehicle = 104 201 RLUs, 95% CI = 19 633 to 188 769 RLUs, *P* = .02). By contrast, the mean luciferase activity of bone extracts from mice treated with the single dosing regimen of zoledronic acid was not statistically significantly different from that of vehicle-treated animals (152 692 versus 147 894 RLUs, difference = 4797 RLUs, 95% CI = −83 460 to 93 054 RLUs, *P* = .67).

Discussion

There is a growing body of evidence from preclinical research showing that bisphosphonates exhibit antitumor activity, both in vitro and in vivo (2). However, there is much debate about the clinical relevance of these experimental findings because the high

doses of bisphosphonates used in most animal studies are incompatible with the dosing regimens that have been approved for the treatment of cancer patients with skeletal metastases. Given that the skeletal retention of bisphosphonates is related to the rate of bone turnover in breast cancer patients with bone metastases (11) and that bone turnover in rodents is three to five times higher than that in humans (12), it could be argued that a fivefold higher bisphosphonate dose in animal models of bone metastasis should mimic the clinical situation. Yet, most animal studies have used even higher doses of bisphosphonate (13). For example, in many animal models of multiple myeloma and breast and prostate cancers, it has been shown that zoledronic acid at total monthly cumulative doses that range from 30 to 150 mg reduces skeletal tumor growth (13). By contrast, the approved monthly dose of zoledronic acid for the treatment of cancer patients with skeletal metastases is 4 mg infused over 15 minutes; higher doses are not feasible because of concerns about renal toxicity (3). Therefore, we reasoned that a chemotherapeutic approach that emphasized dose density (i.e., the administration of a bisphosphonate over shorter treatment intervals) rather than dose escalation could be an effective way to minimize skeletal tumor burden in this mouse model of metastatic breast cancer, and perhaps, in the clinic.

Our results show that clinically relevant doses of bisphosphonates produced meaningful antitumor effects in an animal model of breast cancer bone metastasis, as long as the bisphosphonate was administered at a low dosage on a daily or weekly dosing schedule. Our results also suggest that, in the clinical setting, bisphosphonate therapy with a long dosing interval could reduce osteolysis by inhibiting bone resorption, whereas therapy with a more frequent dosing interval could also directly affect the growth of tumor cells resident in bone. Our contention that bisphosphonate therapy with a long dosing interval may inhibit cancer-induced bone loss is supported by our finding that mice with established bone metastases that were treated on the treatment protocol with a single clinical dose of zoledronic acid had less bone destruction, but not less skeletal tumor burden, than vehicle-treated animals. Moreover, the radiographic analysis of mice treated on the preventive protocols with weekly zoledronic acid or the single dose of zoledronic acid clearly showed the presence of dense transverse lines on the tibial metaphysis. Similar dense metaphyseal lines were previously noted in children with severe osteogenesis imperfecta after cyclic administration of the bisphosphonate pamidronate, where they were thought to arise after temporary interruption of growth plate cartilage resorption at the time of a pamidronate infusion (14). In agreement with these findings (14), the metaphyseal lines we observed in the bones of mice in our breast cancer metastasis model consisted of trabecular bone that had not been destroyed because a single or weekly bisphosphonate administration had temporarily interrupted osteoclast-mediated bone resorption; when bone growth later resumed, the growth plate moved away from these horizontal trabeculae, which then became visible as dense transverse lines on radiographs. However, we surmise that, concomitant with bone growth, breast cancer cells may have started to stimulate osteoclast-mediated bone resorption, leading to bone destruction. Conversely, a daily treatment with zoledronic acid induced a continuous inhibition of bone resorption, leading to an almost complete inhibition of skeletal tumor burden. A contin-

uous daily treatment with clodronate also reduced skeletal tumor burden, although the extent of inhibition was much less than that observed with zoledronic acid. These results are in accordance with the "vicious cycle" theory (4), in which breast cancer cells stimulate osteoclast-mediated bone resorption and bone-derived growth factors released from resorbed bone stimulate tumor growth. The results also suggest that bisphosphonates (as exemplified here by zoledronic acid and clodronate) inhibit bone resorption, which subsequently deprives breast cancer cells of bone-derived growth factors that are required for tumor cell proliferation.

Aside from our observation that bisphosphonates may render the bone a less favorable microenvironment for tumor growth by reducing osteoclast-mediated bone resorption, we used a continuous or frequent intermittent low-dose therapy with zoledronic acid to investigate whether bisphosphonates also have the potential to exert a direct antitumor effect in vivo. We found that although the different dosing regimens of zoledronic had similar inhibitory effects on bone resorption in the treatment protocols, only a daily or weekly regimen of zoledronic acid reduced skeletal tumor burden. If bisphosphonate treatment decreased skeletal tumor burden solely by reducing bone loss, we would have expected the single-dose regimen of zoledronic acid to have inhibited skeletal tumor growth more than what we observed. It is interesting that Gao et al. (15) recently reported that a preventive treatment regimen with zoledronic acid (used at dosage of 30 μg/kg/wk, which was similar to the 20 μg/kg/wk dosage that we used) inhibited not only the formation of bone tumors in transgenic mice that spontaneously develop leukemia and osteolytic lesions but also the formation of soft tissue tumors. These findings (15) suggest that a frequent zoledronic acid dosage may directly inhibit tumor growth. Indeed, we showed that a daily or weekly preventive regimen of zoledronic acid or a daily preventive regimen of clodronate interfered with the homing of luciferase-expressing B02 breast cancer cells to bone in vivo. van der Pluijm et al. (10) also recently reported that preventive treatment of nude mice with the bisphosphonate olpadronate (given in a daily clinical dosing regimen at 23 μg/kg) inhibited the early development of luciferase-expressing MDA-MB-231 breast cancer cells (MDA-231-B/luc[+]) in bone. However, in this animal model, the inhibitory effect of olpadronate on skeletal tumor growth was transient, and tumor growth eventually resumed (although there was a substantial reduction in osteolysis) (10). These results may be explained by the extramedullary growth of MDA-231-B/luc^{+} cells that was observed in the surrounding soft tissues (10), which may have masked the inhibitory effect of olpadronate on skeletal tumor burden. A similar masking of the inhibitory effect of the bisphosphonate ibandronate on skeletal tumor burden has been observed in 5TGM1 and ARH-77 murine models of myeloma, in which tumor growth is not confined to bone (2). Conversely, we found that when tumor growth is restricted to bone, as in our breast cancer model, a continuous dosing regimen of zoledronic acid or clodronate or a frequent intermittent dosing regimen of zoledronic acid reduced both the progression of osteolysis and the homing of tumor cells to bone.

Our results are reminiscent of those obtained in a randomized trial of dose-dense versus conventionally scheduled chemotherapy in the adjuvant treatment of node-positive breast cancer patients, in which dose density was found to be more effective than dose

escalation for reducing residual tumor burden (16). It is therefore possible that continuous or frequent intermittent low-dose therapy with zoledronic acid or clodronate allows prolonged exposure of the bone marrow to bisphosphonates, thus enabling a direct effect on tumor cells that reside in bone. The homing of tumor cells in bone likely involves early metastatic processes such as tumor cell invasion (4). In vitro, bisphosphonates inhibit breast cancer cell invasion (2). However, to our knowledge, there is no in vivo evidence that bisphosphonates directly inhibit tumor cell invasion. A daily or weekly preventive regimen of zoledronic acid or a daily preventive regimen of clodronate could therefore inhibit the homing of metastatic cells to bone by reducing tumor cell invasion. Additional mechanisms, such as the inhibition of tumor cell extravasation and proliferation within the bone microenvironment, may also be involved in reducing the homing of tumor cells to bone. For example, it was recently shown that the activation of bone turnover in athymic mice favored prostate cancer localization and growth in the skeleton, whereas zoledronic acid treatment reduced the development of these skeletal lesions (17). A continuous or frequent intermittent low-dose preventive therapy with zoledronic acid or clodronate could also reduce bone turnover in our animal model, thereby inhibiting the homing of B02 breast cancer cells in bone. Such potential inhibitory mechanisms using continuous or frequent intermittent bisphosphonate therapy merit a fuller investigation.

The main limitation of our study is that the bone metastases were induced by injecting human breast cancer cells into the systemic circulation in immunocompromised mice. Such an approach does not recapitulate all the steps that are required for tumor cells to spread from the primary tumor to distant organs and does not take the role of the immune system into account. An animal model that more likely reflects the clinical reality does exist, in which mouse 4T1 breast cancer cells implanted in the mammary gland spontaneously metastasize to bone, lungs, and liver in immunocompetent syngeneic mice (18). In that model, Hiraga et al. (18) found that zoledronic acid (used at a 60-mg total monthly cumulative dose) reduced 4T1 metastasis to visceral organs and bones. It would be interesting to examine the effect of a continuous or frequent intermittent low-dose preventive bisphosphonate therapy on spontaneous metastasis in this animal model.

Our results provide some support for an adjuvant role of bisphosphonates in breast cancer. In this respect, it was recently reported that the addition of oral clodronate (1600 mg daily for 2 years) to standard treatment for primary operable breast cancer statistically significantly reduced the risk of bone metastases by 31% over a 5-year period (19). Two larger phase III trials of bisphosphonates as adjuvant therapy for primary breast cancer, each enrolling more than 3000 patients with early-stage breast cancer, are underway. The National Surgical Adjuvant Breast Project B-34 trial will determine whether oral clodronate (1600 mg daily for 3 years) administered alone or in combination with chemotherapy and/or hormonal therapy reduces the incidence of skeletal and nonskeletal metastases or improves overall or relapse-free survival. The Southwest Oncology Group 0307 trial will compare zoledronic acid (4 mg administered intravenously every 4 weeks for 6 months, then every 3 months for 2.5 years), clodronate (1600 mg taken orally daily for 3 years), and the bisphosonate ibandronate (50 mg taken orally daily for 3 years) as adjuvant therapy for primary breast cancer. The first results from these clinical trials are expected to be available in 2008. On the basis of our preclinical results reported here and those obtained for clodronate and for dose-dense chemotherapy in clinical trials (16,19), we anticipate that the use of dose-dense bisphosphonate therapy as adjuvant treatment of primary breast cancer will decrease the risk of bone metastases.

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Notes