A Cathepsin K Inhibitor Reduces Breast Cancer–Induced Osteolysis and Skeletal Tumor Burden

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Abstract

Osteoclasts mediate bone destruction in breast cancer skeletal metastases. Cathepsin K is a proteinase that is secreted by osteoclasts and degrades bone. Here, immunohistochemistry revealed that cathepsin K was expressed not only by osteoclasts but also by breast cancer cells that metastasize to bone. Following intratibial injection with cathepsin K–expressing human BT474 breast cancer cells, tumor-bearing mice treated with a clinical dosing regimen of cathepsin K inhibitor (CKI; 50 mg/kg, twice daily) had osteolytic lesions that were 79% smaller than those of tumor-bearing mice treated with the vehicle. The effect of CKI was also studied in a mouse model in which the i.v. inoculation of human B02 breast cancer cells expressing cathepsin K leads to bone metastasis formation. Drug administration was started before (preventive protocol) or after (treatment protocol) the occurrence of osteolytic lesions. In treatment protocols, CKI (50 mg/kg, twice daily) or a single clinical dose of 100 μg/kg zoledronic acid (osteoclast inhibitor) reduced the progression of osteolytic lesions by 59% to 66%. CKI therapy also reduced skeletal tumor burden by 62% compared with vehicle, whereas zoledronic acid did not decrease the tumor burden. The efficacy of CKI at inhibiting skeletal tumor burden was similar in the treatment and preventive protocols. By contrast, CKI did not block the growth of s.c. B02 tumor xenografts in animals. Thus, CKI may render the bone a less favorable microenvironment for tumor growth by inhibiting bone resorption. These findings raise the possibility that cathepsin K could be a therapeutic target for the treatment of bone metastases. [Cancer Res 2007;67(20):9894–902]

Introduction

Bone metastases are common complications of breast cancer (1, 2). They can be fatal or may rapidly impede the quality of life (1, 2). Bone-residing breast cancer cells do not directly destroy bone (2). Instead, they secrete molecules like parathyroid hormone–related protein, interleukins (IL-6, IL-8, and IL-11), and prostaglandins that stimulate osteoclast activity, leading to osteolysis (2–6).

Osteoclasts are large multinucleated cells formed by fusion of mononuclear monocyte-macrophage progenitors whose differentiation is principally regulated by receptor activator of nuclear factor-κB ligand and macrophage colony-stimulating factor (7). Osteoclasts are the bone resorptive cells. They resorb bone by secreting protons and a lysosomal cysteine proteinase, cathepsin K (7). Protons dissolve bone mineral and provide an acidic microenvironment enabling cathepsin K to degrade the demineralized collagenous matrix (7). The most compelling evidence that cathepsin K is involved in osteoclast-mediated bone resorption comes from the demonstration that cathepsin K–knockout mice display an osteopetrotic phenotype associated with a severe impairment of the resorptive activity of osteoclasts (8).

The observation that osteoclasts play a pivotal role in the formation of osteolytic lesions has provided the rationale for using bisphosphonates as potent inhibitors of osteoclast-mediated bone resorption in the treatment of breast cancer patients with bone metastases (1). Bisphosphonates bind avidly to bone mineral, and those containing a nitrogen moiety in their structure target osteoclast farnesyl diphosphate synthase, a key enzyme in the mevalonate pathway (9, 10). The inhibition of farnesyl diphosphate synthase by nitrogen-containing bisphosphonates prevents the prenylation of small GTPases that are essential for osteoclast activity and survival, thereby causing a reduction of bone resorption (1). Yet, in the clinic, bisphosphonate treatment is only palliative and does not provide a life-prolonging benefit to the majority of patients with advanced cancer (1). New therapeutics targeting osteoclast activity are therefore required. They could be used in combination with bisphosphonates to more efficiently treat breast cancer patients with bone metastases.

This need could be fulfilled by cathepsin K inhibitors (CKI). They have successfully been used in the treatment of bone loss associated with osteoporosis (11). In addition, cathepsin K is expressed by human breast cancer cells in skeletal metastases (12), suggesting that CKIs could target not only osteoclasts but also tumor cells. Here, we present experimental evidence that a CKI, alone or in combination with nitrogen-containing bisphosphonate zoledronic acid, reduces breast cancer–induced osteolysis and skeletal tumor burden in animals.

Materials and Methods

Drugs and reagents. CKIs and zoledronic acid [1-hydroxy-2-(1*H*-imidazole-1-yl)ethylidene-bisphosphonic acid, used as a disodium salt] were obtained from Novartis Pharma AG. The structure of CKI has previously been reported (11, 13). CKI was dissolved in 65% (v/v) cremophor EL and 35% (v/v) absolute ethanol, and this solution was used as the vehicle for *in vitro* and *in vivo* experiments. Zoledronic acid was dissolved in PBS.

Breast cancer cell lines. Human BT474 breast carcinoma cells were obtained from the American Type Culture Collection. Human B02 breast carcinoma cells are a subpopulation of the MDA-MB-231 breast cancer cell

Note: C. Le Gall and A. Bellahcène contributed equally to this work.

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line specifically selected for its high efficiency to metastasize to bone after i.v. inoculation (14). A mouse mammary carcinoma cell line (CCL-51) stably transfected to express cathepsin K has been provided by Novartis and was used here as a positive control for Western immunoblotting experiments. Characteristics of the BT474, B02, and CCL-51 breast cancer cell lines have been described elsewhere (13–15). BT474, MDA-MB-231, B02, and CCL-51 cells are routinely cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO_2 incubator.

Reverse transcription and PCR. Total RNA from BT474, B02, and MDA-MB-231 cells was extracted using Total RNA Isolation System (Promega). cDNA was synthesized using Moloney murine virus-1 reverse transcriptase (Promega). A 399-bp fragment of cathepsin K was amplified using 5′-CAGCAAAGGTGTGTATTATGATGAAAGC as the forward primer and 5′-ATGGGTGGAGAGAAGCAAAGTAGGAAGG as the reverse primer. PCR reactions were run using a program consisting of 1 cycle at 94°C for 5 min, and then 40 cycles of 94°C for 30 s, 54°C for 15 s, and 72°C for 30 s.

Breast cancer tissue specimens. The autopsy files of the Department of Pathology (Pr. J. Boniver, Centre Hospitalier Universitaire of Liège, Belgium) were searched for diagnosis of disseminated breast cancer with histologically proven bone metastases during the period of 1991 to 1998. Slides were retrieved and clinical history was obtained. Two breast cancer patients deceased of disseminated disease, including bone metastases, were selected for this study (case A: primary breast carcinoma with bone, liver, and lung metastatic lesions; case B: primary breast carcinoma with bone, liver, and renal metastatic lesions). Twelve additional specimens of bone metastasis were also randomly selected for the present study. Soft tissue metastases were fixed in formalin, dehydrated, and paraffin embedded. Formalin-fixed bone specimens were decalcified with a solution of EDTA and hydrochloric acid (Decalcifier II, Surgipath Europe Ltd.) or with a solution of formalin (20%) containing 5% (v/v) nitric acid. Paraffin-embedded tissue blocks were sectioned at 5 μm. Slides were then processed for immunostaining.

Antibodies and immunodetection of cathepsin K. The presence of cathepsin K in human breast cancer tissue specimens, experimental bone metastases, and s.c. tumor xenografts was immunodetected with a rabbit polyclonal antibody (Biovision) followed by immunoperoxidase staining (ABC Vectastain Elite kit, Vector Laboratories, Inc.) according to the supplier's instructions. Tissue sections were first deparaffinized in xylene and rehydrated in graded ethanols. Blocking of the endogenous peroxidase activity was carried out by immersing the slides in 1.2% hydrogen peroxide (H_2O_2) in methanol for 30 min. For antigen retrieval, the slides were heated at 95°C during 40 min in 0.01 mol/L citrate buffer (pH 6.0), then incubated with normal goat serum (1:20) to block the nonspecific serum-binding sites. The anti–cathepsin K antibody (1:1,000 in PBS) was added and incubated overnight at 4°C in a humidified chamber. After washing in PBS, slides were incubated for 30 min with a biotinylated goat anti-rabbit antibody (1:200), washed again with PBS, then incubated with the avidin-biotin-peroxidase complex. Peroxidase activity was developed by addition of a solution of 3,3′-diaminobenzidine tetrahydrochloride (Vel) and 0.03% H_2O_2 in PBS. Slides were then counterstained with Carazzi's hematoxylin and washed in distilled water. After dehydration in graded alcohols, the slides were coverslipped. Negative control experiments included omission of the first antibody and preincubation of slides with a 100 molar excess of cathepsin K before antibody use.

The immunodetection of cathepsin K in BT474, B02, MDA-MB-231, and CCL-51 whole protein extracts was done by Western blotting with an anti–cathepsin K mouse monoclonal antibody (Novocastra) diluted 1:500 in TBS containing 0.05% (v/v) Tween 20. The remainder of the experimental procedure was as previously described (14).

Scoring of cathepsin K immunostaining in human breast cancer tissue specimens. The immunostaining intensity was evaluated independently by two investigators (A.B. and V.C.). The intensity of the staining was scored arbitrarily as follows: negative (−), weak (1+), moderate (2+), and strong (3+). In case of disagreement between examiners, slides were reviewed and a consensus opinion was obtained.

***In vitro* tumor cell invasion assay.** This assay was done essentially as previously described (6, 14, 15). Tumor cell invasion experiments were conducted using Bio-Coat cell chambers (Becton Dickinson) consisting of 24-well companion plates with cell culture inserts containing 8-μm pore size filters coated with Matrigel (37 μg/filter). Untreated or CKI-treated B02 cells (0.25×10^5/500 μL) were seeded in culture inserts (upper chamber) and the chemoattractant (10% FBS) was placed in each well of a companion plate (lower chamber). After a 24-h incubation at 37°C in a 5% CO_2 incubator, noninvading cells were removed and the invading cells on the under surface of the filter were fixed and stained. Membranes were mounted on glass slides and the cells from 10 random microscopic fields (×400 magnification) were counted. All experiments were run in duplicate, and invasion was expressed as the number of invading cells per square millimeter.

Animals. All procedures involving animals including housing and care, method of euthanasia, and experimental protocols were conducted in accordance with a code of practice established by the local ethical committee (CREEA, Lyon, France). Studies were routinely inspected by the attending veterinarian to ensure continued compliance with the proposed protocols. Four- or 10-week-old female BALB/c homozygous (*nu/nu*) athymic mice were obtained from Charles River.

Animal model of intraosseous tumorigenesis. For intraosseous tumor xenograft experiments in anesthesized 10-week-old nude mice, a small hole was drilled with a 30-gauge sterile needle through the left tibial plateau with the knee flexed. Using a new sterile needle fitted to a 50-μL sterile Hamilton syringe (Hamilton Co.), a single-cell suspension (5×10^5 in 30-μL PBS) of BT474 cells was carefully injected in the bone marrow cavity. Fourteen days after tumor cell inoculation, CKI (50 mg/kg, twice daily) or the vehicle was given to animals by i.p. injection during 28 days. On day 42 after tumor cell inoculation, radiographs of anesthetized mice were taken with the use of MIN-R2000 films (Kodak) in an MX-20 cabinet X-ray system (Faxitron X-ray Corp.). The osteolytic lesion area on radiographs was measured using a Visiolab 2000 computerized image analysis system (Explora Nova) and the extent of bone destruction per animal was expressed in square millimeters, as previously described (6, 14, 15). Anesthetized mice were killed by cervical dislocation after radiography on day 42.

Animal model of bone metastasis. The bone metastasis experiments in mice were conducted as previously described (6, 14–16). B02 cells (5×10^5 in 100-μL PBS) were inoculated into the tail vein of anesthetized nude mice. In this model, animals usually develop bone metastases 18 days after tumor cell inoculation, as judged by radiography (6, 14–16). Radiographs of animals were taken 18, 25, and 32 days after tumor cell inoculation, as described above. At sacrifice (day 32), metastatic hind limbs were collected for peripheral quantitative computed tomography (CT), histology, and histomorphometric analysis.

For treatment protocols, mice were first analyzed by radiography on day 18, and tumor-bearing mice were distributed among the different treatment groups to balance these groups for the extent of bone destruction at baseline. Metastatic animals were then treated with CKI, alone or in combination with zoledronic acid. For the preventive protocols, CKI treatment of animals began on the day of tumor cell inoculation (day 0). CKI (50 mg/kg) or the vehicle was given twice daily by i.p. injection from day 18 to day 32 (treatment protocols) or from day 0 to day 32 (preventive protocols). A single dose (100 μg/kg) of zoledronic acid was given to mice by s.c. injection on day 18 (treatment protocol only). This bisphosphonate dose is equivalent to the clinical dose of 4 mg every 3 to 4 weeks, after adjustment for the different molecular weights of the commercial clinical formulation (290.1, free acid monohydrate) and the research-grade compound (401.6, disodium salt, 4.75 hydrate) used in this study.

Animal model of s.c. tumorigenesis. B02 breast cancer cells (10^6) were inoculated s.c. (200 μL) into the right flank of nude mice, as previously described (6). Twenty-one days after tumor cell inoculation, when tumor xenografts became palpable, mice were randomized into two groups: one group received CKI (50 mg/kg, twice daily), administered i.p., whereas the other group received the vehicle only. Tumor size was assessed by external measurement of the length (L) and width (W) of tumor xenografts. Tumor

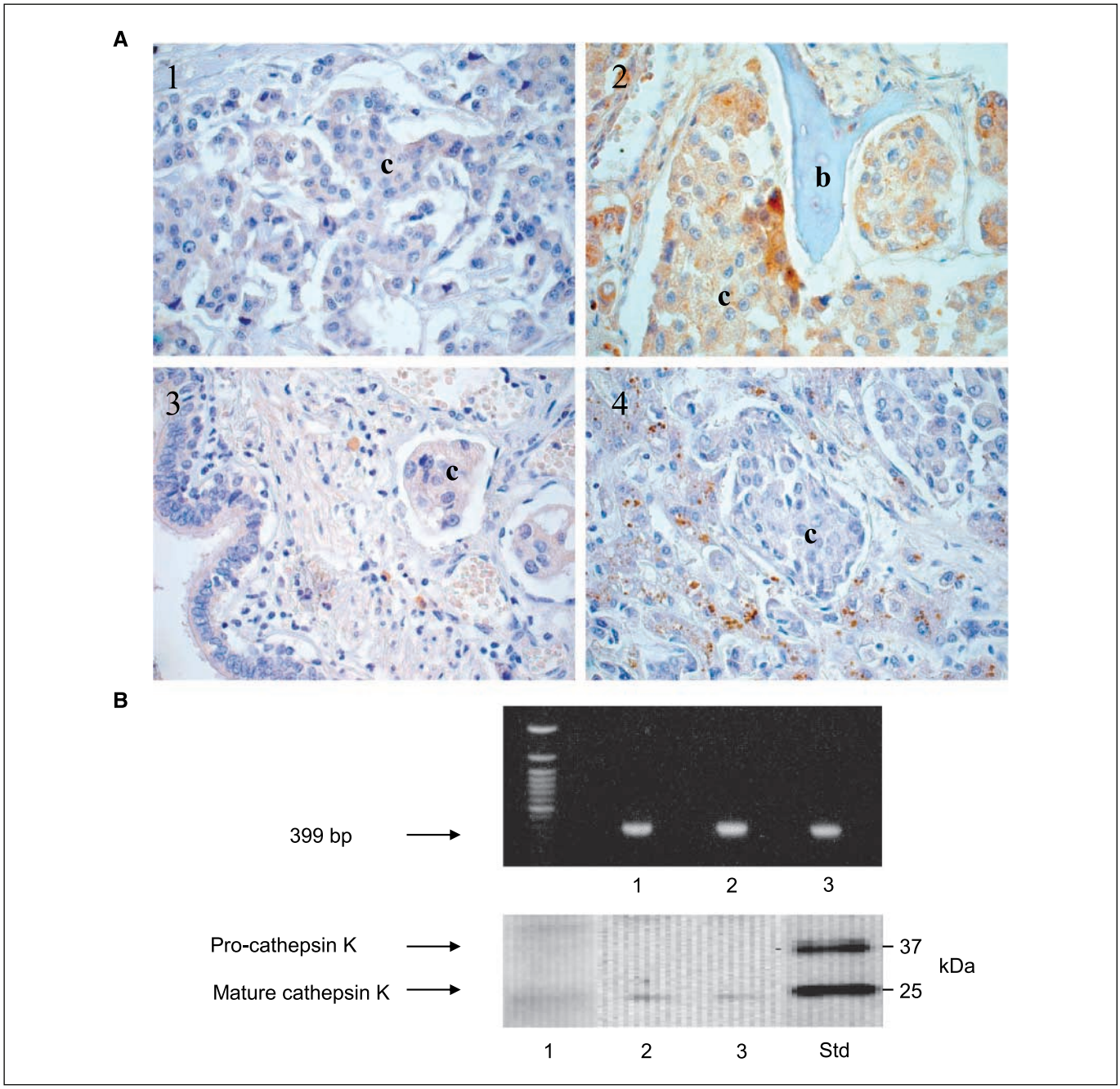

Figure 1. *A,* immunostaining of cathepsin K in cancer cells of a primary breast tumor and matching metastatic lesions from the same patient (case A). *1,* primary breast tumor. *2,* bone metastasis. *3,* lung metastasis. *4,* liver metastasis. In the skeletal metastasis, breast cancer cells (*c*) adjacent to bone (*b*) strongly expressed cathepsin K when compared with that observed in the primary tumor and matching lung and liver metastases. Original magnification, ×400. *B, top,* cathepsin K mRNA expression in human BT474, B02, and MDA-MB-231 breast cancer cells (*lanes 1–3*, respectively). Reverse transcription-PCR fragments were separated on a 2% agarose gel and stained with ethidium bromide. *Bottom,* immunodetection by Western blotting of cathepsin K in whole protein extracts from human BT474, B02, and MDA-MB-231 breast cancer cells (*lanes 1–3*, respectively) and murine CCL-51 mammary carcinoma cells stably transfected to overexpress cathepsin K (*Std*).

volume (TV; expressed in cubic millimeters) was calculated by using the following equation: $TV = (L \times W^2) / 2$.

Peripheral quantitative CT. Three-dimensional reconstruction of metastatic tibiae were done with an XCT Research SA+ scanner (Stratec Medizintechnik) fitted with a 0.5-mm collimator, as previously described (16).

Bone histology, histomorphometry, and immunohistochemistry. Hind limbs from animals were fixed and embedded in methylmethacrylate. Sections (7 μm) of undecalcified long bones were then cut with a microtome (Polycut E, Reichert-Jung) and stained with Goldner's trichrome. Histologic and histomorphometrical analyses were done on Goldner's trichrome–stained longitudinal medial sections of tibial metaphysis with the use of a Visiolab 2000 computerized image analysis system, as previously described (6, 14–16). Histomorphometric measurements [i.e., bone volume to tissue volume (BV/TV) and tumor burden to soft tissue volume (TB/STV) ratios] were done in a standard zone of the tibial metaphysis, situated at 0.5 mm from the growth plate, including cortical and trabecular bones.

Statistical analysis. Data were analyzed with the StatView 5.0 software using ANOVA followed by a Fisher's protected least significant difference (PLSD) test. $P < 0.05$ was considered statistically significant.

Results

Cathepsin K expression in human breast cancer bone metastases and breast cancer cell lines. As a first step toward studying the role of cathepsin K in breast cancer bone metastases, we did immunohistochemistry for cathepsin K expression in primary breast tumors, metastatic lesions, and breast cancer cell lines. Primary breast tumors and their matching metastatic lesions from two patients were initially studied (see cases A and B in Materials and Methods). As illustrated in Fig. 1*A* (case A), cathepsin K was strongly expressed in breast cancer cells of a bone metastasis (*image 2*) as opposed to that observed in the primary tumor (*image 1*) and matching lung and liver metastases (*images 3* and *4*, respectively). Similar results were obtained with case B (data not shown). This observation led us to extend our study and investigate the expression of cathepsin K in bone metastatic specimens from 12 patients with advanced breast cancer. The scoring of the cathepsin K staining intensity in these bone metastatic specimens showed a moderate to strong staining in tumor cells from 11 out of 12 bone metastases, whereas a weak staining of tumor cells was observed in 1 bone metastatic specimen. For all of the bone metastatic specimens studied, the intensity of the cathepsin K staining in osteoclasts was stronger than that observed in tumor cells. Moreover, human breast cancer cell lines (BT474, B02, and MDA-MB-231) also expressed cathepsin K, as judged by both PCR and Western immunoblotting (Fig. 1*B*). Because of the expression of cathepsin K in breast cancer cells and osteoclasts, we next examined whether a CKI could inhibit breast cancer–induced bone destruction in animals.

Effect of a CKI on bone destruction in an animal model of intraosseous tumorigenesis. We used an experimental model in which immunocompromised mice display radiographic evidence of osteolytic lesions 14 days after intratibial inoculation of human BT474 breast cancer cells (13). Tumor-bearing mice were treated with CKI (50 mg/kg, twice daily) or the vehicle from day 14 to day 42 after tumor cell inoculation. This dosing regimen of CKI was calculated on the basis of the daily 50-mg clinical dose of CKI that is given in phase II trials for the treatment of osteoporosis (11).

Radiographic analysis on day 42 revealed that tumor-bearing animals treated with CKI had osteolytic lesions (mean ± SD, 0.43 ± 0.4 mm^2/mouse; n = 8 mice) that were 79% smaller [95% confidence interval (95% CI), 62–95%; P = 0.016] than those of tumor-bearing mice treated with the vehicle (mean ± SD, 2 ± 1.4 mm^2/mouse; n = 8 mice; Fig. 2). The inhibitory effect of CKI on cancer-induced bone destruction was confirmed by three-dimensional micro-CT reconstruction of metastatic tibiae (Fig. 2).

To further assess the therapeutic efficacy of CKI on bone destruction, experiments were next conducted in a mouse model of bone metastasis caused by human B02 breast cancer cells, in which CKI treatment started before (preventive protocol) or after (treatment protocol) immunocompromised animals develop osteolytic lesions.

Effect of a CKI on the progression of established breast cancer bone metastases (treatment protocol). We used a mouse model of breast cancer bone metastasis in which animals display radiographic evidence of osteolytic lesions in hind limbs 18 days after i.v. inoculation of B02 tumor cells (6, 14–16). The effect of CKI (50 mg/kg, i.p., twice daily) was studied on the progression of established osteolytic lesions by using a treatment protocol in which drug administration to tumor-bearing mice was initiated on day 18 after tumor cell injection. We then compared the effect of this CKI dosing regimen with that of a single dose of bisphosphonate zoledronic acid (100 μg/kg of body weight) administered s.c. to animals on day 18 after tumor cell injection. The effect of CKI in combination with zoledronic acid was also examined. The dosage of 100 μg/kg zoledronic acid was calculated to be equivalent to the 4-mg clinical i.v. dose given to breast cancer patients with bone metastases (see Materials and Methods for further details on the dose).

Radiographic analysis on day 32 after tumor cell injection revealed that tumor-bearing animals treated with CKI had osteolytic lesions that were 66% (95% CI, 53–79%; P < 0.001) smaller than those of tumor-bearing mice treated with the vehicle (Table 1; Fig. 3*A*). Similarly, tumor-bearing animals that were treated with zoledronic acid had osteolytic lesions that were 59% (95% CI, 48–69%; P < 0.001) smaller than those of vehicle-treated animals (Table 1; Fig. 3*A*). The inhibitory effect of zoledronic acid on progression of osteolytic lesions did not differ statistically significantly from that observed with CKI. In addition, the use of CKI in combination with zoledronic acid did not further reduce the progression of osteolytic lesions in metastatic animals (Table 1; Fig. 3*A*). These results were confirmed by three-dimensional micro-CT reconstruction of metastatic hind limbs (Fig. 3*A*).

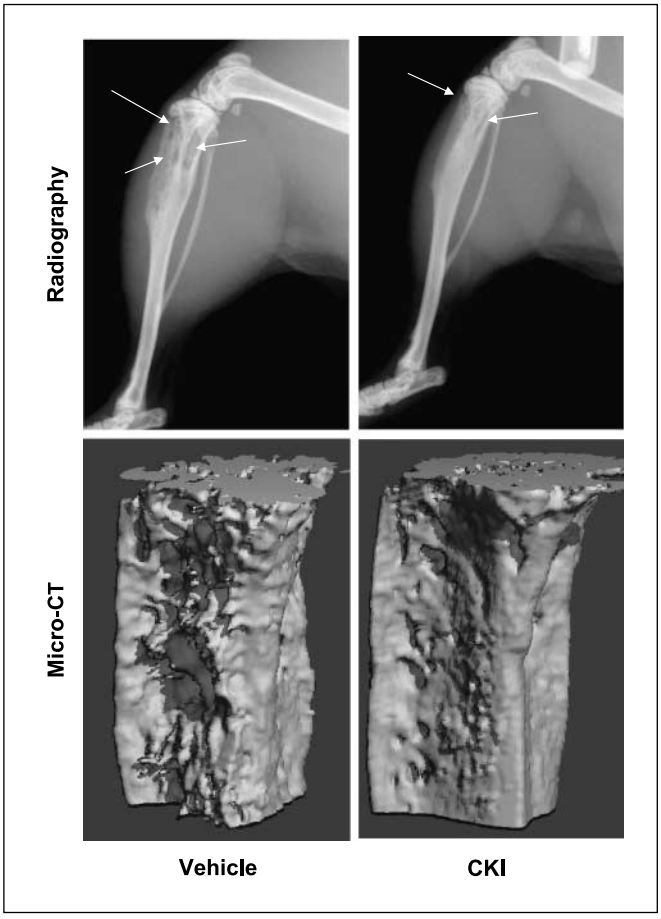

Figure 2. Effect of a CKI on experimental bone metastasis of human BT474 breast cancer. BT474 cells were injected in the tibial bone marrow cavity. Fourteen days after tumor cell inoculation, the CKI (50 mg/kg, twice daily) or the vehicle was administered to animals by i.p. injection during 28 d. *Top* and *bottom,* representative radiographs and three-dimensional micro-CT reconstruction of hind limbs from mice 42 d after tumor cell inoculation, respectively. *Top, arrows,* osteolytic lesions.

Table 1. Effect of a CKI, alone or in combination with zoledronic acid, on the progression of established breast cancer bone metastases

Treatment*	Radiography (mm^2/mouse)	Histomorphometry †	
		BV/TV (%)	TB/STV (%)
Vehicle	10.4 ± 3.9 (n = 34)	11.8 ± 5.1 (n = 34)	81.7 ± 20 (n = 33)
CKI	3.6 ± 3.3 (n = 23) ‡	18.8 ± 8 (n = 18) ‡	32.3 ± 37.7 (n = 18) ‡
Zoledronic acid (single)	4.3 ± 2.2 (n = 18) ‡	26.6 ± 7.8 (n = 14) ‡	81.4 ± 11.6 (n = 14)
CKI + zoledronic acid (single)	3.5 ± 2.4 (n = 14) ‡	22.4 ± 6.8 (n = 10) ‡	27.1 ± 38.7 (n = 10) ‡

*Drug administration was initiated after bone metastases had formed (i.e., 18 d after tumor cell injection). All measurements were made 32 d after tumor cell injection. Results are the mean ± SD of three to five separate experiments. CKI, 50 mg/kg, twice daily; zoledronic acid, single dose of 100 μg/kg administered on day 18.

†For histomorphometry, n is the number of legs with bone metastasis.

‡P < 0.001, compared with the vehicle-treated group by ANOVA followed by a Fisher's PLSD test.

Histomorphometric analysis of hind limbs with metastases showed that mice treated with CKI, zoledronic acid, or the combination of CKI with zoledronic acid had statistically significantly higher BV/TV ratios (indicating a prevention of bone loss) than vehicle-treated animals (Table 1). For instance, treatments with CKI, zoledronic acid, and CKI + zoledronic acid increased the BV/TV by 59% (95% CI, 44–96%), 125% (95% CI, 91–163%), and 102% (95% CI, 69–135%), respectively, compared with vehicle (P < 0.001 for all three regimens). Zoledronic acid (alone or in combination with CKI) was statistically significantly more effective (P = 0.001 and P = 0.05, respectively) than CKI alone in increasing the BV/TV ratio (Table 1). This higher BV/TV ratio was due mainly to the increased trabecular bone area that can be viewed on Goldner's trichrome–stained tissue sections of bones from animals treated with zoledronic acid, alone or in combination with CKI (Fig. 3*A*).

CKI, alone or in combination with zoledronic acid, statistically significantly decreased the TB/STV ratio (a measure of the skeletal tumor burden) by 60% (95% CI, 40–84%; P < 0.001) and 74% (95% CI, 44–98%; P < 0.001), respectively, compared with vehicle (Table 1; Fig. 3*A*). The TB/STV ratio for CKI alone did not differ statistically significantly from that obtained with CKI in combination with zoledronic acid (Table 1; Fig. 3*A*). By contrast, a single dose of zoledronic acid decreased the TB/STV ratio by only 0.5% (95% CI, −0.7–11%; Table 1; Fig. 3*A*).

CKI-treated animals did not have a loss of the body weight at the end of the treatment protocol when compared with vehicle-treated animals [19 ± 1.5 g (n = 9) and 18.9 ± 1.4 g (n = 8), respectively].

Effect of a CKI on the formation of experimental breast cancer bone metastases (preventive protocol). We next studied the effect of this dosing regimen of CKI (50 mg/kg, twice daily) on the formation of bone metastases by using a preventive protocol in which drug administration was initiated at the time of B02 breast cancer cell inoculation. Zoledronic acid is only approved for the treatment of patients with advanced cancer (1) and is not yet registered for use in the preventive setting.

Radiographic monitoring of the formation of osteolytic lesions in animals 18, 25, and 32 days after tumor cell inoculation indicated that CKI substantially reduced bone metastasis formation as early as day 25 (Fig. 3*B*). In the treatment protocol, CKI did not reduce the formation of osteolytic lesions on day 25 (data not shown). Yet, on day 32, the extent of inhibition of bone destruction in the treatment protocol [66% inhibition (95% CI, 53–79%)] was similar to that observed in the preventive protocol [61% inhibition (95% CI, 39–84%); Tables 1 and 2].

Histomorphometric analysis of metastatic hind limbs revealed that mice treated with CKI had a BV/TV ratio increased by 155% (95% CI, 81–237%; P < 0.001) compared with vehicle (Table 2). A preventive dosing regimen of CKI was more effective than a treatment dosing regimen in increasing the BV/TV ratio (Tables 1

Table 2. Effect of a CKI on the formation of breast cancer bone metastases

Treatment*	Radiography (mm^2/mouse)	Histomorphometry †	
		BV/TV (%)	TB/STV (%)
Vehicle	7.7 ± 2.2 (n = 14)	8.4 ± 2.7 (n = 11)	82.8 ± 18.3 (n = 11)
CKI	3 ± 2.9 (n = 13) ‡	21.4 ± 9.2 (n = 9) ‡	39.1 ± 43 (n = 9) §

*Drug administration was initiated at the time of tumor cell injection. All measurements were made 32 d after tumor cell injection. Results are the mean ± SD of two separate experiments. CKI, 50 mg/kg, twice daily.

†For histomorphometry, n is the number of legs with bone metastasis.

‡P = 0.007, compared with the vehicle-treated group by ANOVA followed by a Fisher's PLSD test.

§P < 0.001, compared with the vehicle-treated group by ANOVA followed by a Fisher's PLSD test.

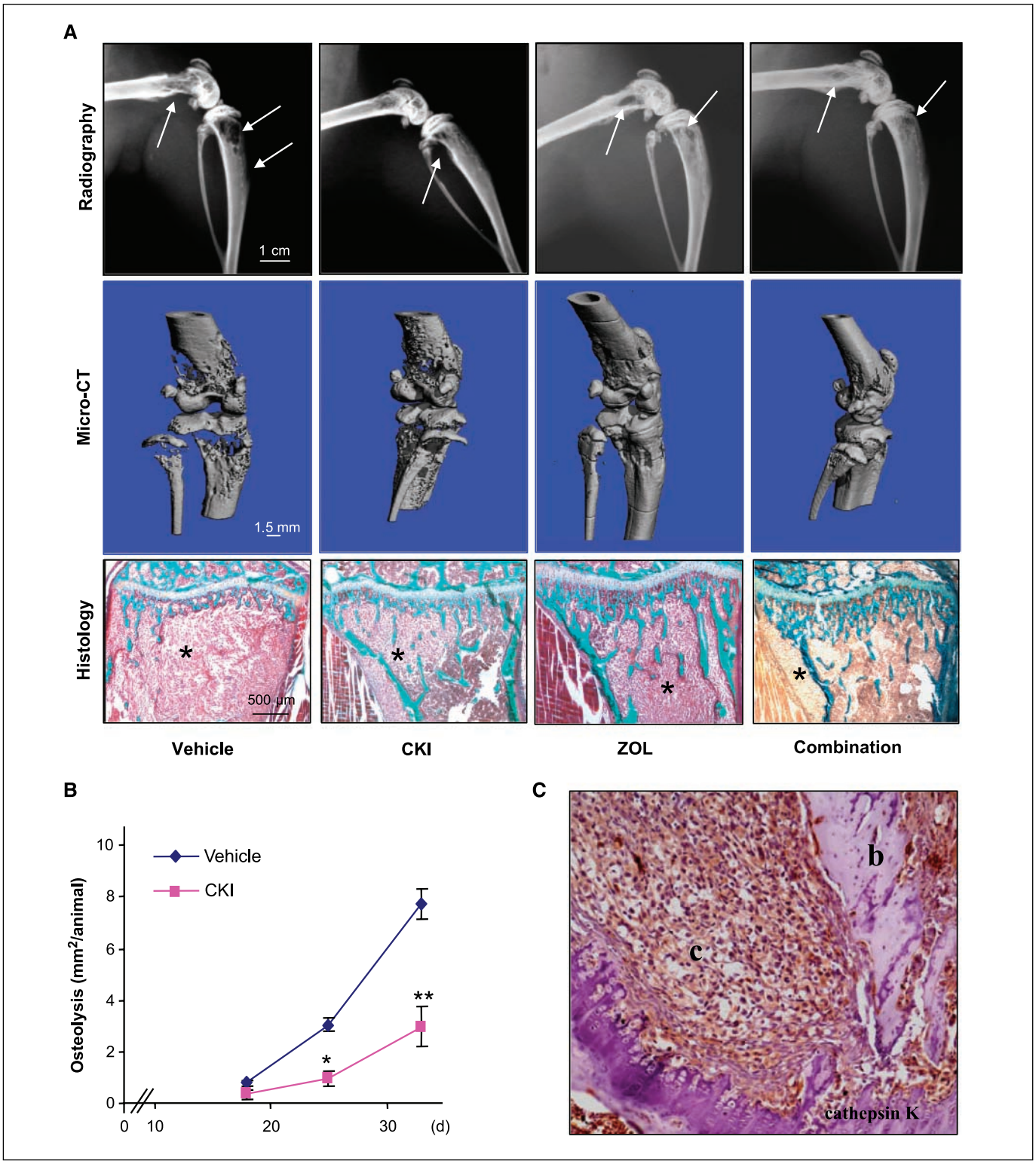

Figure 3. *A,* effect of a CKI, alone or in combination with zoledronic acid, on the progression of established breast cancer osteolytic lesions. B02 breast cancer cells were inoculated i.v. into nude mice. Eighteen days after tumor cell inoculation, animals bearing bone metastases received treatment with vehicle, CKI (50 mg/kg, twice daily), bisphosphonate zoledronic acid (*ZOL*; 100 μg/kg, one single dose on day 18), or CKI in combination with zoledronic acid. *Top* and *middle,* representative radiographs and three-dimensional micro-CT reconstruction of hind limbs from mice 32 d after tumor cell inoculation, respectively. *Bottom,* representative bone histology of Goldner's trichrome–stained tibial metaphysis from metastatic animals. *Top, arrows,* osteolytic lesions. *Bottom,* bone is stained green whereas bone marrow and tumor cells (*) are stained red. *B,* effect of a CKI on the formation of breast cancer bone metastases. B02 breast cancer cells were inoculated i.v. into nude mice. The vehicle or CKI (50 mg/kg, twice daily) was administered to animals from the time of tumor cell inoculation to the end of the protocol. The extent of osteolytic lesions in animals was monitored by radiography on days 18, 25, and 32. *Points,* mean of two separate experiments (13–14 animals per group); *bars,* SD. *, $P < 0.05$; **, $P < 0.001$, compared with vehicle-treated animals by ANOVA followed by a Fisher's PLSD test. *C,* immunostaining of cathepsin K in experimental bone metastasis. B02 breast cancer cells (*c*), which were present in the bone marrow cavity, strongly expressed cathepsin K. *b,* bone.

and 2). These results may be explained by the total cumulative dose of CKI administered to animals in the preventive setting (3,200 mg), which was 2.3-fold higher than in the treatment setting (1,400 mg).

Compared with the vehicle, a preventive CKI dosing regimen also reduced the skeletal tumor burden by 54% (95% CI, 16–93%; $P = 0.007$; Table 2). However, the efficacy of CKI at inhibiting skeletal tumor burden in this preventive setting did not differ significantly from that observed in the treatment setting [62% inhibition (95% CI, 40–84%); Table 1]. We would have expected the preventive dosing regimen to have inhibited skeletal tumor burden more than what we observed because the cathepsin K immunostaining of bones bearing metastatic lesions revealed that B02 breast cancer cells strongly expressed cathepsin K *in situ* (Fig. 3*C*). We therefore examined whether the dosing regimen of CKI used in our bone metastasis model could directly affect the growth of s.c. B02 tumor xenografts in animals.

Effect of a CKI on the growth of s.c. breast cancer xenografts *in vivo*. We used a mouse model of tumorigenesis in which human B02 breast cancer cells were injected s.c. to immunocompromised mice. At the time the volume of tumor xenografts reached ~200 mm^3 (21 days after tumor cell injection), animals were treated with CKI (50 mg/kg, i.p., twice daily) or the vehicle for 32 days (day 52). At the end of the protocol (day 52), the total cumulative dose of CKI given to each mouse was 3,200 mg.

Compared with the vehicle, CKI did not inhibit the growth of s.c. tumors in animals (Fig. 4*A*), whereas the same total cumulative dose of CKI reduced skeletal tumor growth (Table 2). Immunohistochemistry of s.c. tumors revealed that cathepsin K was strongly expressed in B02 cells (Fig. 4*A, inset*).

Effect of a CKI on breast cancer cell invasion *in vitro*. Tumor cell invasion is an early process occurring during breast cancer bone metastasis formation (2). It involves the degradation of the basement membrane by tumor-derived proteases. It has previously

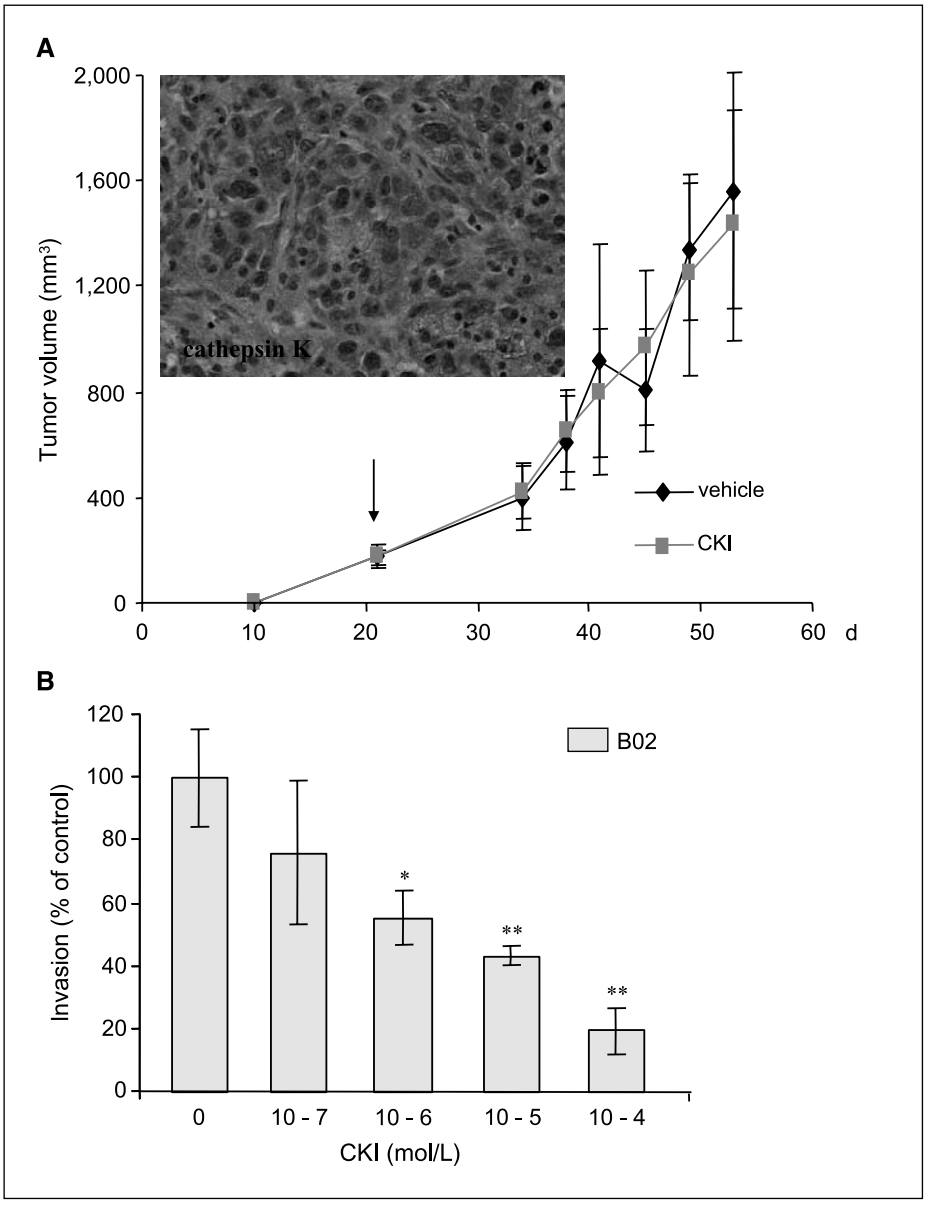

Figure 4. *A,* effect of a CKI on the growth of s.c. breast cancer xenografts. B02 cells were inoculated s.c. into the right flank of nude mice. At the time (day 21) tumors reached 176 mm^3 (*black arrow*), animals were treated i.p. with the vehicle or CKI (50 mg/kg, twice daily) until the end of the protocol (day 52). The CKI did not inhibit the growth of B02 breast cancer cells *in vivo*. *Points,* mean of five to seven animals per group; *bars,* SD. *Inset,* immunostaining of cathepsin K in s.c. tumor xenografts. Cathepsin K was strongly expressed by B02 cancer cells. *B,* dose-dependent inhibitory effect of CKI on B02 tumor cell invasion *in vitro*. *Columns,* mean percentage of the control from three separate experiments; *bars,* SD. *, $P < 0.05$; **, $P < 0.001$, compared with control by ANOVA followed by a Fisher's PLSD test.

been proposed that cathepsin K expressed by prostate cancer cells may contribute to the invasive potential of these cells (17). We therefore investigated whether CKI could interfere with breast cancer cell invasion.

The pharmacokinetic profile of a single dose of 50 mg/kg CKI revealed that concentrations in the bone marrow reached 10^{-5} mol/L. CKI concentrations in the range of 10^{-7} to 10^{-4} mol/L were therefore chosen to study the effect of the drug on B02 cell invasion *in vitro*. As shown in Fig. 4*B*, CKI dose-dependently inhibited B02 cell invasion through basement membrane Matrigel, reaching half-maximal inhibitory concentration at 10^{-5} mol/L.

Discussion

In breast cancer skeletal metastases, bone destruction is mediated by osteoclasts (2). Osteoclasts resorb bone by secreting protons and cathepsin K. Protons dissolve bone mineral and provide an acidic microenvironment, enabling cathepsin K to degrade the demineralized collagenous matrix (7). Matrix metalloproteinases are also involved in this resorptive process (18, 19). However, cathepsin K is a key proteinase in bone resorption (7, 8).

The results presented here first show that cathepsin K is expressed not only by osteoclasts but also by breast cancer cells. Similar findings showing the presence of cathepsin K in breast and prostate cancers have previously been reported (12, 17). In addition, we found that breast cancer cells in bone metastases overproduced cathepsin K compared with the expression observed in the primary tumor and soft tissue metastases of the same patient. Although more patients with advanced cancer need to be investigated to clearly address this question, our results are in accordance with the osteomimicry theory (20, 21), suggesting that cancers metastasizing to bone must express bone-related genes to grow and thrive in the bone microenvironment. Because cathepsin K was expressed by osteoclasts and bone-residing breast cancer cells, we therefore reasoned that a therapeutic approach using a CKI could be an effective way to treat breast cancer bone metastases.

Our results show that a clinically relevant dose of CKI substantially reduced osteolysis in two different mouse models of breast cancer bone metastasis. Moreover, we showed that CKI therapy produced meaningful antitumor effects in bone. We surmise that this reduction of skeletal tumor burden is due to the antiresorptive activity of CKI, which, in turn, deprives breast cancer cells of bone-derived growth factors that are required for tumor growth. This assumption is first supported by our finding that the preventive dosing regimen of CKI that inhibited skeletal tumor burden did not inhibit the growth of s.c. tumor xenografts in animals. Moreover, using the experimental model of B02 breast cancer bone metastasis, we found that CKI had a similar inhibitory effect on skeletal tumor burden in the treatment and preventive protocols. Yet, B02 cells residing at the bone metastatic site did express cathepsin K. If a decreased skeletal tumor burden on CKI therapy was the result of a combined effect of the drug on B02 cells and osteoclasts, we would have expected the preventive dosing regimen to have inhibited skeletal tumor burden more effectively than what we observed. Our results are therefore reminiscent of those obtained in different mouse models of bone metastasis in which it was found that osteoprotegerin, a direct inactivator of osteoclasts, causes a reduction of skeletal tumor burden, whereas it does not inhibit the growth of tumor cells in soft tissues (22–25). Thus, it is most likely that CKI renders the bone a less favorable microenvironment for tumor growth by inhibiting osteoclast-mediated bone resorption.

The function of cathepsin K in breast cancer cells is currently unknown. Our results show that CKI inhibited B02 cell invasion *in vitro*, suggesting that cathepsin K could contribute to the invasive potential of B02 breast cancer cells. As discussed above, the clinical dosing regimen of CKI that we used did not, however, exhibit direct antitumor activity *in vivo*. Perhaps, a higher dosing of CKI would have been required to target tumor-derived cathepsin K *in vivo*. However, given that bone turnover in rodents is three to five times higher than in humans (26), we chose to use a dose of 50 mg/kg (twice daily) CKI that corresponds to a realistic clinical situation. We anticipate that higher doses of CKI, which are well above the current clinical dosing regimens, could generate side effects. For example, in a recent placebo-controlled trial, 5-, 10-, 25-, and 50-mg daily doses of a CKI (balicatib) or placebo were given to 675 postmenopausal women with osteopenia/osteoporosis, and the results obtained at 12 months show that balicatib increases the bone mineral density (27). However, there was a higher incidence of skin adverse events (mainly pruritus) with a daily dose of 50-mg balicatib (27).

Our contention that drugs like CKI should be used in preclinical studies at doses relevant to human clinical testing is reminiscent of the use of bisphosphonates in preclinical studies, in which high doses of these drugs not only reduce skeletal tumor burden by inhibiting bone resorption but also exhibit direct antitumor activity (28). There is much debate about the clinical relevance of these experimental findings because high doses of bisphosphonates are not feasible in humans as a result of concerns about renal toxicity (1). Moreover, the bisphosphonate dosing regimens that have been approved for the treatment of cancer patients with skeletal metastases to date have shown no convincing antitumor effects (1). In this respect, using our mouse model of B02 breast cancer bone metastasis, we have recently reported that a single dose of 100 μg/kg zoledronic acid, calculated to be equivalent to the 4-mg i.v. dose given to patients, does not inhibit skeletal tumor burden (16). We confirmed here that tumor-bearing mice that were treated with a single clinically relevant dose of 100 μg/kg zoledronic acid had less bone destruction, but not less skeletal tumor burden, than vehicle-treated animals. By contrast, if zoledronic acid is administered at a low dosage over shorter treatment intervals, the same total cumulative dose of 100 μg/kg zoledronic acid does exhibit direct antitumor activity (16). For instance, we have shown that a weekly dosing regimen of 50 μg/kg zoledronic acid substantially reduces skeletal tumor burden. These results are explained by the prolonged exposure of the bone marrow to the bisphosphonate, enabling a direct effect on tumor cells that reside in bone (16). It is interesting that Kim et al. (29) recently reported that zoledronic acid (used at a dosage of 25 μg/kg, twice daily) in combination with imatinib (a protein tyrosine kinase inhibitor) and paclitaxel (a chemotherapeutic agent) produced a significant preservation of bone structure and decreased skeletal tumor burden in experimental bone metastasis of human prostate cancer when compared with each individual drug separately. Because CKI and zoledronic acid have different molecular targets in osteoclasts (9–11), we used them as a combination therapy in the hope that a greater inhibitory activity on the progression of established bone metastases might be attainable. We found that a single dose of 100 μg/kg zoledronic acid did not statistically significantly improve the efficacy of CKI on inhibition of tumor-induced osteolysis and skeletal tumor burden. Conversely, a weekly dosing regimen of 50 μg/kg zoledronic acid was so effective at inhibiting skeletal tumor burden when used as a single-agent therapy that the combination with CKI

did not further decrease the tumor burden (unpublished results). Although doses and schedules of administration of CKI and zoledronic acid need to be optimized, our results suggest that combination therapy might be an effective way to maximize the antiresorptive (and antitumor) effects of these drugs.

In conclusion, our preclinical study shows for the first time that a CKI reduces breast cancer–induced osteolysis and skeletal tumor burden. Our findings also support a promising future for the use of CKIs in combination with other molecularly targeted antiosteoclastic agents in the treatment of experimental bone metastases and, perhaps, in the treatment of breast cancer patients with advanced disease.

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References

1. Brown JE, Neville-Webbe H, Coleman RE. The role of bisphosphonates in breast and prostate cancers. Endocr Relat Cancer 2004;11:207–24.
2. Mundy GR. Metastasis to bone: causes, consequences, and therapeutic opportunities. Nat Rev Cancer 2002;2: 584–93.
3. Bendre SM, Gaddy-Kurten D, Mon-Foote T, et al. Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis *in vivo*. Cancer Res 2002;62:5571–9.
4. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003;3:537–49.
5. Hiraga T, Myyoui A, Choi ME, Yoshikawa H, Yoneda T. Stimulation of cyclooxygenase-2 expression by bone-derived transforming growth factor-β enhances bone metastases in breast cancer. Cancer Res 2006;66: 2067–73.
6. Boucharaba A, Serre CM, Grès S, et al. Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer. J Clin Invest 2004;114:1714–25.
7. Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504–8.
8. Saftig P, Hunziker E, Wehmeyer O, et al. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. Proc Natl Acad Sci U S A 1998;95:13453–8.
9. Kavanagh KL, Guo K, Dunford JE, et al. The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs. Proc Natl Acad Sci U S A 2006; 103:7829–34.
10. Rondeau JM, Bitsch F, Bourgier E, et al. Structural basis for the exceptional *in vivo* efficacy of bisphosphonate drugs. Chem Med Chem 2006;1:267–73.
11. Grabowska UB, Chambers TJ, Shiroo M. Recent developments in cathepsin K inhibitor design. Curr Opin Drug Discovery Dev 2005;8:619–30.
12. Littlewood-Evans AJ, Bilbe G, Bowler WB, et al. The osteoclast-associated protease cathepsin K is expressed in human breast carcinoma. Cancer Res 1997; 57:5386–90.
13. Grosios K, Altmann E, Gasser JA, Stumm M, Zimmermann J. Efficacy of the bisphosphonate Zometa and the selective cathepsin K inhibitor AFG495 administered alone or in combination in a murine xenograft model of breast cancer cell induced osteolysis. Proc Am Assoc Cancer Res 2005;46:Abs2910.
14. Pécheur I, Peyruchaud O, Serre CM, et al. Integrin αvβ3 expression confers on tumor cells a greater propensity to metastasize to bone. FASEB J 2002;16: 1266–8.
15. Peyruchaud O, Serre CM, NicAmhlaoibh R, Fournier P, Clézardin P. Angiostatin inhibits bone metastasis formation in nude mice through a direct anti-osteoclastic activity. J Biol Chem 2003;278:45826–32.
16. Daubiné F, Le Gall C, Gasser J, Green J, Clézardin P. Antitumor effects of clinical dosing regimens of bisphosphonates in experimental breast cancer bone metastasis. J Natl Cancer Inst 2007;99:322–30.
17. Brubaker KD, Vessella RL, True LD, Thomas R, Corey E. Cathepsin K mRNA and protein expression in prostate cancer progression. J Bone Min Res 2003;18: 222–30.
18. Delaissé JM, Andersen TL, Engsig MT, Henriksen K, Troen T, Blavier L. Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclast activities. Microsc Res Techniq 2003;61:504–13.
19. Everts V, Korper W, Hoeben KA, et al. Osteoclastic bone degradation and the role of different cysteine proteinases and matrix metalloproteinases: differences between calvaria and long bone. J Bone Min Res 2006;21: 1399–1408.
20. Yeung F, Law WK, Yeh CH, et al. Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. J Biol Chem 2002;277: 2468–76.
21. Barnes GL, Hebert KE, Kamal M, et al. Fidelity of Runx2 activity in breast cancer cells is required for the generation of metastases-associated osteolytic disease. Cancer Res 2004;64:4506–13.
22. Zhang J, Dai J, Qi Y, et al. Osteoprotegerin inhibits prostate cancer-induced osteoclasto-genesis and prevents prostate tumor growth in the bone. J Clin Invest 2001;107:1235–44.
23. Morony S, Capparelli C, Sarosi I, Lacey DL, Dunstan CR, Kostenuik PJ. Osteoprotegerin inhibits osteolysis and decreases skeletal tumor burden in syngeneic and nude mouse models of experimental bone metastasis. Cancer Res 2001;61:4432–6.
24. Yonou H, Kanomata N, Goya M, et al. Osteoprotegerin/osteoclastogenesis inhibitory factor decreases human prostate cancer burden in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice. Cancer Res 2003;63:2096–102.
25. Jones DH, Nakashima T, Sanchez OH, et al. Regulation of cancer cell migration and bone metastasis by RANKL. Nature 2006;440:692–6.
26. Bauss F, Wagner M, Hothorn LH. Total administered dose of ibandronate determines its effect on bone mass and architecture in ovariectomized aged rats. J Rheumatol 2002;29:990–8.
27. Adami S, Supronik J, Hala T, et al. Effect of one year treatment with the cathesin-K inhibitor, balicatib, on bone mineral density in postmenopausal women with osteopenia/osteoporosis. J Bone Min Res 2006;21:Abs1085.
28. Clézardin P, Ebetino FH, Fournier PGJ. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res 2005;65:4971–4.
29. Kim SJ, Uehara H, Yazici S, et al. Modulation of bone microenvironment with zoledronate enhances the therapeutic effects of STI571 and paclitaxel against experimental bone metastasis of human prostate cancer. Cancer Res 2005;65:3707–15.