Sagopilone Inhibits Breast Cancer Bone Metastasis and Bone Destruction Due to Simultaneous Inhibition of Both Tumor Growth and Bone Resorption

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Abstract

Purpose: Bone metastases have a considerable impact on quality of life in patients with breast and other cancers. Tumors produce osteoclast-activating factors, whereas bone resorption promotes the growth of tumor cells, thus leading to a "vicious cycle" of bone metastasis. Sagopilone, a novel, fully synthetic epothilone, inhibits the growth of breast cancer cells *in vitro* and *in vivo*, and here we report its activity in the MDA-MB-231(SA) breast cancer bone metastasis mouse model.

Experimental Design: The potency of sagopilone was determined in treatment models simulating the adjuvant (preventive) and metastatic (therapeutic) settings in the clinic.

Results: We showed that sagopilone inhibited tumor burden and bone destruction, in addition to reducing tumor-induced cachexia and paraplegia. The reduction in osteolytic lesions, tumor growth in bone, and weight loss was statistically significant in the preventive model compared with the vehicle group. In the therapeutic model, sagopilone treatment significantly lowered the number of activated osteoclasts and significantly reduced the osteolytic lesion area, bone volume loss, and bone resorption compared with vehicle treatment while simultaneously inhibiting tumor burden. An *in vitro* assay confirmed that sagopilone inhibited osteoclast activation without cytotoxic effects, whereas paclitaxel resulted in lower inhibition and high levels of cytotoxicity.

Conclusions: Sagopilone seems to inhibit the vicious cycle at both the tumor growth and bone resorption stages, suggesting the possibility for substantial benefit in the treatment of patients with breast cancer at risk from bone metastases or with bone lesions already present. Phase II clinical trials with sagopilone in patients with breast cancer are ongoing.

Bone is the most common site for a distant metastasis in women with breast cancer (1), with a reported incidence of up to 75% (2, 3) and an average survival time of ~2 years after diagnosis (3). Symptoms of bone metastases include skeletal complications and bone pain, which affects up to 80% of patients and greatly impacts on their quality of life (4).

Primary breast tumors express osteolytic and osteoblastic factors, stimulating different types of bone metastases, with osteolytic lesions occurring more commonly (5, 6). The development of osteolytic bone metastases has been described as a vicious cycle, with increased osteoclast activity implicated as the predominant mechanism of bone destruction (6). Tumor cells produce factors, such as parathyroid hormone-related protein, which are responsible for an increase of osteoclast activity and consequent bone resorption. Growth factors stored in bone and released during bone resorption in turn stimulate tumor cell growth, thus perpetuating the vicious cycle. The quality of life of breast cancer patients could be considerably increased by a potent inhibitor of bone metastasis (7), and a drug that could simultaneously and effectively inhibit both tumor cell growth and osteoclast activity would have great potential in the treatment of these patients.

A commonly used drug in the treatment of metastatic breast cancer is the taxane paclitaxel, which, like other microtubule-stabilizing agents, inhibits tumor cell division by inducing a mitotic block at the metaphase-anaphase transition, thereby leading to apoptosis (8). Taxane therapy has limitations, however, including the development of drug resistance (9), and the response rates of bone metastases in breast cancer patients to single-agent paclitaxel range from 0% to 30% (10, 11), although there are few detailed reports in the literature.

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Translational Relevance

Sagopilone, a novel, fully synthetic epothilone is a microtubule-stabilizing agent showing, in addition to its strong antiproliferative activity in several *in vitro* and *in vivo* tumor models, also *in vitro* antiresorptive potency on human osteoclasts. These characteristics of sagopilone were confirmed by the effects of sagopilone in a bone metastasis mouse model in which this compound simultaneously inhibited tumor growth and bone resorption. Despite the advances in the treatment of metastatic breast cancer, drugs that are effective in preventing and inhibiting both tumor growth and bone resorption are rare on the market. Thus, our preclinical results suggest that sagopilone might be effective against bone metastasis in patients with breast cancer.

The epothilones comprise a novel class of microtubule stabilizers that are structurally distinct from the taxanes yet have similar mechanisms of action, leading to cell cycle arrest and apoptosis. Certain epothilones, however, are less susceptible to P-glycoprotein–mediated efflux mechanisms than the taxanes. Sagopilone (ZK-EPO), the first fully synthetic epothilone in clinical development, was rationally designed to overcome the limitations of other microtubule stabilizers (12). It has shown *in vitro* and *in vivo* activity with balanced tolerability against a broad range of tumor models, including superior activity compared with paclitaxel in many breast cancer cell lines (12–14),[3] suggesting that sagopilone may have potential in the treatment of breast cancer.

In this study, we evaluated the activity of sagopilone or paclitaxel compared with vehicle treatment against osteolytic lesions and bone metastases in a human xenograft model of breast cancer bone metastasis, MDA-MB-231(SA).

Materials and Methods

Cell lines and culture

Two treatment scenarios were evaluated in the mouse breast cancer bone metastasis model MDA-MB-231(SA). The first scenario (preventive model) simulated the adjuvant treatment of a breast cancer patient with a high risk of developing bone metastases, and the second (therapeutic model), the treatment of a patient with advanced metastatic breast cancer and established osteolytic lesions.

The breast cancer cell line MDA-MB-231(SA), which strongly metastasizes to bone (15), was a gift from Prof. Theresa Guise (University of Virginia, Charlottesville, VA). Cells were cultivated in high-glucose DMEM (Biochrom AG) containing 10% heat-inactivated FCS (Biochrom AG), 2% glutamine (PAA Laboratories GmbH), and 1% nonessential amino acids (PAA Laboratories). Injection of the cells into the left ventricle of nude mice results in the development of bone metastases. For intracardiac inoculations, cells were trypsinized and resuspended in PBS (Biochrom AG) to a final concentration of 5×10^5 cells/100 μL (preventive model). For the therapeutic model, a MDA-MB-231(SA)/luc cell line was generated by stable transfection of the parental MDA-MB-231(SA) cell line with a pRev CMV_Luc2 vector. These cells were cultured with 250 μg/mL hygromycin B (Invitrogen Ltd.) and inoculated intracardially at a concentration of 1×10^5 cells/100 μL.

Animals

Animal studies were conducted in accordance with the guiding principles in the care and use of animals.[4] Female athymic nude *(nu/nu)* mice (Harlan Winkelmann GmbH) were maintained under germ-free conditions accredited by the American Association for Accreditation of Laboratory Animal Care or the European equivalent.

For intracardiac inoculations, 5-wk-old female athymic nude mice were anesthetized with an i.p. application of 5% Rompun (Bayer HealthCare AG)/10% Ketavet (Pfizer) in 0.9% NaCl, 0.1 mL/10 g body weight. With the use of an insulin syringe (BD Micro-Fine+Demi U-100; Becton Dickinson GmbH), 5×10^5 MDA-MB-231 (SA) (preventive model) or 1×10^5 MDA-MB-231(SA)/luc (therapeutic model) cells in 100 μL PBS were inoculated into the left cardiac ventricle of anesthetized mice. At sacrifice, whole blood was collected via the vena cava under anesthesia, and the tartrate-resistant acid phosphatase (TRACP) 5b concentration was determined in serum obtained by the centrifugation of blood samples (100,00 × *g* for 3 min) with the use of serum gel tubes (Sarstedt).

Treatment of bone metastases *in vivo*

In the preventive treatment model, mice were inoculated with a tumor cell suspension of MDA-MB-231(SA) on day 0. Treatment with sagopilone (10 mg/kg single i.v. dose) or vehicle was initiated on day 5, by which time tumor cells were expected to have spread to bone (16). Animal body weights were monitored daily from day 13, and at sacrifice (day 19), animals were assessed by radiography, and hind limbs, kidneys, and adrenal glands were collected for histologic examination. Details of the analytic methods are given below.

In the therapeutic model, after inoculation of MDA-MB-231(SA)/luc tumor cells at day 0 and X-ray randomization into treatment groups according to lesion size on day 12, treatment was initiated on day 13 with vehicle, sagopilone (10 mg/kg single-dose i.v.; day 13) or paclitaxel (9 mg/kg in 0.9% NaCl i.p. once daily; days 13-17; Bristol-Myers Squibb GmbH & Co. KGaA). Body weight and the onset of paraplegia were monitored daily from day 13. Before sacrifice (day 22), bone lesions were assessed by radiography and microcomputed tomography (microCT) under Rompun/Ketavet anesthesia. In addition, cancer cell dissemination was analyzed by bioluminescence imaging (BLI) also on day 22. Bones (hind limbs) and soft tissues (kidneys, adrenal glands) were collected for histologic analysis, and serum samples were collected for analysis of biochemical markers of bone turnover. The methods used for imaging and histologic analyses are detailed below.

In vivo analytic methods

Radiography and measurement of osteolytic lesion area. Hind limbs of animals were X-rayed with a digital Faxitron small animal X-ray cabinet (Faxitron X-Ray) at 35 kV tube voltage, 0.3 mA current, and 3 s exposure time. Quantitation of lesion area was done with the use of image analysis software (analySIS; Soft Imaging System GmbH).

MicroCT measurement of bone volume. MicroCT imaging was done with the use of a TomoScope microCT scanner (VAMP GmbH) in high-quality mode (40 kV tube voltage, 90 s scan time, 80 μm spatial resolution) without application of a contrast agent. Femur and tibia bone volume was calculated from reconstructed microCT images with the use of Amira image analysis software (Mercury Computer Systems Inc.).

Bioluminescence imaging. Noninvasive whole-body imaging was done to monitor luciferase-expressing MDA-MB-231(SA)/luc cells with the use of a cooled charge-coupled device camera (NightOWL LB; Berthold Technologies). Before analysis, mice were injected i.v. with 100 μL luciferin (45 mg/mL in PBS; Synchem OHG) and anesthetized with 1%

[3] Winsel et al. Sagopilone (ZK-EPO), a novel epothilone, induces cell cycle arrest and apoptosis, leading to tumor growth inhibition in multiple breast cancer models. In preparation.

[4] http://www.the-aps.org/about/opguide/appendix.htm#care

to 3% isofluran (CuraMED Pharma GmbH). Photon emission was measured over an integration time of 1 min and recorded as pseudocolor images that were quantified with the use of WinLight software (Berthold Technologies).

Ex vivo analytic methods

TRACP 5b measurement. Serum concentrations of osteoclast-derived TRACP 5b were measured with the use of a solid phase immunofixed enzyme activity (MouseTRAP) assay according to the manufacturer's instructions (Immunodiagnostic Systems GmbH).

Bone histology and histomorphometry. At sacrifice, hind limbs were fixed in 4% neutral buffered formalin (Merck & Co. Inc.) for 2 to 3 d, decalcified in 10% EDTA (Sigma-Aldrich), dehydrated, and embedded in paraffin. Tissue sections (4 μm) were stained with H&E, orange G, and phloxine with the use of standard protocols. Total tumor area was determined in longitudinal midsections of tibiae and femora without knowledge of experimental groups. Histomorphometric analyses were done with the use of Soft Imaging System Cell^F (Olympus Soft Imaging GmbH). For TRACP staining, 4 μm sections were incubated in a substrate solution (naphthol AS-BI phosphate in ethylene glycol monoethyl ether; Sigma-Aldrich) and a staining solution (sodium nitrite and pararosaniline chloride; Sigma- Aldrich), and were counterstained with hematoxylin. The osteoclast number per millimeter tumor bone interface was determined in longitudinal midsections of tibiae and femora without knowledge of experimental groups.

Osteoclast activity assay

The osteoclast activity assay was done by Pharmatest Services Ltd. Human osteoclast precursor cells were cultured for 7 d without test compounds to allow osteoclast differentiation, and the amount of TRACP 5b released into the culture medium was determined at day 7 as an index of the number of osteoclasts formed. Sagopilone (2.5-50 nmol/L) or paclitaxel (2.5-200 nmol/L) was added at day 7, and mature osteoclasts were cultured for an additional 3 d, allowing them to resorb bone. The level of carboxy-terminal cross-linking telopeptide of type I collagen is a measure of the collagen degradation activity of human osteoclasts and was measured at day 10 to determine bone resorption during days 7 to 10. Cytotoxicity was determined by the level of dying cells in the culture medium at day 10 with the use of the Toxilight BioAssay kit (Lonza). Trans-epoxysuccinyl- L-leucylamido-(4-guanidino) butane (E64), a cysteine protease inhibitor that shows selectivity for cathepsin B and is known to inhibit osteoclast activity (17), was used as a control compound at 1.0 μmol/L.

Statistical methods

The results are reported as mean ± SD. Comparisons with control and comparisons of sagopilone versus paclitaxel were done by exact Wilcoxon tests. An α-level of 0.05 was used. Because of the exploratory nature of the study, no adjustments for multiplicity were made.

Results

Sagopilone significantly reduces tumor-induced cachexia, osteolytic lesions, and tumor growth in bone in a preventive model of breast cancer bone metastasis. Sagopilone treatment was analyzed in a preventive setting in the MDA-MB-231(SA) model, simulating the adjuvant treatment of a breast cancer patient at risk of bone metastases. Mice treated with sagopilone had significantly higher body weight, with a mean of 22.3 ± 2.1 g compared with 20.1 ± 2.8 g in the vehicle group ($P = 0.0433$), indicating a significant reduction in tumor-induced cachexia.

Sagopilone treatment in this model also significantly reduced the area and number of osteolytic lesions compared with vehicle treatment as measured by radiography (Fig. 1A). Untreated mice had a mean osteolytic lesion area of 8.1 ± 4.8 mm^2 compared with 0.1 ± 0.2 mm^2 in sagopilone-treated animals ($P < 0.001$; Fig. 1B), and the mean number of lesions was 21.2 ± 11.9 in vehicle-treated mice compared with 0.7 ± 0.9 in sagopilone-treated mice (Fig. 1C). Additionally, none of the mice in the sagopilone

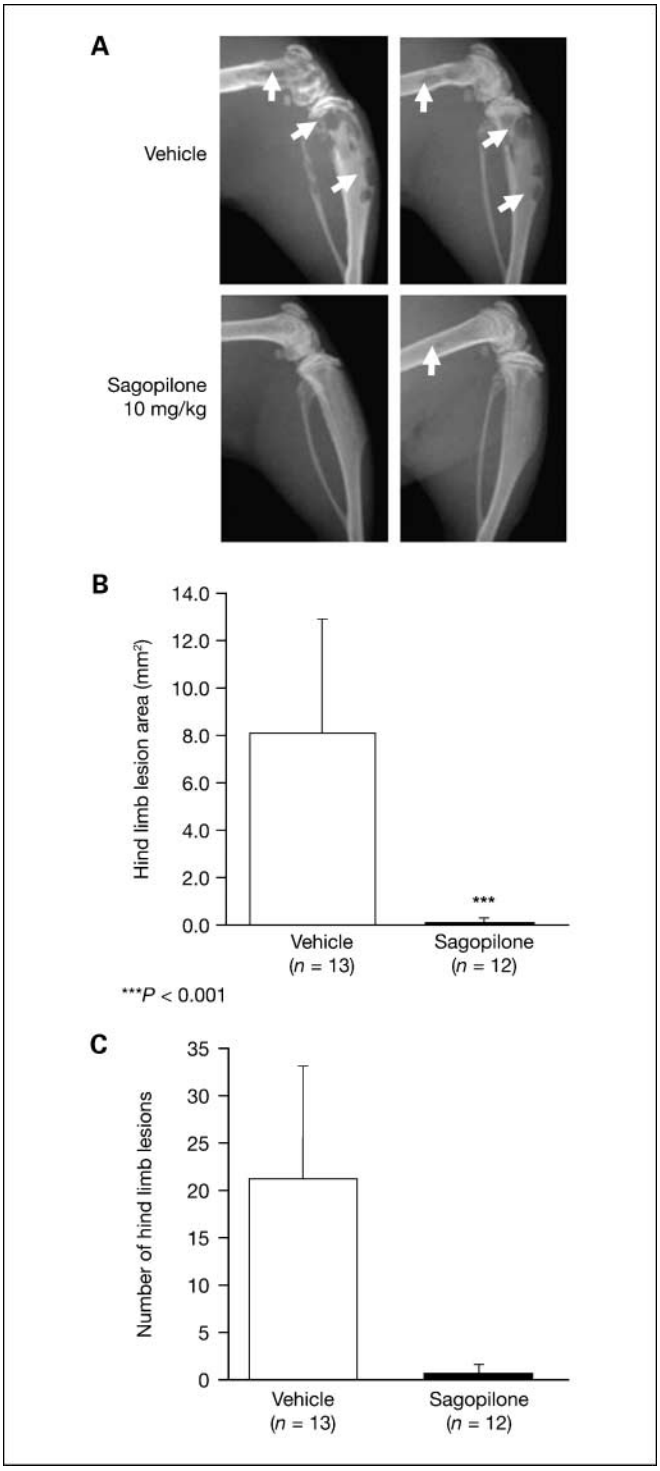

Fig. 1. Sagopilone significantly reduces osteolytic lesions in a preventive MDA-MB-231(SA) treatment model of breast cancer bone metastasis. *A*, representative radiographs of hind limbs of vehicle-treated and sagopilone-treated animals 19 d after tumor cell inoculation (*arrows*, extent of osteolytic lesions). Osteolytic lesion area (*B*) and lesion number (*C*) in hind limbs. Values, mean + SD.

group (n = 15) had visceral metastases compared with five of the vehicle-group animals (n = 15; data not shown).

Sagopilone completely inhibited tumor growth in bone in this setting as assessed by histomorphometry (Fig. 2). In vehicle-treated mice, tumors fill the bone marrow cavity, replace normal cellular elements, and eventually lead to the destruction of cortical bone (Fig. 2A). Sagopilone treatment resulted in a complete reduction of the mean tumor area compared with a tumor size of 6.3 ± 5.5 mm^2 in the vehicle group (Fig. 2B).

Sagopilone and paclitaxel reduce tumor-induced cachexia and paraplegia in a therapeutic model of breast cancer bone metastasis. A second schedule was evaluated in the MDA-MB-231(SA) model to simulate the therapeutic treatment of a patient with advanced metastatic breast cancer involving osteolytic bone lesions. Similar to the results seen with sagopilone in the preventive treatment model, sagopilone or paclitaxel treatment also showed a trend for reduced tumor-induced cachexia in this therapeutic setting, with mean ± SD body weights of 18.8 ± 2.2 g and 19.3 ± 1.7 g in the sagopilone and paclitaxel groups, respectively, compared with 17.6 ± 1.8 g for vehicle-treated animals. Both agents also considerably reduced the number of paraplegic animals compared with vehicle treatment, with ~10% of animals in the sagopilone or paclitaxel treatment groups affected versus 70% of vehicle-treated animals (data not shown).

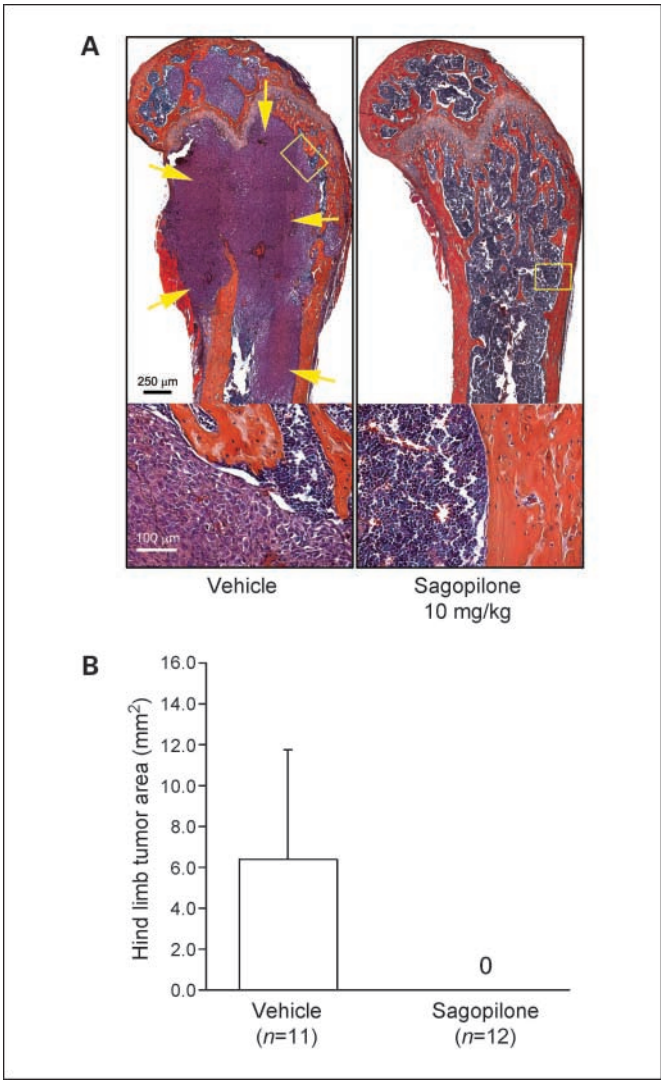

Fig. 2. Sagopilone eradicates tumor growth in bone in a preventive MDA-MB-231(SA) treatment model of breast cancer bone metastasis. *A*, representative H&E-stained bone sections of femurs of vehicle-treated and sagopilone-treated animals (*arrows*, extent of the tumor; *insets*, visualization of bone and tumor morphology; magnification, ×10; *insets*, ×20). *B*, tumor area in the hind limbs of vehicle-treated and sagopilone-treated animals. Values, mean + SD.

Sagopilone significantly reduces osteolytic lesions in the therapeutic MDA-MB-231(SA) treatment model. Radiography assessments 22 days after tumor inoculation in the therapeutic model indicated that sagopilone significantly reduced osteolytic lesions compared with vehicle (Fig. 3A and B). The mean area of hind limb osteolytic lesions was significantly lower in sagopilone-treated animals (1.16 ± 0.84 mm^2) compared with the vehicle group (4.23 ± 2.06 mm^2; $P < 0.001$) and compared with the paclitaxel-treated animals ($P = 0.002$; Fig. 3B). Paclitaxel also reduced the lesion area (2.78 ± 0.83 mm^2) when compared with vehicle treatment, although this decrease was not statistically significant (Fig. 3B).

The analysis of skeletal changes, as measured by microCT, closely correlated with the radiography results (Fig. 3C and D). Bone destruction was again mainly evident at the ends of distal femora and proximal tibiae (Fig. 3C), and whole body images also revealed lesions in the hips, spine, forelimbs, scapula, and calvaria of tumor-bearing animals (data not shown). Quantitation of hind limb total bone volume with the use of microCT data (Fig. 3D) indicated that bone destruction in sagopilone-treated animals (bone volume, 94.06 ± 5.57 mm^3) was significantly reduced compared with both vehicle-treated (bone volume, 87.85 ± 3.70 mm^3; $P = 0.03$) and paclitaxel-treated animals (bone volume, 86.16 ± 3.19 mm^3; $P < 0.001$). In contrast, bone destruction in the paclitaxel-treated group seemed to be similar to the vehicle group ($P > 0.05$).

Sagopilone reduces tumor burden and bone metastases in the therapeutic MDA-MB-231(SA) model setting. Assessment of luciferase-expressing tumor cells by BLI at the end of the study (day 22) was used to measure tumor progression in bone as well as the spread of tumor cells in other tissues. The bioluminescence signal indicated tumor cells in the region of the hind limbs, forelimbs, spine, and brain (Fig. 4A). A significant decrease in bioluminescence signal intensity was observed in the sagopilone treatment group (167551 ± 205993 cts/s) as well as in the paclitaxel-treated group (366622 ± 258612 cts/s) compared with the vehicle group (2604798 ± 2372678 cts/s; $P = 0.011$ for vehicle versus paclitaxel and $P < 0.002$ for vehicle versus sagopilone; Fig. 4B), indicating that both treatments reduced tumor burden.

The bioluminescence data were confirmed by the histologic examination of tumors in bone (Fig. 4C and D) and visceral organs, confirming a correlation between both methods. In paclitaxel-treated and vehicle-treated mice, the tumor area in bone extended throughout the marrow cavity and replaced normal cellular elements (Fig. 4C). Quantitation of tumor size in multiple sections revealed a significantly lower tumor area in bone in the sagopilone-treated animals (1.27 ± 0.98 mm^2) compared with the vehicle group (8.16 ± 5.22 mm^2; $P < 0.001$) and compared with paclitaxel-treated animals ($P = 0.005$), thus reflecting the antitumor effect of sagopilone in bone metastases (Fig. 4D). The tumor area in bone of paclitaxel-treated animals (4.52 ± 2.33 mm^2) was not statistically lower than in the vehicle group. In addition, a lower number of sagopilone-treated

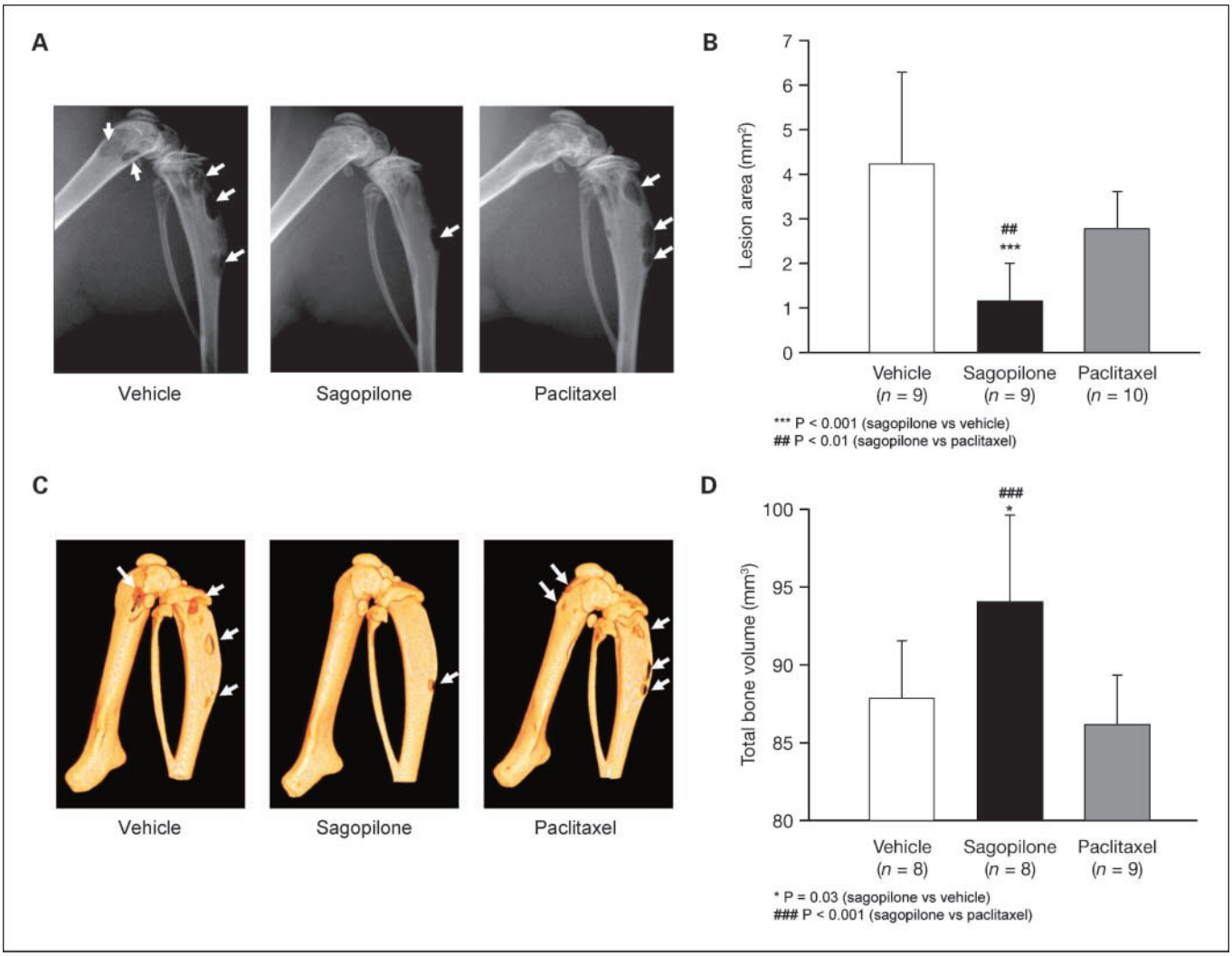

Fig. 3. Sagopilone significantly reduces osteolytic lesion area and loss of bone volume compared with paclitaxel in the MDA-MB-231(SA) model when used as therapeutic treatment. *A,* X-rays taken at day 22 after tumor cell inoculation (*arrows*, extent of osteolytic lesions) and (*B*) corresponding lesion area. *C,* MicroCT scans showing representative three-dimensional images and (*D*) corresponding quantitation of total bone volume of both hind limbs. Values, mean + SD.

animals (20%), and no paclitaxel-treated animals, exhibited adrenal gland metastases compared with the vehicle group (43%; data not shown).

***Sagopilone inhibits the activation of osteoclasts* in vivo.** TRACP 5b is expressed in bone-resorbing osteoclasts and is therefore considered to be a useful marker of bone resorption rate (18). We measured the serum TRACP 5b concentration and analyzed TRACP 5b staining in hind limbs (Fig. 5A), where it correlates with the number of activated osteoclasts. The osteoclast cell count indicated a statistically significant increase of activated osteoclasts in the vehicle (10.57 ± 2.42) group compared with paclitaxel-treated (7.40 ± 2.94; $P = 0.002$) and sagopilone-treated animals (3.67 ± 2.20; $P < 0.001$; Fig. 5B). Moreover, there was a significantly lower number of activated osteoclasts in sagopilone-treated mice compared with paclitaxel-treated animals ($P < 0.001$). Additionally, serum TRACP 5b concentrations were significantly lower in sagopilone-treated mice (5.59 ± 0.96 U/L) and paclitaxel-treated mice (5.24 ± 0.48 U/L) compared with the vehicle group (8.93 ± 1.76 U/L; $P < 0.001$ in both cases; Fig. 5C), suggesting a considerable reduction in the bone resorption rate after sagopilone or paclitaxel treatment.

***Sagopilone inhibits the resorption activity of human osteoclasts* in vitro.** Sagopilone significantly inhibited osteoclast activity in a dose-dependent manner in an *in vitro* human osteoclast activity assay (Fig. 6A) but was not cytotoxic to osteoclasts (Fig. 6B). In comparison, whereas paclitaxel also showed an inhibitory effect on osteoclast activity although to a lesser extent (Fig. 6C), this was associated with significant cytotoxic effects at several dose levels (Fig. 6D). The reference inhibitor E64 significantly inhibited osteoclast activity and showed no cytotoxic effects, showing that the assays were done successfully and the results obtained are reliable.

Discussion

In the present study, we have shown that sagopilone, the first fully synthetic epothilone in clinical development, has high activity and is well tolerated *in vivo* in the MDA-MB-231(SA)

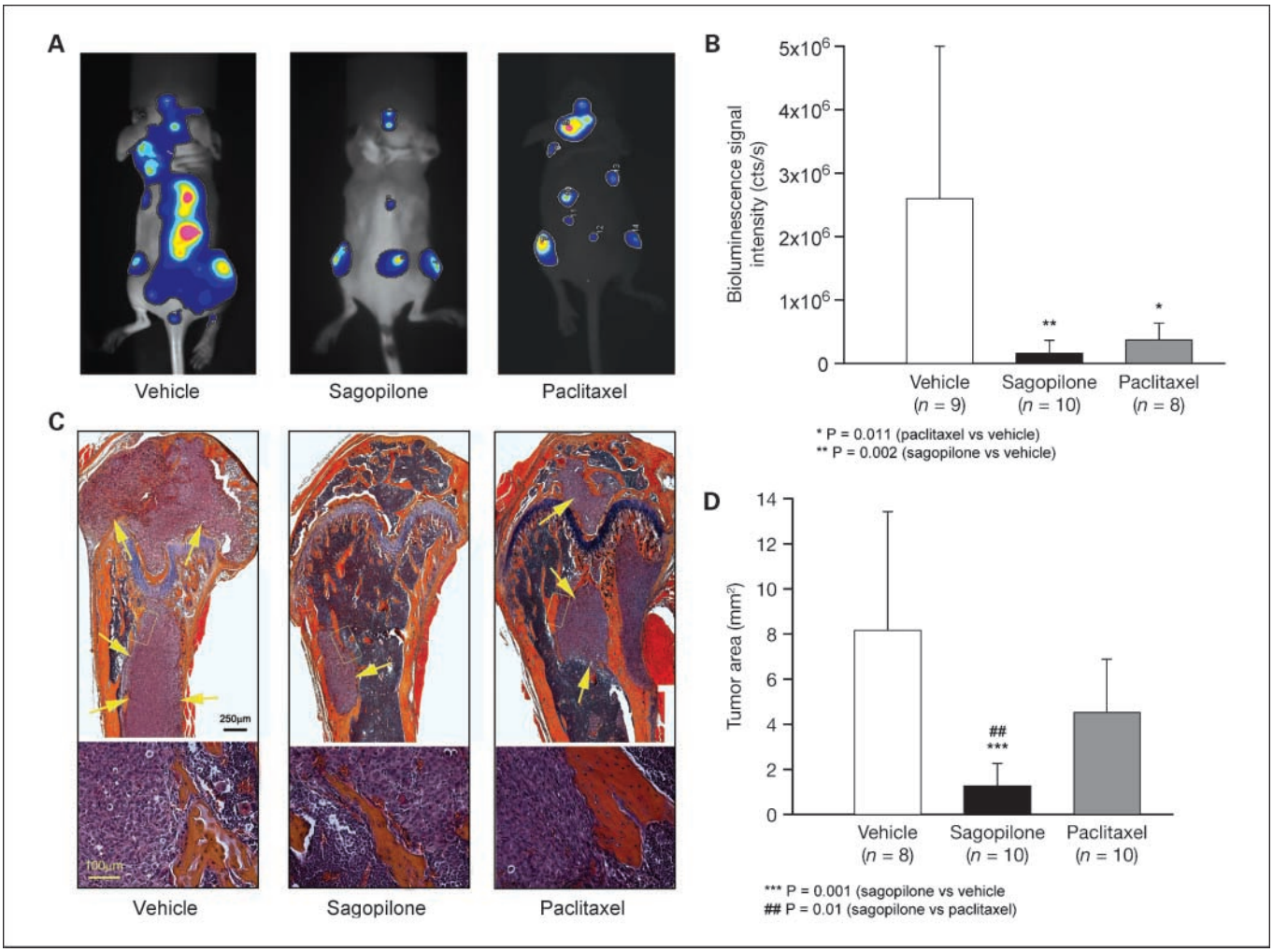

Fig. 4. Sagopilone and paclitaxel considerably reduce tumor burden in a therapeutic MDA-MB-231(SA) treatment model of breast cancer bone metastasis. *A*, bioluminescence images indicating the tumor burden and (*B*) corresponding bioluminescence intensity quantitation. *C*, representative H&E-stained bone sections of femurs of vehicle-treated, sagopilone-treated, and paclitaxel-treated animals (*arrows*, extent of the tumor; *insets*, visualization of the bone and tumor morphology; magnification, ×10; *insets*, ×20) and (*D*) corresponding bone tumor area quantitation. Values, mean + SD.

breast cancer bone metastasis xenograft model when used as both a preventive and therapeutic treatment against bone metastases. The action of sagopilone against bone metastases seems to be 2-fold, combining the inhibition of tumor cell growth with the inhibition of osteoclast activation and bone resorption. In the development of breast cancer bone metastases, tumor growth leads to bone resorption and vice versa (5), and compounds that can inhibit the process at both stages, such as sagopilone, may therefore provide a promising therapy option against bone metastases.

In our preventive treatment model, tumor cells had already spread to the bone, but treatment was started before the development of osteolytic lesions. This scenario aimed to simulate the adjuvant setting in the clinic with local or distant breast cancer progression without diagnosed bone metastases. Patients with a high risk of developing bone metastases include those with positive lymph node status or immunocytochemical evidence of bone marrow involvement (19, 20). Our *in vivo* data suggest that sagopilone may increase metastasis-free survival by reduction of cancer dissemination combined with an almost complete eradication of metastatic tumors in bone and the inhibition of osteolysis. The effects of this treatment include reduced tumor-induced cachexia, which can impact on quality of life. Thus, adjuvant treatment with sagopilone may be effective in patients who have an aggressive tumor with high risk of bone metastasis and could signal a possible trend towards fewer pathologic fractures, reduced bone pain, and less need for radiotherapy treatment. The results of the preventive study gave first evidence of the dual action of sagopilone on osteoclasts and tumor cells. Thus, the compound was tested in the more aggressive therapeutic setting compared with paclitaxel, which was approved for the treatment of metastatic breast cancer in 1994 (21).

In the therapeutic MDA-MB-231(SA) breast cancer bone metastasis model, tumor growth in bone was already severe and osteolytic lesions were clearly visible by radiography, indicating an extremely active vicious cycle. This model resembles the clinical situation of a patient with advanced metastatic breast cancer exhibiting severe osteolytic lesions and tumor growth in bone. In the clinic, therapy for patients with established bone metastasis is mainly palliative and aims to increase quality of life by reducing factors, such as tumor-associated cachexia (22), which has been noted as an indicator of poor prognosis

in various indications, including lung and pancreatic cancer (23–25). Skeletal complications, including spinal compression, also increase the degree of morbidity and impact on quality of life (26) and can lead to permanent neurologic damage, including paraplegia (25, 27). Sagopilone substantially reduced cachexia and paraplegia *in vivo*, suggesting a potential for increased quality of life in the clinic.

In the therapeutic model, sagopilone treatment again significantly reduced overall tumor burden compared with both vehicle and paclitaxel treatment, whereas the reduction seen with paclitaxel compared with the vehicle group was not significant. The strong antitumor effect is particularly due to the pharmacokinetic profile of sagopilone. It is quickly taken up by the cells and incorporates more efficiently in the tumor cells as compared with paclitaxel (13). Inhibition of tumor cell growth in sagopilone-treated animals, indicated by BLI and histomorphometry assessments, led to a subsequent decline in bone resorption compared with the vehicle group, with significantly reduced osteoclastic lesion area and bone destruction monitored by radiography and microCT. With regard to the osteolytic lesion area and bone volume, sagopilone had a significantly better effect on reducing bone destruction when compared with paclitaxel. In contrast, the osteolytic lesion area and bone volume in paclitaxel-treated animals did not differ significantly from vehicle-treated animals. Sagopilone treatment also resulted in the significant reduction of activated osteoclasts in TRACP-stained bone sections compared with the vehicle and paclitaxel-treated animals, as well as reduced serum TRACP 5b levels compared with the vehicle group, indicating decreased bone resorption. Research is currently underway in non–tumor-bearing animals to confirm that this is not an indirect effect of reduced tumor cell growth. The reduced serum TRACP 5b concentration in paclitaxel-treated animals may be due to the destruction of osteoclasts outside the tumor-bone interface. Our *in vitro* results, which indicate that paclitaxel is cytotoxic to osteoclasts, also provide an explanation for the decrease in overall bone resorption rate with paclitaxel treatment. The inhibition of bone resorption by paclitaxel is therefore likely to be a result of nonspecific cytotoxic effects on osteoclasts, unrelated to osteoclast activation by tumors.

As a sensitive and specific marker for bone resorption activity, TRACP 5b is used in the clinic to monitor treatment response in breast cancer patients with bone metastases (28). These results suggest that sagopilone effectively interrupts the vicious cycle at two different positions, inhibiting both tumor growth and bone resorption, whereas paclitaxel seems to mainly interrupt the cycle by inhibiting tumor growth. Together, the tolerability and efficacy data for the therapeutic treatment model suggest great promise for sagopilone as a treatment for patients with advanced metastatic breast cancer and bone involvement.

The *in vivo* inhibition of bone resorption shown by sagopilone was mirrored *in vitro* in a bone resorption assay with the use of human osteoclasts. However, the exact molecular mechanism behind this activity is still unknown and requires further study. The doses of the *in vitro* assay were chosen according to the pharmacokinetic data in mice. The serum levels of the compounds in mice quickly decrease after i.v. application and the compounds accumulate in the tumor sagopilone, incorporating more efficiently in the cells compared with paclitaxel. Hall et al. reported that paclitaxel also has *in vitro* effects on osteoclastic bone resorption at therapeutically relevant concentrations and that 10 μmol/L paclitaxel had cytotoxic effects on osteoclasts (29). Our *in vitro* assay indicated that sagopilone inhibits human osteoclast activity and bone resorption to a greater extent than paclitaxel but without the cytotoxic effects associated with paclitaxel treatment. The inhibition of the growth and activity of nonproliferating end cells, such as osteoclasts, by sagopilone may partly be due to interference with nonmitotic microtubule-dependent functions, which include exocytosis, cell morphology, polarization, and adhesion. Indeed, the inhibition of endothelial cell adhesion has previously been reported with paclitaxel (30). Normal adhesion is required for adequate osteoclast function because osteoclasts are activated by contact with the mineralized bone matrix (31). Moreover, under nonadherent conditions, the formation of multinuclear osteoclasts is decreased, thus leading to coverage of a smaller area of bone and further reduction in activity (32). The exocytosis of extracellular vesicles, important for several processes during normal bone resorption, including degradation of the bone matrix (29), may also be blocked by sagopilone.

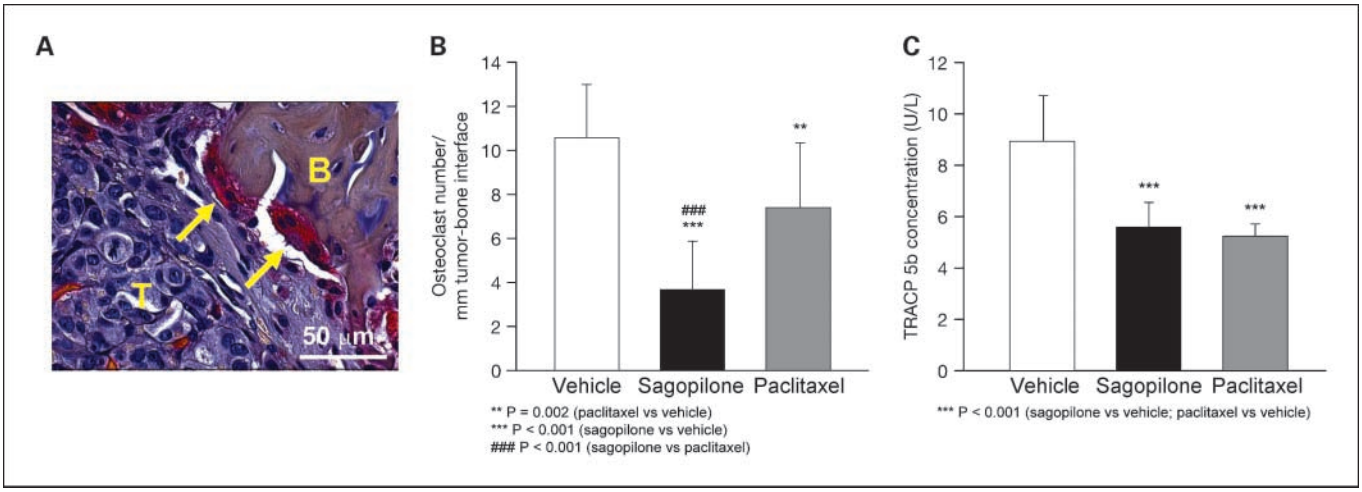

Fig. 5. Sagopilone-treated animals have a significantly lower number of activated osteoclasts and bone resorption rate compared with vehicle-treated and paclitaxel-treated mice. *A,* TRACP staining of bone (*arrows*, osteoclasts; *B*, bone; *T*, tumor; magnification, ×40). *B,* osteoclast number per millimeter tumor-bone interface calculated in TRACP-stained sections. *C,* serum TRACP 5b concentration. Values, mean + SD.

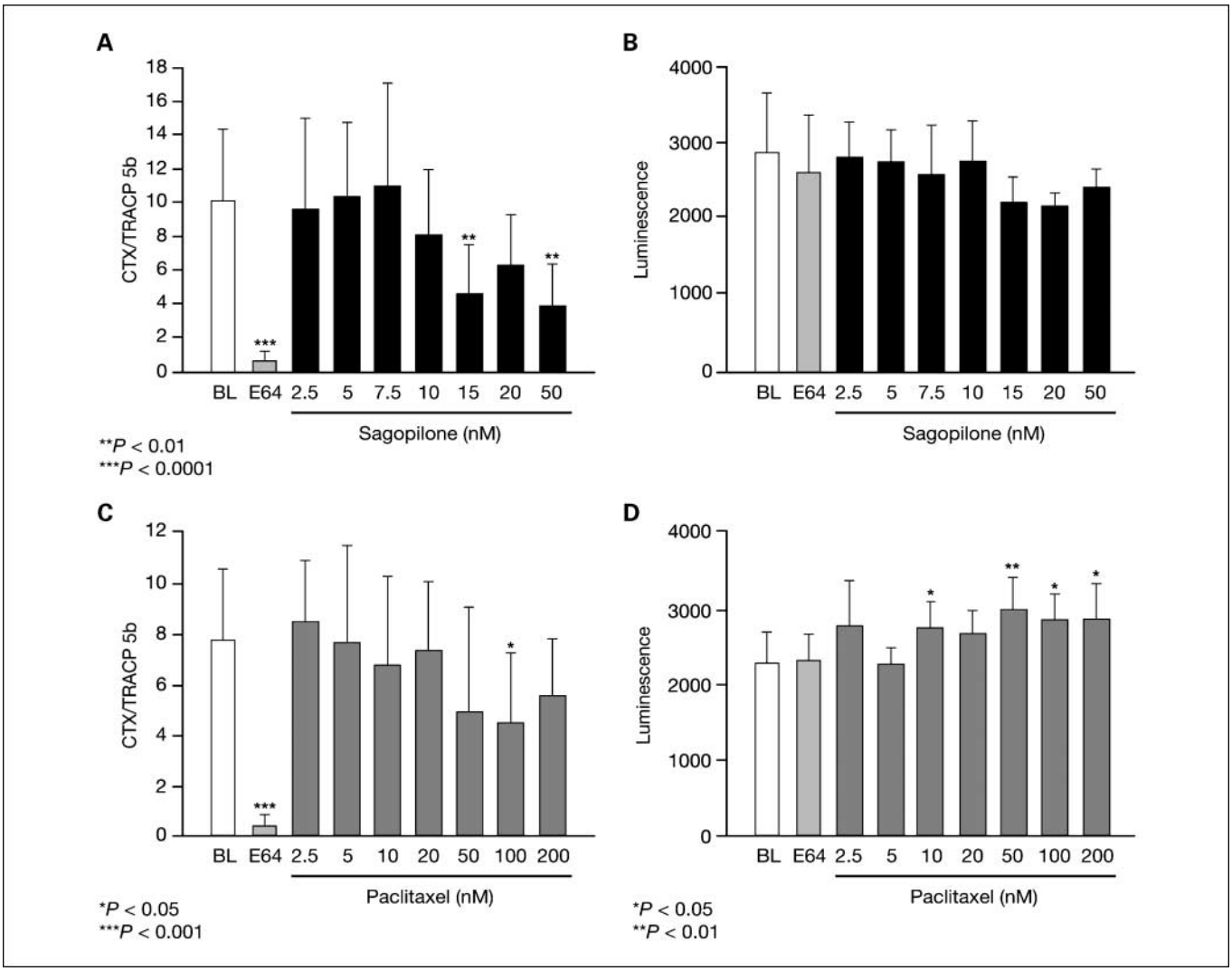

Fig. 6. Sagopilone reduces the resorption activity of human osteoclasts with no cytotoxic effects in a human osteoclast activity assay. The mean osteoclast activity in sagopilone-treated (*A*) and paclitaxel-treated (*C*) cells expressed as the resorption index (CTX concentration at day 10/TRACP 5b concentration at day 7). Cytotoxicity in sagopilone-treated (*B*) and paclitaxel-treated (*D*) cells determined as the luminescence released from dying cells during the culture period. Values, mean + SD. CTX, carboxy-terminal cross-linking telopeptide of type I collagen; BL, baseline.

In this study, the efficacy of sagopilone against bone metastases *in vivo* was assessed with the use of a combination of different imaging techniques: BLI to monitor the dissemination of cancer cells and tumor burden; X-ray for quantitative analysis of bone lesions; and microCT, which accurately reflects a three-dimensional view of the skeleton, for morphologic changes. We found that microCT also allows quantitative analysis of tumor volume in bone, similar to its use in other studies to accurately quantify bone loss (33, 34). Treatment effects determined by microCT measurements in the sagopilone and paclitaxel treatment groups correlated with the X-ray data. In terms of measuring efficacy against tumor burden, BLI and histologic examination showed a good correlation in this study. However, BLI is only suitable for semiquantitative assessment of tumor progression in bone, and histologic examination is essential for measuring structural bone changes, including cortical destruction, and effects on bone marrow. In the clinic, imaging techniques for early detection of metastases are indispensable because most serum markers, such as the bone resorption marker TRACP 5b, are insufficiently sensitive to detect early bone metastases, although they can serve as early indicators of treatment response (35).

Despite promising results with bisphosphonates (36), a broader spectrum of compounds to treat and prevent bone metastases is needed. A study that directly compares sagopilone with clodronate in the MDA-MB-231 bone metastasis model shows similar effects of both compounds on bone and additional effects of sagopilone on tumor (37). Proof-of-concept has already been shown for sagopilone in phase II trials of patients with platinum-resistant ovarian cancer (38) and prostate cancer (39). The results presented here highlight the potential of sagopilone for the treatment of patients with advanced metastatic breast cancer, including bone metastases, and also those with breast or other cancers who have a high risk of developing bone metastases. These potentially include prostate cancer patients, particularly those treated with androgen deprivation therapy, who suffer from skeletal complications, including fractures (40). The ongoing sagopilone phase II clinical trial program will clarify how the dual action of sagopilone against

both tumor growth and osteoclast activity *in vivo* translates into efficacy and tolerability in the clinic.

Disclosure of Potential Conflicts of Interest

All authors are employed by Bayer Schering Pharma AG. U. Klar, J. Hoffman, P. Hauff, and S.-M. Käkönen have an ownership interest in Bayer Schering Pharma AG.

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