

# Doxycycline Decreases Tumor Burden in a Bone Metastasis Model of Human Breast Cancer<sup>1</sup>

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## Abstract

Bone is one of the most frequent sites for metastasis in breast cancer patients, often resulting in significant clinical morbidity and mortality. Increased matrix metalloproteinase (MMP) activity of tumor cells correlates with a higher invasive and metastatic potential. Members of the tetracycline family of antibiotics, including doxycycline, have potential treatment value for bone metastasis; they inhibit cancer cell proliferation, and they are also potent MMP inhibitors and are highly osteotropic. Doxycycline treatment in an experimental bone metastasis mouse model of human breast cancer MDA-MB-231 cells resulted in a 70% reduction in total tumor burden when compared with placebo control animals. In tumor-bearing animals, the amount of doxycycline incorporated into the radius/ulna as assessed by ELISA was lower than in non-tumor-bearing animals. In doxycycline-treated mice, bone formation was significantly enhanced as determined by increased numbers of osteoblasts, osteoid surface, and volume, whereas a decrease in bone resorption was also observed. Doxycycline treatment may be beneficial for breast cancer patients with or at risk for osteolytic bone metastasis; it greatly reduces tumor burden and could also compensate for the increased bone resorption associated with the disease.

## Introduction

Bone is one of the most frequent sites for metastasis of breast and prostate cancer. Bone metastases are often associated, especially in breast cancer, with extensive osteolysis of mineralized collagenous bone matrix (1) and subsequent hypercalcemia, resulting in significant clinical morbidity. The process of bone degradation by osteoclasts can be halted *in vitro* by specifically inhibiting either the activity of lysosomal cysteine proteinases, the cathepsins, or the MMPs<sup>3</sup> (2). MMPs are a large class of closely related proteinases, the activity of which is tightly regulated in physiological situations where extracellular remodeling must occur (3), including in normal bone remodeling. 92 kDa type IV collagenase (MMP-9) has been localized primarily to the osteoclasts by *in situ* hybridization (4), whereas osteoblasts additionally secrete interstitial collagenase (MMP-1) and 72 kDa type IV collagenase (MMP-2; Ref. 5). In addition, the invasive and metastatic potential of cancer cells is strongly associated with the synthesis, release, and activation of MMPs (6). Thus, blocking the activity of MMPs may not only result in inhibition of bone resorption but also in shrinkage and containment of the tumor. The members of the tetracycline family of antibiotics are potent MMP inhibitors, most probably by their ability to chelate Zn<sup>2+</sup> from the active site of the MMPs (7). Doxycycline is one of the more potent MMP inhibitors of

the tetracycline family; it is also better absorbed and has a longer half-life than the parent compound, tetracycline. In gingival extracts from doxycycline-treated rats infected with *Porphyromonas gingivalis*, bone loss returns to normal values accompanied by an inhibition of the degradation of type I collagen (8). Moreover, doxycycline may not only prevent MMP-mediated osteolysis but also metastasis-related tumor cell growth. Tetracyclines are capable of inhibiting cell proliferation in a concentration-dependent fashion in bone metastasis-related cancer cells (9) and many different other cancer cell types *in vivo* and *in vitro* (10, 11) through an induction of a G<sub>1</sub> cell cycle arrest (12). Because of their osteotropism, tetracyclines have specific therapeutic value in the treatment of metastatic bone diseases. In the present study, we investigated the effect of doxycycline treatment on bone metastasis in an experimental mouse model using intraarterial injections of human breast cancer MDA-MB-231 cells. The histology of this model closely resembles the characteristics of osteolytic bone metastasis from human breast cancer.

## Materials and Methods

**Cell Line.** The human breast adenocarcinoma MDA-MB-231 cell line obtained from the American Type Culture Collection (Rockville, MD) was maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin sodium, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B; Invitrogen Canada Inc., Burlington, Ontario, Canada).

**Animals.** All protocols for animal studies were reviewed and approved by the Animal Research and Ethics Board of McMaster University. Per treatment group, 10 female inbred nude (BALB/c *nu/nu*) mice (Charles River, St. Constant, Quebec, Canada) 5 weeks of age (15–20 g) were used. Per experiment, control animals not injected with tumor cells were also included. Intracardiac injections of MDA-MB-231 cells were done according to Arguello *et al.* (13). Mice were anesthetized by isoflurane inhalation, and the cells (0.1 ml of cell suspension containing 1 × 10<sup>5</sup> cells) were injected into the left ventricle of the heart using a 26-gauge needle inserted percutaneously near the midline. Twenty-eight days after injection of the cells, radiographic scans of all mice in the prone and lateral position (4 and 7 min, respectively, each at 35 kVp) were taken under anesthesia (150 mg/kg ketamine HCl/10 mg/kg xylazine HCl injected i.p.) using a Faxitron X-ray system model 43855A (Faxitron; X-Ray Corp., Wheeling, IL). The animals were sacrificed, and the heart, lungs, liver, adrenals, and ovaries were dissected, fixed in formalin, and embedded in paraffin. Both tibiae, femora, and humeri and the spinal column were also dissected, fixed in formalin, decalcified using Decalcifier I (Surgipath, Winnipeg, Manitoba, Canada) and embedded in paraffin.

**Treatment.** Doxycycline-containing pellets (10 mg/pellet with a timed-release of 21 days; Innovative Research of America, Sarasota, FL) were implanted s.c. 3 days before cancer cell injections. Placebo pellets were used in control animals. At the same time, pellets containing 0.25 mg of 17β-estradiol (21 day-release) were implanted.

**Histomorphometry.** Tumor burden in the long bones and the spinal column of each mouse were measured using a stereological technique (14). Longitudinal sections (thickness 4 µm) were cut through the middle part of the bone and stained with H&E. Using a point grid with a point area of 0.02292 mm<sup>2</sup>, the points overlying tumor tissue were counted and corrected for mag-

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<sup>3</sup> The abbreviation used is: matrix metalloproteinase.

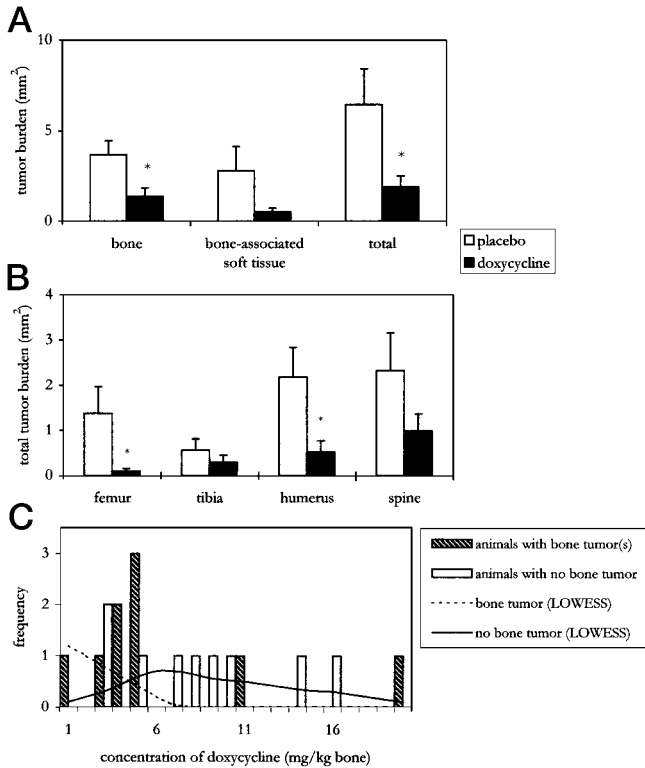


Fig. 1. Effect of doxycycline treatment on tumor burden in a mouse bone metastasis model of human breast cancer (MDA-MB-231). Mice received 21-day, timed-release pellets containing 10 mg of doxycycline 3 days before intracardiac MDA-MB-231 injections. Mice were sacrificed 28 days later. Longitudinal sections of the spine, femora, humeri, and tibiae of tumor-bearing animals ( $n = 14$  in placebo group;  $n = 9$  in the doxycycline group) were analyzed to determine tumor burden in bone and bone-associated soft tissue tumor burden (A) and total tumor burden per evaluated bone (B). The amount of doxycycline in the right radius/ulna of each doxycycline-treated mouse was determined using ELISA. C, frequency distribution of doxycycline concentrations measured in the radius/ulna of doxycycline-treated, tumor cell-injected mice. The original frequency distributions were smoothed according to the method of LOWESS. Mice that did not develop bone tumors were found to have higher concentrations of doxycycline incorporated into the radius/ulna than tumor-bearing animals. \*, significantly different from corresponding placebo group ( $P < 0.05$ ). Data represent means of the combined values of all animals in each of the groups in three separate experiments; bars, SE.

Table 1 Effect of doxycycline treatment on bone volume in mouse bone metastasis model of human breast cancer (MDA-MB-231)

Histomorphometric parameter <sup>a</sup>	Control		Tumor	
	Placebo	Doxycycline	Placebo	Doxycycline
BV/TV (%)	29.5 ± 0.75	29.2 ± 0.97	21.2 ± 1.18 <sup>b</sup>	23.5 ± 1.57 <sup>b</sup>
OV/BV (%)	0.49 ± 0.10	2.28 ± 0.96 <sup>c</sup>	1.00 ± 0.34	1.68 ± 1.52

<sup>a</sup> Longitudinal sections of the femora were analyzed to determine the histomorphometric parameters.

<sup>b,c</sup> Data represent mean ± SE of the combined values of all animals in each of the groups in three separate experiments; <sup>b</sup> significantly different from corresponding control (no tumor) group ( $P < 0.05$ ); <sup>c</sup> significantly different from corresponding placebo group ( $P < 0.05$ ).

nification. The total cumulative tumor area/animal was thus calculated and expressed as bone tumor burden and bone-associated soft tissue tumor burden. Histomorphometric measurements were performed on a cancellous area of the bones, for long bones starting 1 mm below the epiphyseal growth plate. To reduce intrabone variations, at least two sections and 10 microscopic fields with an average of 1833 test points/sample were evaluated ( $\times 100$  magnification). For tumor volume measurements, 10 microscopic fields were screened, whereas bone surfaces were measured on entire sections. The length of bone surface was multiplied by  $\pi/4$  to correct for the obliqueness of the sections. Measurements were performed using a digital color camera attached to a light microscope and Northern Eclipse image-analyzing software (Empix Imaging, Inc., Mississauga, Ontario, Canada). The following parameters were measured:

volume occupied by bone, and osteoid as a fraction of the total tissue volume [BV/TV and OV/TV (%) respectively], number of osteoclasts and osteoblasts per length of trabecular bone surface [ $N.Oc/BS$  and  $N.Ob/BS$  ( $\text{mm}^{-1}$ ) respectively], eroded bone surface, active resorption surface beneath or in contact with osteoclasts, and osteoid surface as a fraction of the trabecular bone surface [ $ES/BS$ ,  $Oc.S/BS$ , and  $OS/BS$  (%), respectively]. The nomenclature and symbols used in conventional bone histomorphometry were expressed according to Parfitt *et al.* (15).

**Tetracycline ELISA.** A commercially available ELISA kit (R-Biopharm GmbH, Darmstadt, Germany) was used to determine the concentration of doxycycline in plasma and to quantify the incorporation into the bone (right radius/ulna of each mouse). Each radius/ulna was decalcified in two steps, each time with 500  $\mu\text{l}$  of 1 M HCl for 8 h to release doxycycline. The combined supernatants of the decalcified bones or 100  $\mu\text{l}$  plasma were purified using a pre-equilibrated  $C_{18}$  (R-Biopharm GmbH) column. Doxycycline was eluted from the column using methanol containing 20 mM oxalic acid and immediately used in the ELISA assay in a dilution of 1:10. The ELISA kit was used according to the manufacturer's protocol using a standard curve of doxycycline ranging from 1,000–100,000 ng/l.

**Statistical Analysis.** Tumor burden data were tested for differences between means using a two-tailed Student's *t* test. Histomorphometric data were tested for differences between medians using a two-tailed Mann-Whitney test. Differences were considered significant at  $P < 0.05$ . Frequency distribution histograms were smoothed using the LOWESS method (using Minitab software; Minitab, Inc., State College, PA).

## Results and Discussion

MMPs play an important role in both bone degradation and in several steps in tumor invasion and metastasis. We have taken advantage of one of the key features of the tetracycline family of antibiotics, *i.e.*, their osteotropism, to deliver and accumulate an MMP inhibitor to bone, a common site for metastasis in breast cancer. We used an osteolytic bone metastasis model typical for human breast cancer first described by Sasaki *et al.* (16).

Six long bones (both femora, tibiae, and humeri), the spinal col-

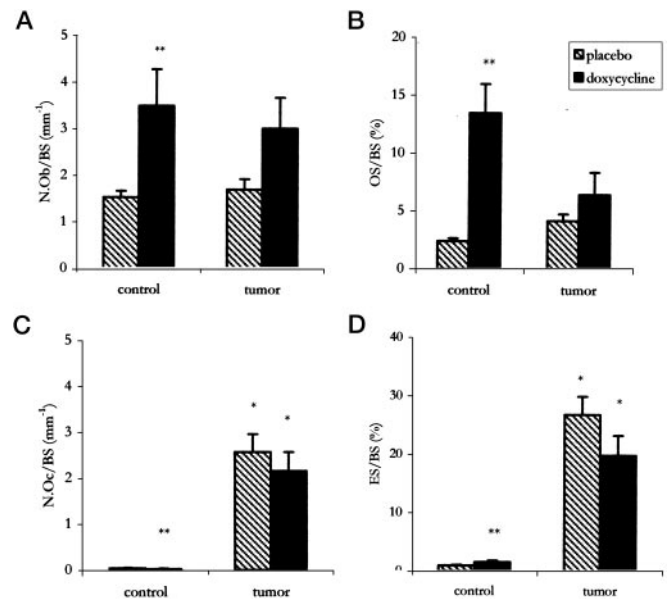
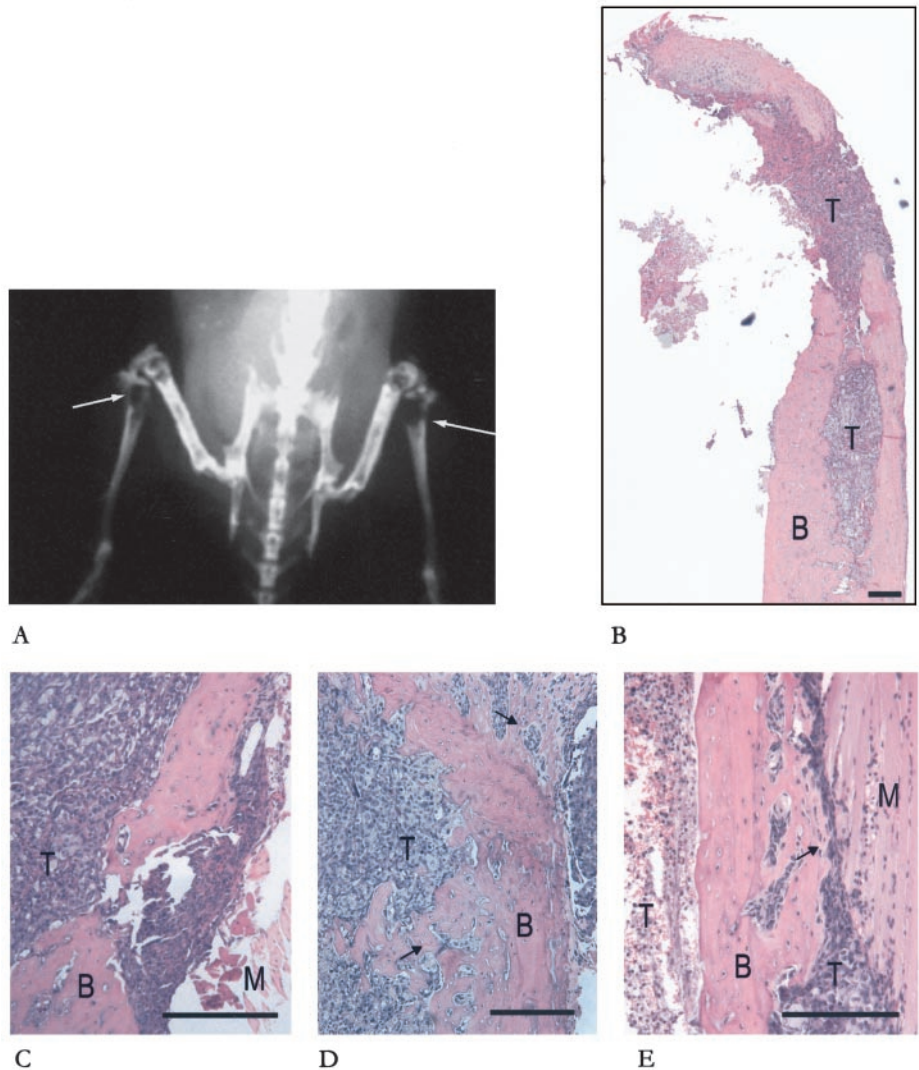


Fig. 2. Effect of doxycycline treatment on bone histomorphometric parameters in a mouse bone metastasis model of human breast cancer (MDA-MB-231). Longitudinal sections of the spine, femora, and humeri were analyzed to determine number of osteoblasts per length of trabecular bone surface ( $\text{mm}^{-1}$ ; A), osteoid surface as a fraction of the trabecular bone surface (%; B), and number of osteoclasts per length of trabecular bone surface ( $\text{mm}^{-1}$ ; C), eroded bone surface as a fraction of the trabecular bone surface (%; D). \*, significantly different from corresponding control group (no tumor cells injected;  $P < 0.05$ ). \*\*, significantly different from corresponding placebo group ( $P < 0.05$ ). Data represent means of the combined values of all animals in each of the groups in three separate experiments ( $n \geq 16$ ); bars, SE.

Fig. 3. Radiological appearance of typical osteolytic lesions (A) in both proximal tibiae of a mouse injected with MDA-MB-231 cells. Histological appearance of H&E sections of skeletal tumors in the left proximal tibia (B) of the same animal as in A is shown. It shows clearly the almost complete destruction of the epiphysis and the breaking of the tumor through the cortical bone. Histological appearance of MDA-MB-231 tumor cells in the humerus of a placebo-treated animal (C) and a doxycycline-treated animal at different magnifications in D and E is shown. It shows the breaking of the tumor through the cortical bone in C and abundant new bone formation as indicated by the arrows in D and periosteal new bone formation in E. T, tumor; B, bone; M, muscle. Bar, 200  $\mu$ m.



umn, and the visceral organs of each mouse were screened for the microscopic presence or absence of tumor. In only a few cases, tumor nodules were found in the heart, at the site of injection, and the lungs, in which case the tumor cells were most probably injected into the right ventricle of the heart, and the animal was excluded from the analysis. The presence of tumor was otherwise restricted to the bone. The treatment protocol of doxycycline with timed-release pellets of 21 days containing 10 mg of doxycycline results in a delivered dose of  $\sim 15$  mg/kg/day. We did not observe any adverse effects of doxycycline treatment in the mice over the 28-day period; toxicity would only be expected at 25–40-fold higher concentrations of doxycycline (17). The average doxycycline concentration in plasma amounted to 0.21 mg/l at day 18 as determined by ELISA, whereas the concentration of doxycycline in the radius/ulna reached 6.2 mg/kg bone (range, 0.97–20.6 mg/kg) at the time of sacrifice. In tumor-positive animals, doxycycline reduced the number of tumor-involved bones of the total of seven bones analyzed by 28%, from an average of  $3.9 \pm 0.5$  bones in the placebo group to  $2.8 \pm 0.6$  in the doxycycline group (data not shown); however, this was not statistically significant. Mice that did not develop bone tumors were found to have higher concentrations of doxycycline incorporated into the radius/ulna than tumor-bearing animals (frequency distribution histogram in Fig. 1C).

Doxycycline treatment did significantly reduce tumor burden (Fig. 1A). In the placebo group, the total cumulative tumor burden/animal

amounted to  $6.43 \pm 2.00$  mm<sup>2</sup> in tumor-bearing animals, whereas the total tumor burden in the doxycycline group was only  $1.88 \pm 0.61$  mm<sup>2</sup>, a reduction of 70%. The reduction in the bone and the bone-associated soft tissue tumor burden was 63 and 81%, respectively. The contribution of the spine and humerus to the total tumor burden/animal was the highest, followed by the femur and tibia, respectively (Fig. 1B). The total tumor burden in each of the bones was lower in the doxycycline-treated mice, but this decrease did not reach significance in the tibiae and spine.

Bone histomorphometry showed that doxycycline significantly increased several parameters of bone formation in the long bones including osteoid volume, osteoid surface, and the number of osteoblasts/bone surface (Table 1; Fig. 2, B and A, respectively). Similar differences were also observed when corresponding bones of tumor-injected mice were evaluated; however, they did not reach significance. These data confirm the published literature on the effect of tetracyclines on bone. Minocycline treatment of streptozotocin-induced diabetic rats results in a more pronounced ruffled border on osteoclasts as compared with many osteoclasts completely lacking a ruffled border and a clear zone in diabetic rats (18). In addition, more active cuboidal osteoblasts, a thicker osteoid layer, and increased collagen synthesis are seen in the treated rats compared with the nontreated diabetic ones (19).

It is known that the presence of breast cancer has a profound effect

on bone. Whereas in control animals hardly any osteoclasts could be found, the number of osteoclasts and the eroded bone surface increased markedly in tumor-bearing bones (Fig. 2, C and D). The bone resorption parameters, eroded surface, and number of osteoclasts were lower in the doxycycline-treated tumor-bearing animals than in the placebo group. H&E sections of the left proximal tibia (Fig. 3B) clearly show the virtually complete destruction of the epiphysis and the breaking of the tumor through the cortical bone, confirming the osteolytic lesions seen on the autoradiogram of the same animal (Fig. 3A). Osteolysis was also apparent on H&E sections of the humerus of a placebo-treated animal (Fig. 3C) and a doxycycline-treated animal as shown in Fig. 3, D and E. In the latter animal, there was also abundant new bone formation.

From the large reduction in tumor burden brought about by doxycycline, it appears more likely that the effectiveness relies on the properties of doxycycline as an inhibitor of tumor cell proliferation and less on its effect as a MMP inhibitor, because only a relative modest decrease in bone resorption was observed. Other osteolytic bone metastasis models, such as the one using orthotopic injections of 4T1 mouse mammary tumor cells (20), will be examined in future studies to confirm our data obtained with the MDA-MB-231 cells. Our results suggest that doxycycline may be useful not only for the treatment of osteolytic but also for the treatment of osteoblastic bone metastasis. However, this remains to be determined using animal models specific for osteoblastic bone metastasis, such as the MCF-7 human breast cancer cell model (20). The mechanism of action of doxycycline is very different from that of the bisphosphonate family of drugs that are used as therapeutic agents in clinical disorders characterized by increased osteolysis, including bone metastasis. Using the same bone metastasis model and a similar treatment protocol, the bisphosphonate risedronate also decreases the tumor burden (16), albeit in a less effective manner than doxycycline. Risedronate, however, has a considerable impact on bone resorption; it is capable of reducing the number of osteoclasts by 75%. On the other hand and in contrast to our data, the bone-associated soft tissue burden increases with risedronate treatment. Similarly, although via a different mechanism of action, recombinant osteoprotegerin, a member of the tumor necrosis factor receptor family that antagonizes OPG-ligand to bind to its receptor, results in an almost complete elimination of osteoclasts in the nude mouse bone metastasis model. This is accompanied by an 80% decrease in tumor area in the bone (21).

A prominent feature of bone metastasis of breast cancer is the uncoupling of bone remodeling, *i.e.*, an increase in bone resorption not followed by an increase in bone formation. We also observed this phenomenon in tumor-bearing bones and show that doxycycline can improve this to some extent by increasing bone formation. In doxycycline-treated tumor-bearing mice, the tissue volume occupied by bone was slightly increased, 23.5% compared with 21.2% in placebo. This was accompanied by an increase in bone formation parameters [number of osteoblasts (Fig. 2A) and osteoid surface (Fig. 2B) and volume (Table 1)] and a concomitant decrease in bone resorption parameters [number of osteoclasts, eroded bone surface (Fig. 2, C and D), and active resorption surface beneath or in contact with osteoclasts (data not shown)].

Whereas the current study clearly demonstrates the benefit of doxycycline when administered from the time of the development of the tumor, future experiments using the same model will also include treatment starting several weeks after the cell injections to determine

whether doxycycline treatment could also be beneficial for patients in whom bone metastasis is already established. In conclusion, doxycycline greatly reduced tumor burden and could also compensate for the increased bone resorption frequently associated with bone metastasis from breast cancer. Although doxycycline was not able to induce significant differences in tumor frequency or in the number of bones involved, treatment might still be very beneficial for breast cancer patients with or at risk for osteolytic bone metastasis.

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