Synergistic effect of bisphosphonate and docetaxel on the growth of bone metastasis in an animal model of established metastatic bone disease

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PRECLINICAL STUDY

Synergistic effect of bisphosphonate and docetaxel on the growth of bone metastasis in an animal model of established metastatic bone disease

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Abstract Bisphosphonates decrease bone resorption and reduce significantly the rate of skeletal complications in patients with metastatic bone disease. Bisphosphonates have also been shown to exhibit antitumor activity in vitro but in vivo results have been equivocal. In the present study, we investigated the effects of bisphosphonate treatment alone or in combination with the cytostatic docetaxel on the growth of breast cancer cells in bone. Tumor gowth was studied in an athymic nude mice model inoculated with MDA-231-B/luc+ breast cancer cells. Two days after the inoculation, mice were treated with risedronate, zolendronate or docetaxel alone or with a combination of risedronate and docetaxel. Bone destruction and tumor growth were evaluated radiographically, histologically and by whole-body bioiluminescent reporter imaging (BLI). Five week treatment with high doses risedronate or zoledronate (37.5–150 μg/kg, 5 times/week), fully protected the bones from osteolysis, but did not affect tumour growth. Docetaxel (2, 4, and 8 mg/kg, 2 times/week) inhibited tumour growth dose-dependently and after 5 weeks treatment with the highest dose, there was no detectable tumour in bone. The combination of a dose of docetaxel (4 mg/kg) that demonstrated only a minimal effect on tumour growth, with risedronate (150 μg/kg), protected bone integrity and nearly completely inhibited the growth of the cancer cells. Risedronate and docetaxel act synergistically to protect bone and decrease tumour burden in an animal model of established bone metastases from breast cancer cells.

Keywords Bisphosphonates · Bone metastasis · Breast cancer · Docetaxel · Risedronate · Zoledronate

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Introduction

Metastatic bone disease is a major cause of morbidity in patients with different cancers including those of the breast and the prostate [1]. Despite differences in the pathogenesis of bone metastases from different cancer types, increased osteoclast-mediated bone resorption is the major mechanism for tumor-induced bone destruction [2, 3]. Bisphosphonates decrease bone resorption and reduce significantly the rate of skeletal complications in patients with metastatic bone disease [4]. In addition, several in vitro studies reported that bisphosphonates have direct antiproliferative and proapoptotic effects on cancer cells and can inhibit the adhesion of cancer cells to mineralized matrices suggesting that these compounds may also have a favorable action on the growth and invasive behavior of cancer cells [5–8]. However, in vivo studies in animal models of bone metastasis have produced equivocal results [9–17].

These apparently discrepant results regarding an antitumor effect of bisphosphonates may be related to the timing of interference with bone turnover during the metastatic process. Decrease of bone turnover by bisphosphonates before colonization of bone by cancer cells, inhibits to a great extent the formation of bone metastases [15, 16]. However, when bisphosphonate treatment is given

after the establishment of bone metastases, it has a minimal effect on the progression of cancer growth despite a substantial reduction of osteolysis. It was hypothesized that cancer cells metastatic to bone after an initial growth phase that depends on their interaction with the local stroma, they become independent of microenvironment's growth support and progress autonomously [15]. For the arrest of growth of established metastases, compounds with mechanisms of action different from that of bisphosphonates will be needed. Previous studies with concomitant administration of bisphosphonates and chemotherapeutics have shown a reduction in metastatic growth to bone [18–23]. However, toxicity of the latter precludes the application of fully effective doses. In the present study, we, therefore, tested the hypothesis that doses of a cytostatic that lack full antitumor efficacy when given alone, can act synergistically with bisphosphonates to reduce the growth of bone metastases from breast cancer cells.

Material and methods

Cell line and culture conditions

Luciferase positive human MDA-MB-231 breast cancer cells (MDA-231-B/luc+), were used for in vivo optical imaging as described previously [24]. MDA-231-B/luc+ cells were cultured in DMEM (Life Technologies, Breda, The Netherlands) containing 4.5 g/l glucose and supplemented with 10% FCS (Life Technologies) and 800 µg/ml geneticin/G418 (Life Technologies).

Animals

Female nude mice (BALB/c nu/nu) were purchased from Charles River (Charles River, Maastricht, The Netherlands). Animals were housed in individual ventilated cages under sterile condition, and sterile food and water were provided ad libitum. Animal experiments were approved by the local committee for animal health, ethics and research of Leiden University and carried out in accordance with European Communities Council Directive 86/609/EEC.

Experimental animal model

MDA-231-B/luc+ cells were harvested at about 80% confluence after changing to geneticin-free medium 24 h before inoculation.

The animals were anesthetized using the isofluorane anesthesia system (XGI-8, Xenogen) and a single-cell suspensions of 1.5×10^5 MDA-231-B/Luc+ cells/10 µl PBS were injected into the right tibiae of 6-week old mice as described previously [24].

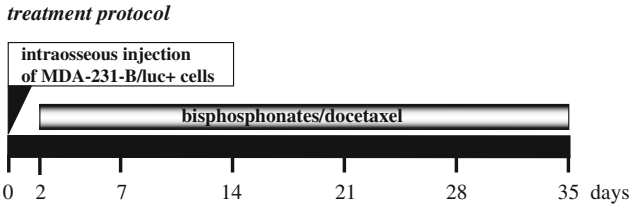

Fig. 1 Schematic representation of the treatment protocol. The left tibial bones of nude mice were injected with MDA-231-B/luc+ cells ("day 0") and 2 days later treatment with i.p. injections of bisphosphonate (5 times/week), docetaxel (2 times/week) or the combination was started. The mice were treated for a total of 35 days

Treatment of the animals started 2 days after intraosseous inoculation of MDA-231-B/Luc+ cells. From this time point (day 0) and during a subsequent period of 5 weeks, they received risedronate or zoledronate (5 times per week (100 µl by i.p. injection)) (dissolved in PBS), docetaxel (2 times per week (50 µl by i.p. injection) (dissolved in DMSO) or a combination of 5 times per week risedronate and 2 times per week docetaxel concurrently. The control animals received vehicle treatment. The different treatment schedules are illustrated in Fig. 1.

Bioluminescent reporter imaging (BLI)

Tumour progression of intraosseous growth was monitored weekly by BLI. For this, the mice were anesthetized as described above and injected i.p. with 2 mg D-luciferin sodium salt (Synchem OHG) dissolved in PBS, and measurements were done 5 min after the injection of D-luciferin. Bioluminescence imaging was acquired with a 15-cm FOV, a medium binning factor, and exposure times of 10–60 s. Imaging data were analyzed by using the program living image (Xenogen). Values are expressed as relative light units (RLU) in photons per seconds.

Radiographs

After the experimental periods, mice were sacrificed by cervical dislocation and the tumor bearing hind legs were removed and assessed for osteolytic lesions by radiography (Kodak X-OMAT TL film, Eastman Kodak Co.) using a Hewlett Packard X-ray system Faxitron 43805 and quantified using NIH Image 1.62b7 software as described earlier [25].

Histology

The skin of the dissected hind legs was removed and the bones were fixed for 24 h in PBS with 4% formaldehyde; subsequently, the bones were decalcified in water containing

10% EDTA, pH 6.4 and embedded in paraffin and submitted to Masson-Goldner staining as previously described [26].

Effects of bisphosphonate treatment on metaphysal dry weight of the tibia

To determine the effectiveness of bisphosphonate treatment, at the end of the experiment, the dry weight of the metaphysis of the right tibia (not inoculated with cancer cells) was measured as previously described [27].

Results

Tumor growth kinetics

Following inoculation of the left tibiae of athymic nude mice with MDA-231-B/Luc+ cells there was a progressive increase in tumor size with an increase of the BLI signal of more than 100-fold ($1.4 \cdot 10^5 \pm 2.5 \cdot 10^5$ to $2.1 \cdot 10^7 \pm 2.3 \cdot 10^7$ RLU) from day 7 to 35 (Fig. 2a). Figure 2b shows representative images of the BLI signal intensity in the tumor baring leg of a control mouse on day 7, 21 and 35, respectively.

Effects of bisphosphonates

We first examined the efficacy of bisphosphonates on normal bone resorption. For this, we measured the dry weight of the metaphyses of contralateral tibiae, which were not inoculated with cancer cells, of the animals after 5 weeks of bisphosphonate treatment. Compared to controls, zoledronate (37.5, 75, and 150 μg/kg) and risedronate (150 μg/kg) (5 times/week) increased significantly the mean metaphysal weight of the tibae of the mice: 112.7 ± 19.2 mg (control); 177 ± 13.9 mg (zoledronate 37.5 μg/kg); 173.7 ± 19.4 mg (zoledronate 75 μg/kg); 186.1 ± 16.7 mg (zoledronate 150 μg/kg) and 155.3 ± 15.0 mg (risedronate 150 μg/kg), respectively ($P < 0.01$ for all bisphosphonate doses). The lack of a dose-dependent effect in the zoledronate treated animals is due probably to already maximal inhibition of osteoclastic resorption by the lowest dose of this bisphosphonate used. Metaphysal weight in the risedronate-treated mice increased to the same extent as that in the zoledronate treated animals, indicating that resorption in these mice, was also maximally inhibited.

Both bisphosphonates prevented destruction of the tumor-bearing tibiae, assessed radiologically and histologically, but had no effect on tumor growth.

Figure 3 depicts representative radiographs, histological sections and BLI pictures of the tumor bearing legs, of these mice. Radiographically, the proximal tibia of the control animal was destroyed, whereas those of the bisphosphonate

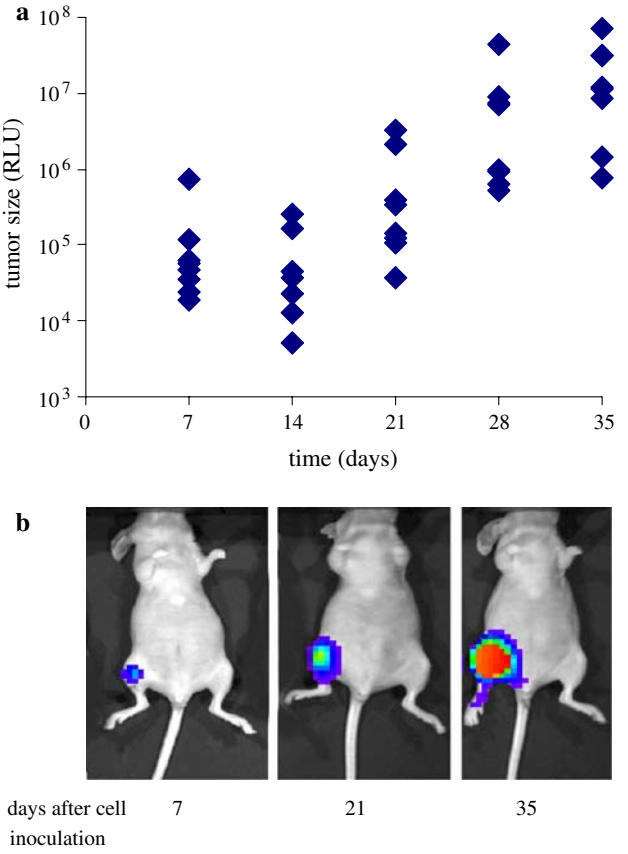

Fig. 2 **a** BLI measurements of tumor growth in control mice, monitored weekly during the 5-week experimental period. Results are expressed as individual mouse values. **b** Representative bioluminescent images of a control mouse at week 1, 3, and 5 after intraosseous inoculation of MDA-231-B/luc+ cells in the tibial bone

treated animals were intact, indicating protection of osteoclast-induced osteolysis by the bisphosphonates. Goldner stained histological sections demonstrated the presence of tumor in the legs of control mice. In bisphosphonate-treated mice there was a clear apparent reduction in the tumor within the bone whereas treatment had no effect on tumor load outside the bone collar. The bone marrow cavity of treated and untreated mice was invaded by the tumor which expanded outside the bone collar. Obviously, as result of their antiresorptive action, the tibial metaphysis of the bisphosphonate treated mice contained significantly more trabecular bone than that of controls. The results of BLI were consistent with the histological findings, as also shown previously [15, 24], and showed no difference in signal intensity between control and bisphosphonate-treated animals and neither bisphosphonate at any dose had any effect on tumor growth (Fig. 3b).

Effects of docetaxel

Figure 4a shows the effect of systemically administered docetaxel (2, 4 or 8 mg/kg, 2 times/week) on tumor growth

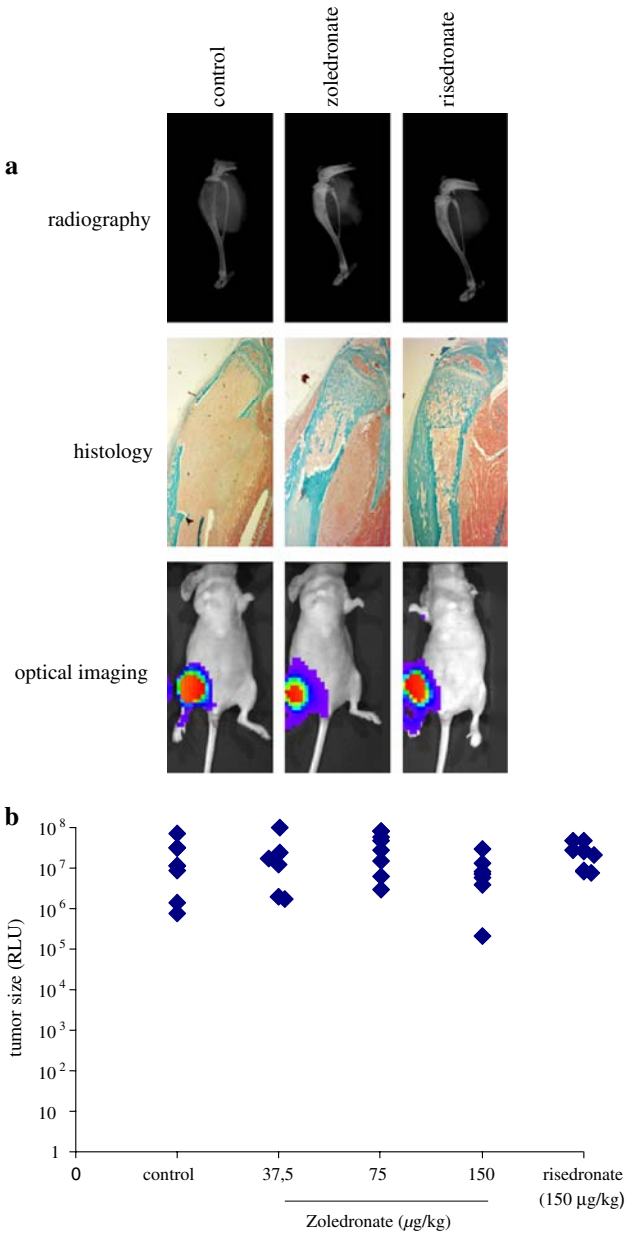

Fig. 3 **a** Representative radiographic, histological and bioluminescent images of a control, zoledronate (150 µg/kg) and risedronate (150 µg/kg) treated mouse after 5 weeks of treatment. **b** Effect of zoledronate (37.5, 75, and 150 µg/kg) and risedronate (150 µg/kg) on tumor growth after 5 weeks of treatment, monitored by BLI measurement

after 5 weeks of treatment. Docetaxel inhibited tumor growth dose-dependently, with no BLI signal being measurable at the highest dose tested. Histological examination of the tibiae corroborated BLI findings. In contrast to controls, the tibiae of mice treated with 8 mg/kg docetaxel were intact and there was no detectable tumor tissue (Fig. 4b). Qualitative evaluation of the metaphyses revealed further that the amount of metaphyseal trabecular bone of docetaxel-treated mice appeared similar to that of controls and less than that of bisphosphonate-treated animals after 5 weeks.

Effects of combined treatment with risedronate and docetaxel

To examine the effect of the concomitant administration of bisphosphonate and docetaxel on tumor growth, docetaxel was given at minimally effective concentrations (4 mg/kg, twice/week) and risedronate at a dose of 150 µg/kg (5 times/week). As shown in Fig. 5a, risedronate alone did not affect tumor growth, as expected, while docetaxel alone failed to reduce tumor growth in five of the seven mice. Treatment with the combination of docetaxel and risedronate, however, resulted in a total absence of BLI signal in six out of seven mice. Histological examination confirmed the optical imaging findings, and only in one animal in the combined treatment group a tumor was present whereas in the other six mice no cancer tissue could be detected. In addition, like in the mice treated with risedronate alone, the tibiae of the animals treated with the combination of docetaxel and risedronate showed no osteolysis and contained a large quantity of trabecular bone (Fig. 5b).

Discussion

We show here that combined treatment with a potent bisphosphonate and a cytostatic, at doses that have minimal effect on tumor growth when given alone, protects skeletal integrity and inhibits the growth of breast cancer cells in an animal model of metastatic bone disease. Animal and human studies have previously shown that increased bone resorption comprises the main mechanism responsible for bone destruction in metastatic disease and is related to the incidence and severity of skeletal complications in patients with malignancies [28, 29]. Breast cancer cells secrete factors, such as PTHrP, which stimulate the formation and activity of osteoclasts leading to bone destruction which causes bone pain, pathological fractures and hypercalcemia [1, 2, 30, 31]. This pathogenetic mechanism provided the rationale for the use of bisphosphonates in the management of patients with various tumours which metastasize to the skeleton, including those of the breast. However, during bone resorption induced by the osteoclasts, factors stored in the matrix of bone are also released in the bone marrow microenvironment and can act on cancer cells and stimulate further their growth as well as the production of bone resorbing factors [2, 31]. It was, therefore, thought that inhibitors of bone resorption, such as the bisphosphonates, may not only protect the integrity of bone at metastatic sites but may also have a favourable effect on the local growth of bone metastases. In addition, several in vitro

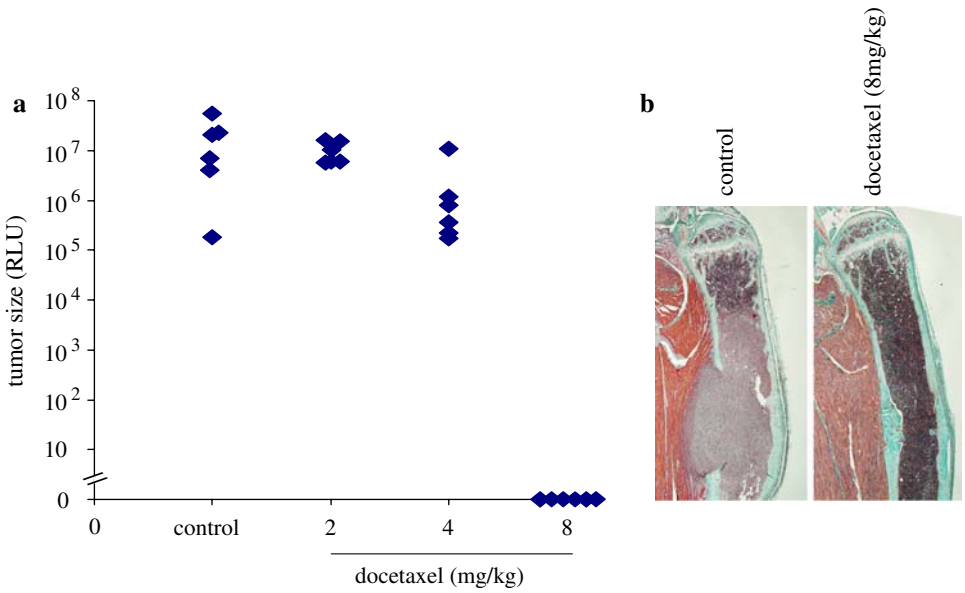

Fig. 4 **a** Effect of docetaxel (2, 4, and 8 mg/kg, 2 times/week) on tumor growth after 5 weeks of treatment, monitored by BLI measurement. **b** Histology of the tibiae of a control and a docetaxel (8 mg/kg) treated animal

studies have shown that bisphosphonates have direct effects on tumour cells, increase their rate of apoptosis, decrease angiogenesis and prevent their attachment on bone matrices [5, 6]. Thus, bisphosphonates, in addition, to their bone protective effect, may also reduce the growth potential of cancer cells in the bone-bone marrow microenvorment.

This attractive hypothesis has been, however, difficult to prove experimentally or clinically and appears to depend on the stage of the metastatic process as well as on the techniques used to assess cancer growth. For example, interference with the bone microenvironment with bisphosphonates before the establishment of bone metastases protects bone integrity and inhibits tumour growth. However, when bisphosphonates are given after the establishment of bone metastases, their effect on tumour growth is minimal as also shown in the present study. Furthermore, in studies reporting a beneficial effect on the tumour burden following bisphosphonate treatment, this is generally evaluated by histology of the area contained within the bones of animal models. However, it has been shown that tumour growth outside the bone collar was not affected by treatment and that the apparent decrease in tumour growth within bone was rather due to the decreased space available due to the preservation of the bone structure [15]. Such histological findings were supported by studies which assessed directly tumour growth by molecular imaging techniques and showed no effect in the overall growth of cancer cells [15]. We confirmed this in the present study and we showed that treatment with the two very potent bisphosphonates risedronate and zoledronate given at high doses with similar antiresorptive potencies to an animal model of established bone metastases were very effective in decreasing bone resorption and preventing bone destruction. However, bisphosphonate treatment given alone had only a minor effect on tumour growth assessed by histology and BLI once the tumour had been established in the bone marrow. In contrast, treatment with high doses of docetaxel did not only preserve the structure of bone but decreased also significantly the growth of the cancer cells within and outside the bone collar.

In recent years the significance of the interactions between tumour cells and cells of the bone marrow in the development of micrometastases to overt metastases has been increasingly recognized [2, 31]. In this process, increased bone resorption plays an important role and promotes the initial growth of cancer cells. However, once these cells develop into macrometastases mechanisms other than bone resorption contribute to their growth potential, such as for example angiogenesis. This sequence of events explains why a bisphosphonate given to animals for prevention of bone metastases is effective whereas when given to models with established metastatic disease has minimal effect on the further growth of the tumour. The lack of an antitumour effect of bisphosphonates on bone metastases in vivo despite the demonstration of such effects in vitro is probably attributed to the specific pharmacokinetics of these compounds. Bisphosphonates are cleared rapidly from the circulation and are taken up preferentially by the skeleton at active remodelling sites where they bind strongly to bone [32, 33]. This action allows only very limited, if any, exposure of the cancer cells in the marrow to bisphosphonates [34]. Therefore, for the adequate management of established metastatic disease in bone, bisphosphonates may have to be combined with other agents which specifically affect tumour growth and progression.

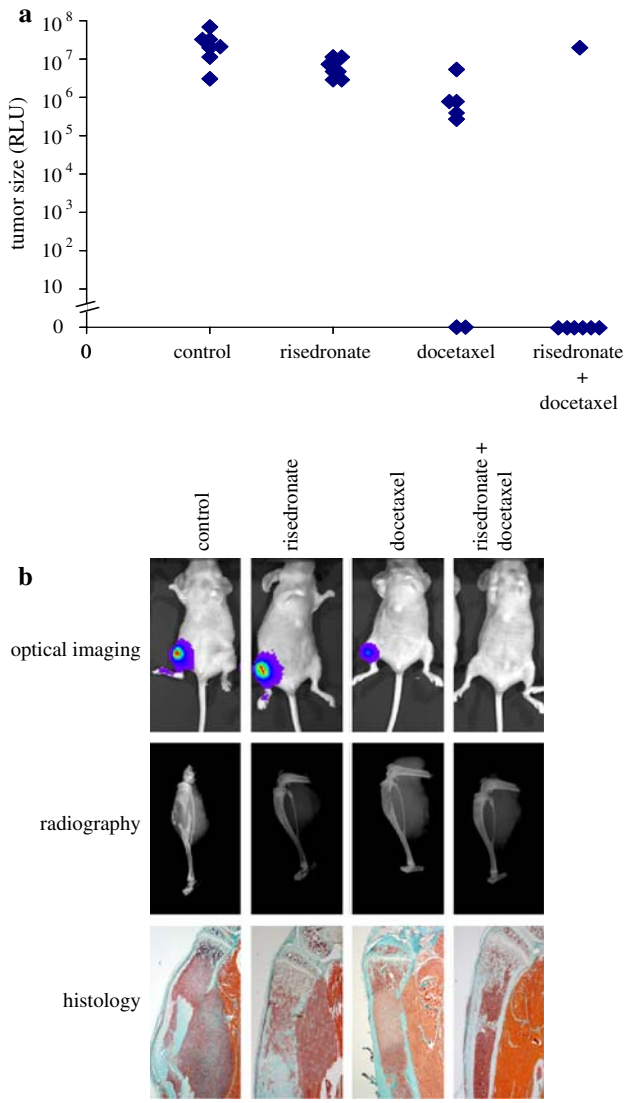

Fig. 5 **a** Effect of risedronate (150 μg/kg, 5 times/week) and docetaxel (4 mg/kg, 2 times per/week) treatment, alone or in combination, on tumor growth after 5 weeks of treatment, monitored by BLI measurement. Difference among groups $P < 0.001$ (one-way ANOVA); combination therapy ($P < 0.001$) and docetaxel ($P < 0.05$) different from risedronate alone; combination therapy different from docetaxel alone ($P < 0.05$). **b** Representative bioluminescent, radiographic and histological images of a control, risedronate (150 g/kg), docetaxel (4 mg/kg) and risedronate + docetaxel treated mouse after 5 weeks of treatment

Previous studies with bisphosphonates in combination with antitumor drugs were effective in decreasing tumour growth in relevant animal models and in vitro evidence of a synergism has been reported [34–37]. The question, therefore, addressed in this study was whether the combination of a bisphosphonate with a dose of a chemotherapeutic that has no effect on tumour growth when given alone, might act synergistically on tumour growth in vivo. Our results showed that a dose of docetaxel that affected tumour growth minimally, when dosed alone, had a profound effect on the growth of breast cancer cells in bone when dosed in combination with risedronate. In all but one of the treated animals with risedronate and the lower dose of docetaxel tumour cells were completely eliminated from bone. Thus, the combined treatment did not only preserve the structural integrity of bone but had a clear antitumour effect demonstrated both histologically and by BLI. Interestingly, trabecular bone of the animals treated with risedronate and docetaxel appeared to be better preserved than the bone of the animals which received the higher docetaxel dose. This should be attributed to the specific action of the bisphosphonate on bone.

In conclusion, bisphosphonates and chemotherapeutics act synergistically to protect bone and decrease tumour burden in an animal model of established bone metastases from breast cancer cells. This approach warrants further investigation in animal and human studies, as it may allow the use of less toxic dose of chemotherapeutics in the management of patients with bone metastases.

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