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Pathogenesis and treatment of skeletal metastasis : studies in animal models

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Chapter 4

Interference with the Microenvironment Support Impairs the *De Novo* Formation of Bone Metastasis *In Vivo*

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Abstract

Interference with the microenvironmental growth support is an attractive therapeutic strategy for repressing metastatic tumor growth. Bone is a highly dynamic tissue that is continuously remodelled by bone resorption and subsequent bone formation. Growth factors supporting bone metastatic growth are released especially during bone resorption. Differently from most of the other tissues, drugs that can limit local turnover, namely bisphosphonates (BPs), are available for bone. Thus, we investigated whether pharmacological interference with bone turnover could repress development and growth progression of experimental bone metastasis. Whole body bioluminescent imaging (BLI) was adopted as a validated and reliable method for sensitive detection, monitoring and quantification *in vivo* of the growth progression of bone metastases induced by intracardiac or intraosseous injection of luciferase-transfected breast cancer cells (MDA-231-B/Luc+). Preventive BP-induced reduction of bone turnover, prior to bone colonization by cancer cells, inhibited by a great extent the number of developing bone metastases. Nevertheless, tumor growth in the few, but still developing bone metastases, was affected only transiently. Reduction of bone turnover was also unable to control growth progression of bone metastases, either induced by hematogenous spreading or by intraosseous inoculation, which were already established when BP-treatment was initiated. This, despite a substantial anti-osteolytic effect induced by BPs. Therefore, bone metastatic cancer cells, after an initial growth phase dependent on the interaction with the local stroma, become independent on microenvironmental growth factor support and progress autonomously. Inhibition of bone turnover may represent a useful adjuvant therapy for cancer patients at risk to develop bone metastasis.

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Introduction

Micrometastases, persisting in various tissues of cancer patients after removal of the primary tumor, represent the pathophysiological basis for cancer relapse as overt metastases. Preferential colonization of certain tissues by cancer cells and their subsequent growth are determined by interaction with the tissue-specific microenvironment (Seed & Soil theory) [1,2]. Therefore, pharmacological interference with the microenvironmental growth support, is becoming an attractive therapeutic strategy for repressing metastatic tumor growth [3].

Bone metastases are common in cancer, especially breast and prostate carcinoma. They cause considerable morbidity characterized by severe bone pain and high incidence of skeletal, neurological and hematopoietic complications (hypercalcemia, fracture, spinal cord compression and bone marrow aplasia [4-9]. Evidence from animal studies supports the concept that the rate of bone turnover is directly related to the occurrence and progression of bone metastases [10-13]. Recently, this view has been further substantiated by a clinical study, showing a strong and highly significant association between the rate of bone resorption and incidence of subsequent skeletal complications in breast and prostate cancer patients [14,15].

The skeleton is a highly dynamic tissue that is continuously renewed through the process of bone remodelling. This occurs at multiple sites in the skeleton by temporary structures called 'basic multicellular units' (BMU) [16,17]. The result is the replacement of old bone with new bone, thus maintaining the structural integrity and the mechanical competence of the skeleton throughout adult life. The number and the activity of these BMUs determine the rate of bone turnover [18]. During bone remodelling osteoblasts and osteoclasts, the cellular components of the BMU, secrete paracrine factors that induce chemotaxis and cell adhesion, support cell survival and growth, and stimulate angiogenesis [19,20]. Furthermore, there is experimental evidence showing that growth factors like transforming growth factor-β (TGF-β)are released from the bone matrix and stimulate the secretion of bone active cytokines, which not only enhance bone resorption, but also stimulate further tumor growth ('vicious circle') [19-25].

Taken together the evidence above suggests that pharmacological suppression of bone turnover may not only protect skeletal integrity at the metastatic site and reduce the incidence of skeletal complications, as has been shown with bisphoshonates (BPs), but may also interfere with the local growth support of cancer cells, thus preventing the development and progression of bone metastases. We tested this hypothesis by BP-induced inhibition of bone turnover in animal models of bone metastasis induced by luciferase-expressing human MDA-MB-231 breast cancer cells. Metastatic tumor development and progression was monitored by whole body bioluminescent reporter imaging (BLI) as described earlier [26].

BLI allows spatio-temporal and quantitative analyses of tumor growth and, due to its sensitivity, is ideally suited to evaluate the effectiveness of therapeutic approaches that target both early stages of metastatic development and advanced metastatic disease. BLI

gives detailed information on localization and growth of minimal metastatic deposits in the bone marrow of experimental animals at a stage largely preceding tumor detection by other methods. Simultaneous assessment of bone destruction by morphometrical analyses (radiographs, histomorphometry) and of tumor burden (BLI) allows discrimination between bone sparing effects (inhibition of tumor-induced osteolysis) and interference with tumor progression (viability and growth). Evidence is presented that bone metastatic cancer cells, after an initial stage that is probably necessary to initiate a self-maintaining autocrine growth, become independent from the microenvironmental growth factor support and, thus, expand autonomously, independent of pharmacological reduction of bone turnover.

Materials and Methods

Cell line and culture conditions

MDA-MB-231 (MDA-231), an estrogen independent human breast cancer cell line, was obtained from the American Type Culture Collection (Rockville, MD, USA). A subclone inducing invariably bone metastases after intracardiac inoculation (bone-seeking clone MDA-231-B) has been established by 4 consecutive sequential cycles of intracardiac inoculation of MDA-MB-231 *in vivo* and subsequent expansion *in vitro* of the cell population recovered from the resulting bone metastases as described earlier [26]. MDA-231-B cells were stably transfected with a CMV-promotor driven mammalian expression vector for luciferase, CMV-luc, and one clone with the highest expression of luciferase expression (MDA-231-B/Luc+) was successfully used for *in vivo* optical imaging as described previously [26]. MDA-231-B/Luc+ cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco-BRL, Breda, the Netherlands) containing 4.5 g of glucose/L and supplemented with 10 % fetal calf serum (Gibco-BRL, Breda, the Netherlands), and 800 µg/ml geneticyne/G418 (Gibco-BRL, Breda, the Netherlands). Cells were regularly certified free of mycoplasma contamination.

All MDA-231-B/Luc+cell cultures were harvested at sub-confluence after being re-fed with fresh medium 24 hours before inoculation preparation. Cell suspensions of MDA-231-B/Luc+(1 x 10^5 cells/100 µl PBS for intracardiac inoculation or 1 x 10^5 cells /10 µl PBS for intraosseous inoculation) were prepared as described previously [26-28].

Animals

Female nude (BALB/c *nu/nu*) mice were purchased from Charles River (L'Arbresle, France). They were housed in individual ventilated cages under sterile condition according to the Dutch guidelines for the care and use of laboratory animals (DEC). Sterile food and water were provided ad libitum. Mice were 6 weeks old when used for the intracardiac or intraosseous inoculation of cancer cells.

For surgical and analytical procedures (intraosseous inoculation, whole body imaging and skeletal radiography) mice were anaesthesised by intraperitoneal injection of a 50 µl

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1:1:1 mixture; Ketamine HCl (Stock solution of 100 mg/ml Nimatek; Vetimex Animal Health B.V., Bladel, The Netherlands.) + Xylazine (2 % Rompun, Bayer AG, Leverkusen, Germany) + PBS (pH 6.8). Intracardiac inoculation of cancer cells was performed under Isofluorane anaesthesia (0.8 L/min, Isofluorane, Air Products, Waddinxveen, The Netherlands) using the Vapex3 system (VetTech Solutions Ltd, United Kingdom). At the end of the experimental period animals were sacrificed by cervical dislocation.

Induction of systemic metastates by intracardiac (i.c.) injection of MDA-231-B/Luc+cells

A single cell suspension of 1 x 10^5 MDA-231-B/Luc+cells /100 µl PBS was injected into the left cardiac ventricle (i.c.) according to the method originally described by Arguello and co-workers [29] and modified for MDA-MB-231 cells by Sasaki and co-workers [30]. The progression of the cancer cell growth was monitored weekly for by optical imaging as described previously [26]. At the same time points, osteolytic bone metastases were monitored by radiographs and analyzed as published earlier [26-28]. After the experimental period, the animals were sacrificed, and selected long bones site of cancer metastasis dissected and processed for further histomorphometric and immunohistochemical analysis (see below).

Intraosseous inoculation (i.o.) of MDA-231-B/Luc+cells

A single cell suspension of MDA-231-B/Luc+cells was injected into the right tibiae as described previously [26-28]. In brief, two holes, 4 to 5 mm apart and each with a diameter of ~ 0.35 mm, were drilled through the bone cortex of the upper right tibia with the aid of a dental drill. Space in the bone marrow was created by flushing out the bone marrow from the proximal end of the shaft. The upper hole was sealed by surgical wax and 1 x 10^5 MDA-231-B/Luc+cells per 10 µl PBS were slowly inoculated via a 30-gauge needle through the lower hole. Finally the lower hole was sealed with surgical wax and the cutaneous wound was sutured. The progression of cancer cell growth was monitored weekly by optical imaging as described previously (see below). At the same time points, osteolytic bone metastases were monitored by radiographs and analyzed as described previously [26-28]. After the experimental period the animals were sacrificed, the tibia site of intraosseous inoculation dissected and processed for further histomorphometrical and immunohistochemical analysis (see below).

Bioluminescent reporter imaging (BLI) and quantification of the bioluminescent signal

Whole body optical imaging of tumors induced by the luciferase-expressing human breast cancer cell line MDA-MB-231, inoculated either i.o. or i.c, was performed as described earlier [26]. After intra-peritoneal administration of 2 mg D-luciferin (Perbio Science Nederland B.V., Etten-Leur, The Netherlands), the animals were immediately transferred to a light-tight chamber and reference gray-scale body-surface images were taken using a intensified charged-coupled device (CCD) camera (C2400-77AH-01, Hamamatsu Photonics K.K.,

Japan) fitted with a 25 mm/0.95 f (optical aperture) objective (Schneider Optik, Kreuznanch, Germany). Five minutes after administration of D-luciferin, photon emission was integrated for a period of 3 minutes and processed using an Argus 20 image processor and M4314 image intensifier (Hamamatsu). Gray-scale images and bioluminescent images were superimposed using OpenLab software (Improvision, Coventry, UK). The relative light intensity was visualized by pseudocolors. Analyses for each metastatic site were performed after definition of the region of interest (= ROI, Openlab software) and quantified as described previously [26]. Values are expressed as relative light units (RLU).

Radiographical Analyses

After intraosseous or intracardiac inoculation of MDA-231-B/Luc+breast cancer cells into 6-week-old nude mice the formation of osteolytic lesions of the in the skeleton was assessed by radiography (Kodak X-OMAT TL film, Eastman Kodak Company, Rochester NY) using a Hewlett Packard X-ray system Faxitron 43805 as described previously [27,28]. Osteolytic areas were measured using NIH-Image 1.62b7 software as described earlier [27,28].

Histomorphometry, Histochemistry and Immunohistochemistry

Dissected long bones were fixed in 4 % paraformaldehyde (pH 6.8), decalcified as described previously and processed for paraffin embedding [27,28]. Longitudinal sections (5 μm) were cut through the sagittal plane of the tibiae containing tumors induced by the i.o. inoculation of MDA-231-B/Luc+cells. Sections were either submitted to Goldner staining or histochemical staining for tartrate resistant acid phospahtase (TRAcP) as described previously [27,28].

Histomorphometric measurements of tumor burden were performed on central sections through the tumor (largest tumor area). Tumor growth in bone could be readily identified by cytokeratin staining alone or in combination with HE staining. Total tumor area, as an estimate of total tumor burden, was measured by image analysis using NIH-Image 1.62b7 Image Analyses Software as described previously by us [27,28]. Subsequently, a distinction was made between the total tumor burden and intra- and extraosseous tumor burden. For this the digital image of the total tumor area was subdivided into an area delimited by the bone cortex or, were this has been partially resorbed as a result of the tumor-induced osteolysis, by a virtual line joining the remnants of the bone cortex, to define 'intraosseous' tumor growth. In addition, extraosseous tumor growth was defined as extramedullary growth of cancer cells (tumor cells growing surrounding the bone cortex or its remnants).

Histomorphometric measurements of trabecular bone volumes were performed after Goldner staining on the same central sections as used for tumor burden measurements (see above). Trabecular bone volume (TBV) was estimated in the proximal tibia by measuring the total area of trabecular structures in an area 0-2 mm distal to the capillary invasion front of the growth plate. TBV is expressed as percentage of the total area that was covered by trabeculae.

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Bisphosphonate treatment

Treatment protocols to establish the effects of olpadronate on the generation and progression of systemic bone metastases induced by i.c. injection of MDA-231-B/Luc+cells were performed according to the following two schemes (Fig. 1):

Preventive protocol: effect of long-term BP treatment on '*de novo*' generation and growth of systemic bone metastases (Fig. 1*A*). Beginning from two days prior to inoculation of MDA-231-B/Luc+cells the mice were treated by daily s.c. injections with 1.6 μM olpadronate/kg/day for the whole duration of the *in vivo* study (48 days). The generation and progression of bone metastases was monitored by BLI and radiography at day 7, 14, 21, 28, 33, 39 and 46. After 48 days of BP treatment (= day 46 from i.c. injection of cancer cells) the animals were sacrificed.

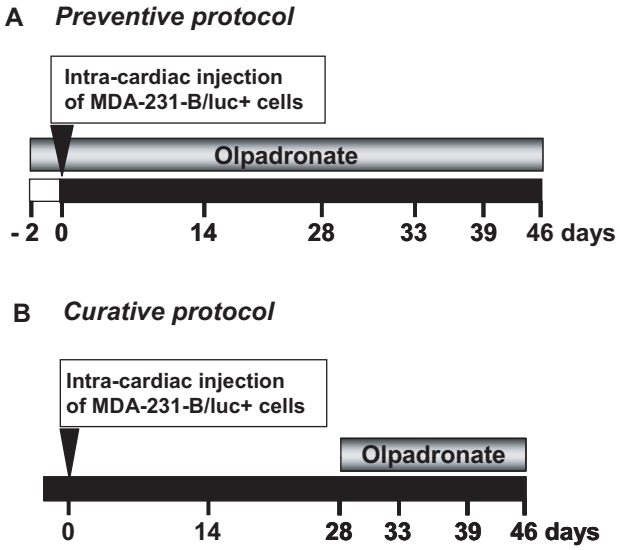


Figure 1 **Preventive and curative protocols for bisphosphonate (BP) treatment in a heart injection model of bone metastasis.** *A*, in the preventive protocol, 4-week-old female BALB/c *nu/nu* mice were treated with 1.6 μM/kg/day olpadronate for two days prior to inoculation of cancer cells. Subsequently 10^5 MDA-231-B/Luc+cells were inoculated into the left cardiac ventricle and the animals were treated by daily s.c. injections with 1.6 μM/kg/d of olpadronate for the duration of the experiment. The formation of bone metastases (and quantification of individual metastasis) was monitored and quantified weekly by whole body bioluminescent reporter imaging (BLI). In the same animals, the formation of osteolytic lesions was followed by radiography. *B*, in the curative protocol, 10^5 MDA-231-B/Luc+cells were inoculated into the left cardiac of ventricle 4-week-old female BALB/c *nu/nu*. Animals were monitored weekly to assess the formation of distant metastases and osteolysis (BLI, radiography). Starting from 28 days after cancer cell inoculation, the animals were equally distributed into two experimental groups and they were given either 1.6 μM/kg/day olpadronate or vehicle. Subsequently the progression of bone metastasis was monitored by BLI and radiography at day 5, 11 and 18. After 18 days of BP treatment (= day 46 from intracardiac injection) the animals were sacrificed.

Curative protocol: effect of short-term BP treatment on growth of established bone metastases (Fig. 1*B*). The presence of established bone metastases was verified by BLI and radiography. 28 days after i.c. injection of MDA-231-B/Luc+cells the animals were equally distributed into two experimental groups based on a comparable bone metastatic tumor burden/mouse, as detected by BLI. Subsequently, to one experimental group 1.6 μM/kg/day olpadronate was given, while to the other vehicle was administered (0.9% NaCl) for the following 18 days. The progression of bone metastasis was monitored by BLI and radiography at day 5, 11 and 18. After 18 days of BP treatment (= day 46 from i.c. inoculation of cancer cells) the animals were sacrificed.

To investigate the effect of BP on tumor development and osteolysis subsequent to intraosseous inoculation of MDA-231-B/Luc+cells, beginning from day 3 after inoculation (day of inoculation = day 0) the animals were treated for a period of 40 days by daily subcutaneous (s.c.) injections of BP dissolved in 0.9 % NaCl (50 μl volume) at the following doses: 16 μM/kg/d pamidronate, 1.6 μM/kg/d olpadronate. These doses were shown previously to complete inhibit osteoclastic bone resorption *in vivo* (31, 32). After this treatment period the animals were sacrificed. Alternatively, in order to achieve constantly elevated blood levels of BP during the entire period of treatment, 16 μM/kg/day of olpadronate dissolved in 0.9 % NaCl, a dose of ten fold higher than the maximal resorption-inhibitory dose *in vivo*, was continuously infused by osmotic minipumps (200 μl, 0.25 μl/hr, model 2004; Alzet Scientific products, Alza Corporation, Mountain View, CA) over a period of 28 days, beginning from day 0 after i.o. inoculation of MDA-231-B/Luc+cells. Osmotic minipumps filled with vehicle solution (0.9% NaCl in water) were implanted in a control group. The animals were sacrificed at the end of the treatment period.

BP effect on the expression of luciferase in MDA-231-B/Luc+cells

To exclude putative bisphosphonate effects on luciferase expression by MDA-231-B/Luc+cells, the breast cancer cells were cultured in the continuous presence of bisphosphonates for up to 48 hours. Continuous treatment of MDA-231-B/Luc+cells with a dose range of bisphosphonates (10-4 – 10-8 M for 24 and 48 hours) did not affect functional expression of the luciferase gene in MDA-231-B/Luc+cells *in vitro* as measured in luciferase assays and semi-quantitative PCR (results not shown).

Statistical Analysis

All data are represented as mean ± s.e.m. for animal studies and mean ± SD for histomorphometrical analyses. Statistical evaluation was carried out by ANOVA or two-tailed Student's t-test.

Results

Effect of BP treatment on the *'de novo'* generation and progression of systemic bone metastases ('Preventive' protocol).

In this experiment we investigated whether pharmacological inhibition of bone turnover prior to inoculation of cancer cells, and continued for the duration of the entire experiment, could prevent the initiation and maintenance of a positive interaction between the bone microenvironment and cancer cells. For this purpose olpadronate was given two days prior to inoculation of MDA-231-B/Luc+cells into the left cardiac ventricle and continued throughout the entire duration of the experiment (48 days) (Fig. 1).

Consistently with our previous findings [26] BLI showed that MDA-231-B/Luc+cells metastasize exclusively to the skeleton. At the end of the experimental period (day 47) olpadronate treatment decreased significantly the number of bone metastases per animal, as detected by BLI, by about 70% (Fig. 2*A*). Radiographic analysis in the same animals showed, from day 40 onwards, almost no osteolytic bone lesions in BP-treated animals as compared to control (Fig. 2*B*).

During the early experimental period olpadronate treatment significantly delayed the progression of the bone metastases, detected by BLI as total tumor burden per animal, (Fig. 2*C*, day 26-33), and tumor burden for each bone metastatic site (Fig. 2*D*, day 26-40). However, this effect was only transient and at later stages both the total burden per mouse (Fig. 2*C*, day 40-47) and the tumor burden per bone metastatic site (Fig. 2*D*, day 47) in the olpadronate treated animals became equivalent to those of the vehicle-treated animals.

Effect of BP treatment on growth of established bone metastases ('Curative' protocol).

In this experiment we investigated the effect of pharmacological inhibition of bone turnover on the bone microenvironmental growth support for cancer metastases that are already in an advanced stage of progression. For this purpose, daily s.c. administration of olpadronate (1.6 μM/kg/d for 18 days) was initiated when bone metastases had already developed (day 28) following i.c. injection of MDA 231-B/Luc+ cells (Fig. 1*B*).

As in the previous experiment MDA-231-B/Luc+cells metastasized exclusively to the skeleton. Olpadronate treatment did not affect the number of bone metastases detected by BLI (Fig. 3A), but significantly reduced at the end of treatment period (day 18) the number of osteolytic lesions per mouse as assessed by radiography (Fig. 3*B*). A discrepancy between BLI and radiography in the number of bone metastases detected in control and olpadronate treated animals can be noticed (Fig. 3*A,B*).

Olpadronate treatment transiently affected (day 5), although not significantly, total tumor burden per animal, as detected by BLI (Fig. 3*C*). However, at later time points tumor growth was not further affected by olpadronate (Fig. 3*C*) and at the end of the experiment it even enhanced significantly tumor burden per bone metastatic lesion (Fig. 3*C,D*).

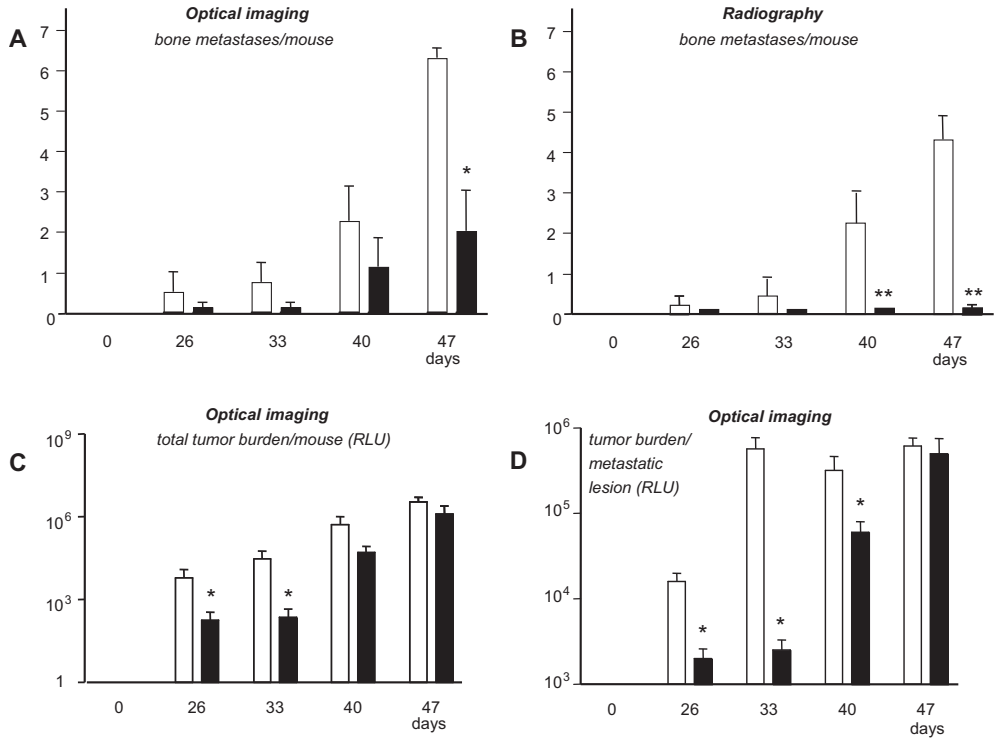


Figure 2 **Prevention of bone metastasis formation by the BP Olpadronate after inoculation of MDA-231-B/Luc+into the left cardiac ventricle of nude mice ('Preventive protocol').** Olpadronate (or vehicle) was given two days prior to inoculation of MDA-231-B/Luc+cells into the left cardiac ventricle and continued for the duration of the experiment (1.6 μM/kg/d for 48 days). Distribution and growth of MDA-231-B/Luc+cells was monitored weekly by BLI and radiography. Under both experimental conditions, MDA-231-B/Luc+cells metastasize exclusively to the skeleton. *A*, number of bone metastases per mouse determined by BLI. *B*, number of bone metastases per mouse determined by radiographical analysis. *C*, Total tumor burden per mouse (BLI). *D*, tumor burden per bone metastatic lesion (BLI). BLI measurements (C and D) are expressed relative light units (RLU). Open bars and closed bars represent Vehicle-treated and olpadronate-treated animals respectively. Values are expressed as means ± s.e.m., n=8. * $p \leq 0.05$, ** $p \leq 0.001$.

As shown in a representative radiograph of an olpadronate treated animal (Fig. 4*A*), there is minimal osteolysis in the left proximal tibia and proximal humerus. However, the photon emission detected by BLI of the same bone areas (Fig. 4*B*) indicates that these bones are site of metastatic growth of MDA-231-B/Luc+cells. Due to the limited intraosseous space, most of this growth seems to have taken place in the soft tissues surrounding bone, as revealed on the radiograph by the tumor shadow present especially around the left humerus (Fig. 4).

Effect of BP-treatment on intraosseous growth of breast cancer cells.

To investigate in more detail the cellular events during inhibition of bone turnover, progression and growth of cancer cells was analyzed in a model of tumor growth in the bone marrow. For

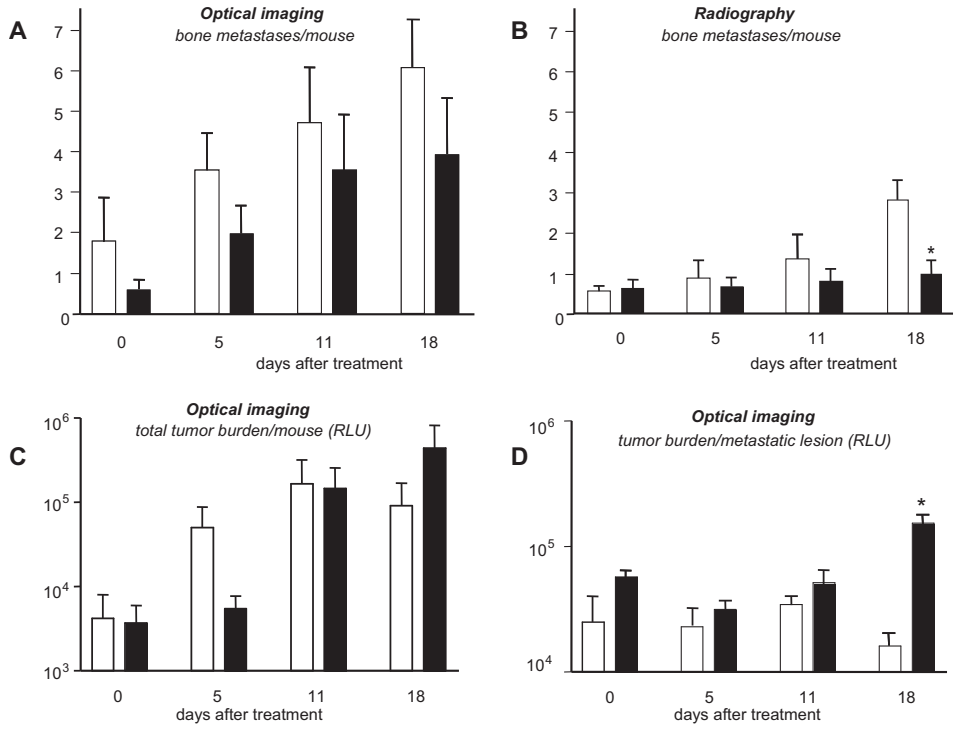


Figure 3 **Effects of olpadronate treatment on existing bone metastases, induced by inoculation of MDA-231-B/Luc+into the left cardiac ventricle of nude mice according to the 'Curative protocol' (see figure 1 B).** Daily s.c. administration of olpadronate (1.6 μM/kg/d for 18 days), was initiated when bone metastases had already developed (day 28) following i.c. injection of MDA 231-B/luc+ cells. The fate and growth of MDA-231-B/Luc+cells were monitored weekly by BLI and radiography. Under both experimental conditions, MDA-231-B/Luc+cells metastasize exclusively to the skeleton. *A*, number of bone metastases per mouse determined by BLI. *B*, number of bone metastases per mouse determined by radiographical analysis. *C*, total tumor burden per mouse (BLI). *D*, tumor burden per bone metastatic lesion (BLI). BLI measurements (figures 2 C and D) are expressed relative light units (RLU). Open bars and closed bars represent Vehicle-treated and olpadronate-treated animals respectively. Values are expressed as means ± s.e.m., n=8. * $p \leq 0.05$.

this purpose MDA-231-B/Luc+cells were inoculated directly into the tibiae of nude mice [26-28], which were then treated by daily s.c. injections with olpadronate or pamidronate.

Continuous BP treatment did not significantly affect growth of the cancer cells as determined by BLI (Fig. 5A,*C*). As expected, both BPs strongly and significantly inhibited the development of radiologically evident osteolytic lesions (Fig. 5*B,C*). Histomorphometric analysis (Fig. 5*D*) confirmed that BPs inhibited cancer-induced bone destruction leading to a significant increase in trabecular bone area associated with a significant decrease in TRAcP+ resorbing osteoclasts at the bone surface (Table 1). As a consequence of this bone sparing effect of BP treatment, the intra-bone tumor burden was significantly decreased, although it was clear that the cancer cells were filling completely the bone marrow spaces

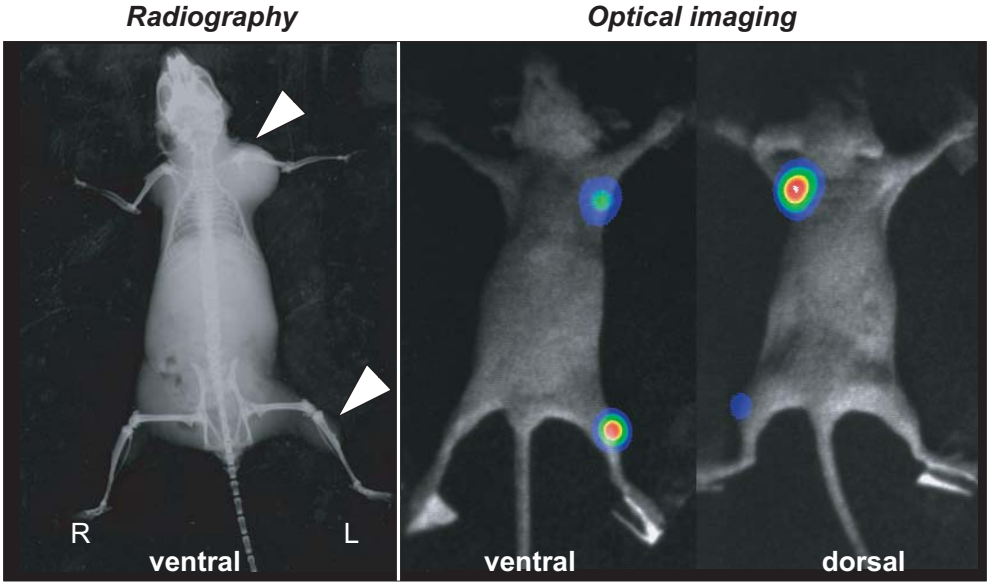


Figure 4 **Representative radiograph and bioluminescent images of an olpadronate treated mouse according to the Curative protocol (18 days of treatment, day 46, see figure 3).** Osteolysis is minimal in the left proximal tibia and proximal humerus (left panel, arrows). However, the photon emission spectra detected by BLI of the same bone areas (central and right panel) indicate that these bones are the site of metastatic growth of MDA-231-B/Luc+cells. Due to the limited intraosseous space, most of this growth seems to have taken place in the soft tissues surrounding bone, as revealed on the radiograph by the tumor shadow present especially around the left humerus.

(Fig. 5*D*). In contrast to the intraosseous tumor burden, no significant inhibition of the overall tumor burden (= intraosseous and in surrounding soft tissues) was observed (Fig. 5*D*), which is in line with the optical imaging data presented in figures 5*A* and *C*.

Table 1 Effects of bisphosphonate treatment on osteoclast numbers at the bone surface of MDA-231-B/Luc+breast cancer cells growing in tibiae of 6-week-old female nude mice.

	No. osteoclast/mm bone surface, mean (SD)	P
Vehicle treated	22 (4)	-
Pamidronate 1.6 µmol/kg/d	5 (1)	< 0.01
Olpadronate 1.6 µmol/kg/d	4 (2)	< 0.01

Note: n = 5 per experimental group. Ps represent bisphosphonate treated vs. vehicle treated animals.

Previously it was found that the intensity of the signal detected by BLI is a function of the tissue composition and depth of the bioluminescent source [33,34]. There is the possibility that changes in the tissue type invaded by the tumor growth and/or representation of bone

tissue, with consequent differences in light penetration and light scattering between the experimental groups (vehicle vs. BP), may have generated artifactual results in determining tumor burden by BLI. To exclude this, the treatment/control ratios of the tumor burden values obtained by BLI and histomorphometrical analyses were compared. No differences in ratios between these two measurement methods were found (0.98±0.16 and 1.17±0.22 treatment/control ratios for pamidronate and olpadronate respectively). This correlation indicates that potential differences in quenching and/or light scattering by bone cannot account for the observed lack of *in vivo* growth inhibitory effects during BP treatment, and confirms the reliability of BLI as method to quantify tumor burden.

Taken together, these results clearly show that BP, despite a significant decrease in intraosseous tumor burden due to their bone-sparing effect, do not affect tumor growth rate, but merely modify the direction of tumor growth. As a result cancer cells invade more conspicuously the soft tissues (muscle, tendon and connective tissue) surrounding the long bones.

To ensure constant delivery of a dose of BP sufficient to inhibit the high bone turnover of rapidly growing animals in a further experiment, olpadronate was administered by osmotic mini-pumps. In line with the data obtained after s.c. administration with BP in the same intraosseous tumor model (Fig. 5*A,C,D*), continuous high dose olpadronate treatment did not affect the tumor growth as detected by BLI (Fig. 5*E*). As expected, continuous olpadronate treatment strongly and significantly inhibited tumor-induced osteolysis, as detected by radiography (Fig. 5*F*). Histomorphometric analysis confirmed these effects of olpadronate by showing a significantincrease in bone volume, a decrease in intra-bone tumor volume, and no significant effect on total tumor burden (Table 2).

Table 2 Histomorphometric analysis of TBV (%), intraosseous tumor volume (mm2), and total tumor volume (mm2) of MDA-231-B/Luc+breast cancer cells growing in tibiae of 6-week-old female nude mice.

	Vehicle, mean (SD)	Olpadronate, mean (SD)	P
TBV	3 (2)	13 (3)	< 0.05
Intra-bone tumor burden	16 (4)	8 (1)	< 0.05
Total tumor volume	39 (6)	25 (6)	n.s.

Note: Olpadronate (or vehicle) was released at a high dose by osmotic mini-pumps (16 µmol/kg/d, 24 hours/d) for 28 days. n = 5, n.s. = not significant.

Discussion

Interference with the microenvironmental growth support is currently being evaluated as a therapeutic strategy for the treatment of metastatic disease. Bone metastasis is a paradigm of the interactions that take place at the tumor-stroma interface [1,2] and evidence from

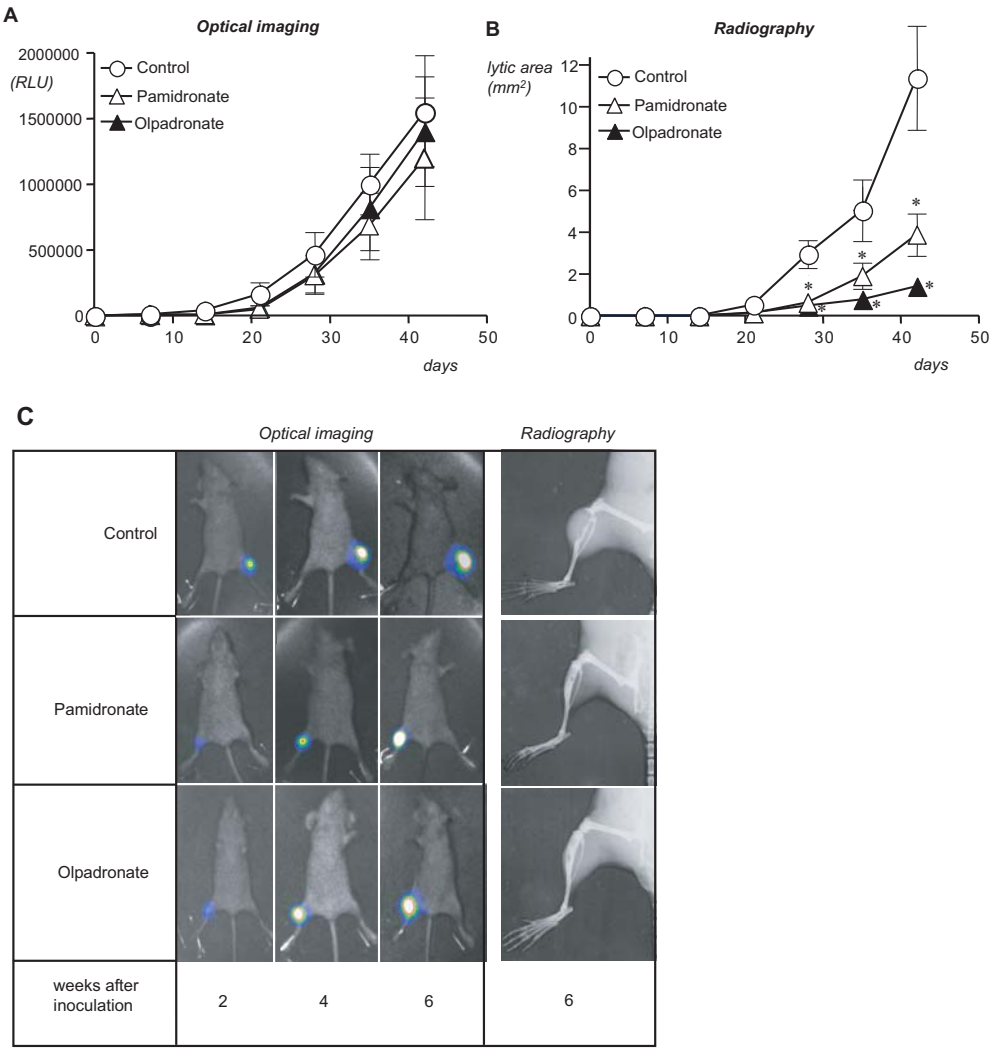
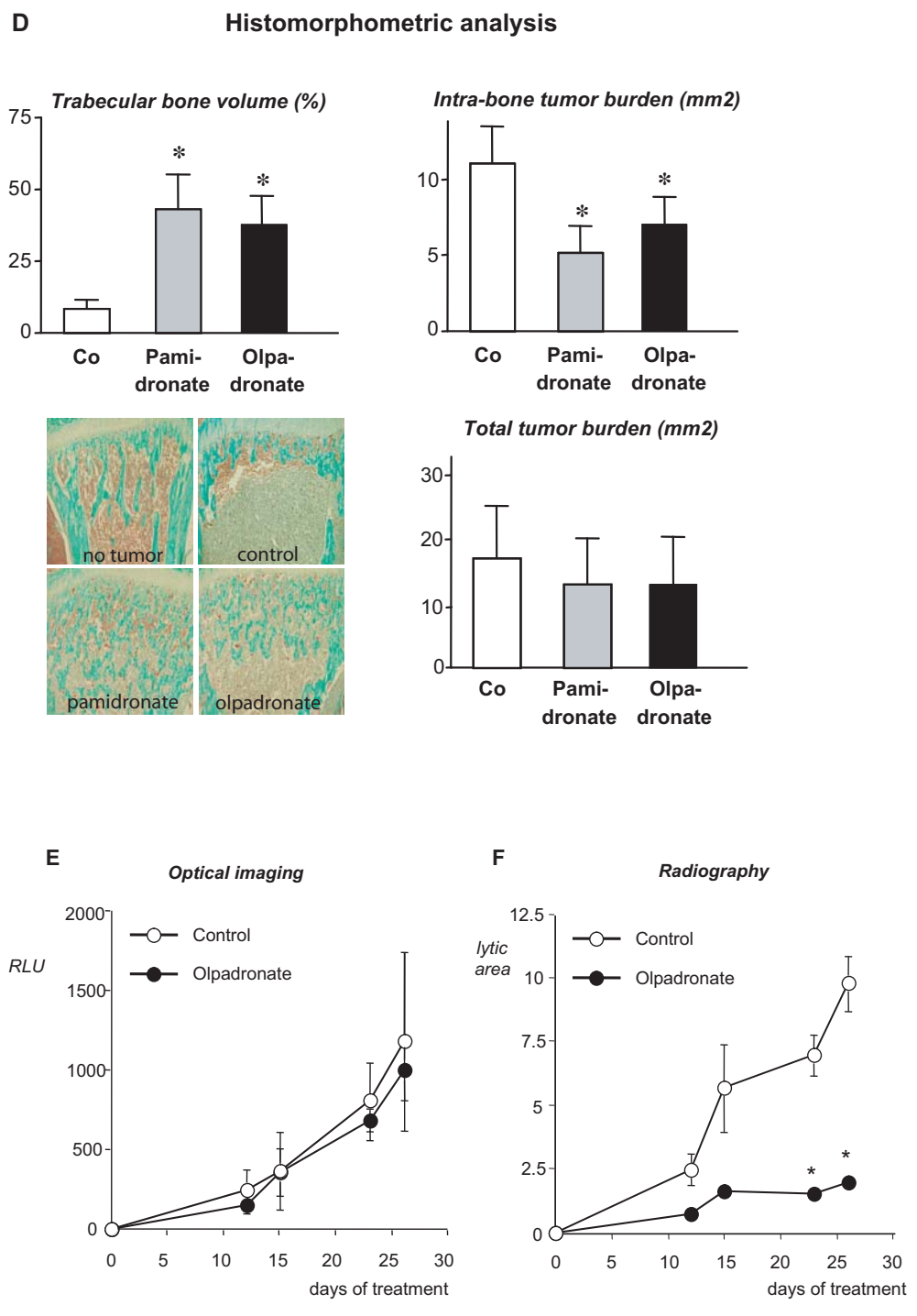


Figure 5 **Effects of BP treatment on tumor growth of MDA-231-B/Luc+ breast cancer cells (BLI+histology) and osteolysis (radiography) in tibiae of 6-week-old female nude mice.** MDA-231-B/Luc+cells were inoculated directly into the left tibia of nude mice, which were subsequently treated by daily s.c. injections with 1.6 μM/kg/d olpadronate (closed triangles), 16 μM/kg/d pamidronate (open triangles) or vehicle (open circles). *A*, growth of breast cancer cells in the bone marrow cavity measured by BLI (RLU). *B*, development of osteolytic areas due to tumor-induced bone destruction as assessed radiographically (lytic area, mm^2). *C*, representative animals of each experimental groups (BLI, radiography). *D*, histomorphometric analysis of intra-bone tumor burden, trabecular bone volumes and total tumor burden (= total of intra-bone + extramedullary tumor burden). *E*, continuous release of high dose of Olpadronate by osmotic mini-pumps (16 μM/kg/d, 24 hours/day) on intra bone tumor growth as detected by BLI. *F*, continuous release of high dose of Olpadronate by osmotic mini-pumps (16 μM/kg/d, 24 hours/day) on development of osteolytic lesions as assessed radiographically (mm^2). Values are expressed as means ± s.d., n = 5, * $p \leq 0.05$.


D
Histomorphometric analysis
Trabecular bone volume (%)
75
50
25
0
Co
Pami-dronate
Olpa-dronate
Intra-bone tumor burden (mm2)
10
5
0
Co
Pami-dronate
Olpa-dronate
no tumor
control
pamidronate
olpadronate
Total tumor burden (mm2)
30
20
10
0
Co
Pami-dronate
Olpa-dronate
E
Optical imaging
RLU
2000
1500
1000
500
0
Control
Olpadronate
0 5 10 15 20 25 30
days of treatment
F
Radiography
lytic area
12.5
10
7.5
5
2.5
0
Control
Olpadronate
0 5 10 15 20 25 30
days of treatment

animal and clinical studies support the notion that bone turnover, particularly bone resorption, contributes substantially to initiation and maintanance of local tumor growth through release of growth factors and bone resorbing cytokines ('vicious cycle'). Differently from other tissues, bone turnover can be reduced by pharmacological means and, thus, animal models of bone metastasis offer the unique opportunity to test *in vivo* the therapeutic efficacy of the interference with the tumor-stroma interface.

In this study we investigated whether inhibition of bone turnover by BP administration could prevent the development of bone metastases and/or repress tumor growth in already developed bone metastases. Our results show unequivocally that inhibition of bone turnover by BPs, prior to the establishment of a micrometastatic spreading induced by intracardiac inoculation of breast cancer cells ('Preventive' protocol), reduces by a great extent, although not completely, the development of bone metastases as detected by BLI. Although not significant, a tendency toward this effect is also present when BP are administered later, after the establishment of a micrometastatic spreading ('Curative' protocol), when some bone metastases had already been generated, but a residual number had still to develop. In this case, the limited entity of this residual number and, thus, the low statistical power, may explain the lack of significance.

The dose of olpadronate given (1.6 µmol/kg body weight/day) has been shown previously to efficiently inhibit bone resorption *in vivo* [32], but in the present study, it did not prevent the formation of bone metastases by >70%. This level of inhibition is consistent with the observation that in the experimental animal not more than 70% of the bone resorption can be inhibited by high dose of BPs [35]. Possibly, this persistently low level of bone resorption is sufficient to provide growth support to a minimal number of micrometastatic foci.

Although preventive BP-inhibition of bone turnover precludes to a great extent the generation of bone metastases from experimentally-induced micrometastatic spread of breast cancer cells, the progression of tumor growth in the small number of still developing metastases, as detected by BLI, is significantly reduced only during early stages. A similar trend is also seen when BP are administered according to the 'Curative protocol' to mice with already developed bone metastases. However, at later stages, bone metastatic growth is even accelerated, leading to a significant increase of tumor burden per metastatic lesion in the 'Curative protocol' after 18 days of BP treatment. This result is in line with earlier observations by others demonstrating that BP treatment, despite preserving trabecular bone, does not inhibit bone metastatic growth, and may even increase skeletal tumor burden [36]. This suggests that bone metastatic growth, once it is initiated and reaches a critical mass, proceeds independently of the bone microenvironmental growth support and is self-maintained by autocrine mechanisms.

The lack of effect by interference with the microenvironmental support on growth progression of already developed bone metastases was corroborated in a further experiment in which bone tumors were induced by intraosseous inoculation of breast cancer cells. Also

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in this case continuous inhibition of bone resorption, achieved by BP administered either s.c. or by sustained high dose release, did not affect tumor growth. Concomitant with BLI, radiographic and histomorphometric analysis could even better dissect the known BP bone sparing effect from an effect on tumor growth that may result either directly from these compounds or indirectly from the inhibition of bone turnover. In fact, histomorphometry revealed that, although the tumor mass confined within the bone marrow cavity was decreased as effect of the reduced intra-medullary space made available by persisting bone trabeculae, the total tumor mass was compensated by growth of cancer cells forced to invade the soft tissues (muscle, tendon and connective tissue) surrounding the bone cortex. Bone tissue may partially segregate the cancer cells within the medullary cavity and prevent them from invading the surrounding soft tissues. Instead, upon inhibition of bone resorption, the invasive growth is most likely forced to modify its direction from intra-medullary to extramedullary. It is important to note that MDA-MB-231 breast cancer cells have been shown to be able to migrate from bone marrow to periosteal surfaces [26], probably through vascular channels thus providing an opening to the periosteum and bone surrounding tissues.

Previous studies investigating the effect of BP-induced inhibition of bone resorption/turnover on bone metastatic growth in comparable animal models have shown contradictory results [30,36-40]. BPs have been reported to reduce the tumor burden in established bone metastases, but not in soft tissue and visceral metastases. This effect seemed to be mediated by induction of apoptosis in the cancer cells [30,37]. Treatment with BP, given prior to the development of evident metastasis, resulted in a marked reduction of the number of bone metastases [30,38-40]. However, in another preventive study it has been reported that BP treatment resulted in an increased tumor burden in the developing bone metastases, but not in visceral metastases [38]. In most of these investigations tumor burden has been quantified indirectly by measuring the osteolytic area on bone radiographs.

The difference between BLI and radiography in the number of bone metastastic foci detected either in the BP- or in the vehicle-treated animals is consistent with our previous report [26] and corroborates further the higher sensitivity, reliability and statistical power of the BLI method. BLI provides a direct indication of tumor viability and growth, while radiography does not detect actual tumor burden, but reveals only osteolysis, which is an indirect, late and inconsistent sign of the presence of a tumor [26]. In fact, determination of the osteolytic area depends not only on tumor cell mass, but also on the ability of the cancer cells to secrete bone-resorbing cytokines. Obviously, this consideration is especially true when bone resorption is pharmacologically inhibited. Furthermore, the determination of the osteolytic area does not take in account extension of the tumor burden in the surrounding soft tissues.

From the considerations above it should be concluded that the determination of the osteolytic area is invalid as a measurement of bone tumor mass. Therefore, the results of previous studies, based exclusively on this method, should be reconsidered. Only histo-

morphometry could avoid the latter pitfall. However, when a parallel histomorphometric analysis was performed, only intraosseous tumor burden was measured. Only one study assessed this analysis to the neighboring tissues. In the latter paper, BP-induced inhibition of bone resorption restrained only intraosseous tumor growth, while increasing tumor burden in the soft tissues [30]. This effect is perfectly in line with the results presented here with both histomorphometry and BLI, a much more reliable and sensitive method for monitoring tumor burden *in vivo*.

In vitro, BPs have been reported to exert direct anti-proliferative and pro-apoptotic effects on cancer cells [41-43], to interfere with cancer cell adhesion to bone matrix proteins [44,45], to inhibit matrix metalloproteinases [46,47], and to diminish cancer cell migration and invasion [48,49]. The results shown here exclude that BP may have a direct cytostatic effect *in vivo*.

Collectively, the results obtained in this investigation substantiate the concept that interference with the bone microenvironmental growth support, namely through inhibition of bone resorption/turnover, may be of therapeutic relevance for inhibiting early developmental steps of bone metastasis. However, they seem to exclude that the same strategy could repress progression of overt bone metastases and, also, that BPs may exert an effect on cancer cell growth, either directly by induction of apoptosis or indirectly by inhibition of angiogenesis.

How can the therapeutic effect of inhibition of bone resorption/turnover on early stages of bone metastasis development be explained? Bone remodelling does not occur throughout the entire bone surface, but takes place at microscopic patches of bone resorption and subsequent bone formation, called basic multicellular units (BMUs). The number and the activity of these BMUs determine the rate of bone turnover. Osteoblasts and osteoclasts, the cellular components in an active BMU, secrete and/or release from bone matrix cytokines, like TGF-β and IGF-1, which physiologically modulate the biology of bone cells. However, they can also act as paracrine growth factors for neighboring cancer cells that may have colonized bone marrow [19,22-25,28,50-55]. Consequently, local growth factor release by an active BMU in close proximity of a bone marrow micrometastasis may evoke the growth and invasive potential of the cancer cells, ultimately leading to the development of a macrometastasis (Fig. 6). We hypothesize that the probability for a bone marrow micrometastasis of becoming a clinically evident bone metastasis should be related to the chance that micrometastatic cancer cells extravasate in the proximity of an active BMU. In contrast, cancer cells extravasating near to a non-remodelling, 'resting' bone surface will persist as 'dormant', and eventually become apoptotic. Accordingly, a higher number and density of active BMUs (high bone turnover rate) should favour the probability that bone marrow micrometastases 'home' near active BMUs. This may explain the clinical and experimental evidence showing an association between high bone turnover rate and the preferential development of hematogenous metastases at sites of active bone remodelling [14,15]. Conversely, a preventive inhibition of bone turnover, e.g., by BP, prior to the generation of a self-maintaining, autocrine

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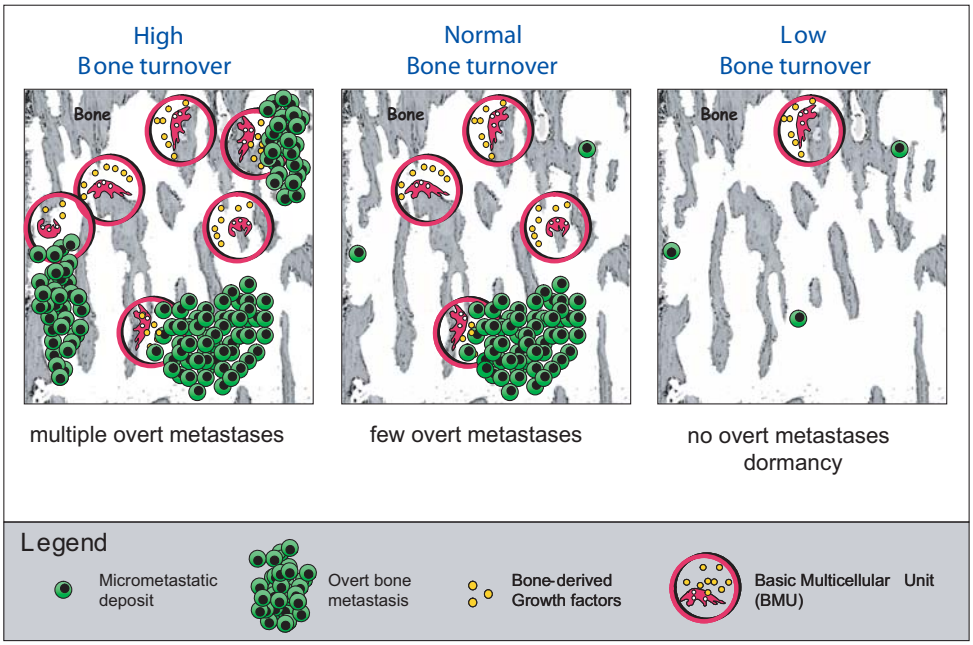

Figure 6 ***Hypothesis regarding the importance of bone turnover rate and the therapeutic relevance for inhibiting early developmental steps of bone metastasis.*** Bone turnover takes place at microscopic patches of bone resorption and subsequent bone formation, called basic multicellular units (BMUs). The number and the activity of these BMUs determine the rate of bone turnover. Osteoblasts and osteoclasts, the cellular components of an active BMU, secrete cytokines from bone matrix, which can act as paracrine growth factors for neighboring cancer cells that may have colonized bone marrow (micrometastatic deposits). Consequently, local growth factor release by an active BMU in close proximity of a bone marrow micrometastasis may evoke the growth and invasive potential of the cancer cells, ultimately leading to the development of an overt bone metastases. Interference with the bone microenvironmental growth support through inhibition of bone resorption/turnover (for instance by BPs), may be of therapeutic relevance for inhibiting early developmental steps of bone metastasis. However, cancer cell growth of overt bone metastases during treatment with BPs is not affected. The probability of a bone marrow micrometastasis to become clinically evident bone metastasis may thus be related to the chance that micro-metastatic cancer cells extravasate in the proximity of an active BMU. In contrast, cancer cells extravasating near to a non-remodelling, 'resting' bone surface will persist as 'dormant', and eventually become apoptotic. Preventive inhibition of bone turnover, e.g., by BPs, prior the generation of a self-maintaining, autocrine cancer cell growth, may arrest the 'vicious cycle' underlying the initial growth support by the BMU and, ultimately, frustrate the development of clinically evident macrometastasis.

cancer cell growth, may arrest the 'vicious cycle' underlying the initial growth support by the BMU and, ultimately, frustrate the development of clinically evident macrometastasis.

Assessment of the bone turnover status may lead to a better identification and stratification of the cancer patients at risk to develop bone metastases. Cancer patients, probably harboring micrometastasis in the bone marrow at the moment of diagnosis and/or surgery, but with high bone turnover rate, either basal (e.g., induced by menopause) or subsequent to surgical or chemical sex steroid deprivation, may benefit from a prophylactic, BP-induced reduction of the bone turnover rate.

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