Review

Actions of bisphosphonates in animal models of breast cancer

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Abstract

The skeleton is the most common site of breast cancer metastases. These bone metastases are usually osteolytic and cause significant morbidity. Bisphosphonates, potent inhibitors of bone resorption, reduce skeletal morbidity in breast cancer patients with bone metastases. Animal studies with bisphosphonates are crucial to understanding the mechanisms by which these compounds affect bone and tumor cells *in vivo*. Such animal models of breast cancer that are used to test the efficacy of bisphosphonates are discussed. These studies may offer insight into the treatment of other tumor types that frequently metastasize to bone.

Keywords: animal models, bisphosphonate, breast cancer, metastases, skeletal metastases

Introduction

Up to one-third of patients with early stage breast cancer will eventually die from the disease, and most of these (~80%) will have bone metastases. Although a majority of these bone metastases are destructive or osteolytic, a significant percentage also causes abnormal bone formation or osteosclerotic lesions. Once tumor has metastasized to bone, the disease is incurable. Because the average survival of breast cancer patients following diagnosis of bone metastases is 24–36 months, the morbidity of bone pain, fracture, hypercalcemia and nerve compression syndromes is longstanding. Therapeutics to treat and prevent these devastating complications of bone metastases are therefore in great demand.

Seed and soil hypothesis

It is well established that the skeleton is the most common site of distant metastases of breast cancer cells. Paget proposed, in 1889, that the affinity of certain cancers to metastasize to bone was due to the fact that the bone provides a 'fertile soil' or environment for the cells to germinate [1]. This seed and soil hypothesis is supported by the fact that bone is a repository for a number of growth factors and that osteoclastic bone resorption releases these growth factors. Histological sections of breast cancer metastases to bone reveal tumor cells adjacent to osteoclasts that are resorbing bone. These observations, combined with the clinical data demonstrating that bisphosphonate inhibitors of bone resorption reduce skeletal morbidity in breast cancer patients, indicate that bone destruction in breast cancer osteolysis is mediated by the osteoclast.

Our laboratory and other laboratories have provided evidence of a 'vicious cycle' involving breast cancer and bone. In this vicious cycle, metastatic breast cancer cells in bone produce factors (such as parathyroid hormone-related protein) that stimulate osteoclastic bone resorption. This production results in the release of growth factors, such as transforming growth factor-β, from the bone matrix [2,3]. Growth factors, in turn, stimulate tumor growth and production of more parathyroid hormone-related protein, resulting in a 'vicious cycle' that further fuels the bone destruction and tumor growth [2,3].

These local tumor–bone cell interactions resulting in osteolysis are the final steps of the journey that a tumor cell navigates from the primary site to the skeleton. The tenacious tumor cell must undergo the multistep process of

the metastatic cascade before it comfortably settles in bone. Specifically, the tumor cell must detach from the primary site, enter tumor vasculature to reach the circulation, survive host immune response and physical forces in the circulation, arrest in a distant capillary bed, escape the capillary bed, and proliferate in the metastatic site.

The events involved in entering the tumor vasculature are similar to those involved with exiting the vasculature in the bone marrow cavity. These events include attachment of tumor cells to the basement membrane, tumor cell secretion of proteolytic enzymes to disrupt the basement membrane, and migration of tumor cells through the basement membrane. Attachment of tumor cells to basement membranes and to other cells is mediated through cell adhesion molecules such as laminin and E-cadherin. Tumor cell secretion of substances such as metalloproteinases facilitates disruption of the basement membranes and enhances invasion. Inherent tumor cell motility or motility in response to chemotactic stimuli are also important factors for tumor cell invasion to the secondary site.

A number of other tumor types frequently metastasize to bone, including prostate, lung, thyroid, and renal cell carcinomata. Multiple myeloma is a primary hematologic malignancy that causes osteoclast-mediated bone destruction but is not a metastatic tumor *per se*. However, many of the tumor cell–bone cell interactions that cause the bone destruction are similar to those implicated in breast cancer osteolysis. Although this review focuses on breast cancer metastases to bone, the studies reviewed here offer general mechanisms that can be applied to other tumor types.

Bisphosphonates: what they are and what they do

Bisphosphonates are synthetic analogs of inorganic pyrophosphate. They are taken up preferentially by the skeleton and are strongly bound to hydroxyapatite on the surface of bone. Bisphosphonates are potent inhibitors of osteoclastic bone resorption. The effects of these drugs are primarily on the bone-resorbing osteoclasts but may also target osteoblasts, macrophages and tumor cells [4–8]. The mechanisms by which bisphosphonates inhibit osteoclast activity, and the relative potencies with which they do so, are dependent on the molecular structure of each compound.

The major mechanism of the nitrogen-containing bisphosphonates to decrease osteoclast number, osteoclast activity and bone resorption is by induction of osteoclast apoptosis [9]. As described by Fleisch in this issue [10], bisphosphonates inhibit farnesyl diphosphate synthase in the mevalonate pathway and thereby prevent protein prenylation of small GTPase signaling proteins required for osteoclast function [11,12]. The degree to which nitrogen-containing bisphosphonates inhibit bone resorption correlates with the capacity to cause apoptosis in cells of the osteoclast lineage, as well as with the capacity to inhibit farnesyl diphosphate synthase and protein prenylation in the osteoclast [11]. The non-nitrogen-containing bisphosphonates, such as clodronate, do not inhibit protein prenylation and have a different mode of action that may involve the formation of cytotoxic metabolites in osteoclasts or inhibition of protein tyrosine phosphatases [12].

Bisphosphonates also affect cells other than osteoclasts in the bone microenvironment. Derenne *et al*. showed that the bisphosphonates pamidronate and zoledronate inhibited interleukin-6-induced production of matrix metalloproteinase-1 by bone marrow stromal cells [13]. *In vitro* studies have also shown that nitrogen-containing bisphosphonates inhibit the adhesion of breast cancer cells to bone and bone matrix [14].

Even more intriguing are those results indicating that bisphosphonates may have direct effects on the tumor cells themselves, as reviewed in detail by Senaratne and Colston in this issue [15]. Bisphosphonates have been shown to decrease the proliferation and viability of human tumor cells lines, as well as to increase the apoptotic index of the human tumor cell lines [13,16]. Numerous investigators have demonstrated that bisphosphonates significantly reduce tumor invasion and adhesion to bone [13,14,16–19].

Bisphosphonates in preclinical animal models of breast cancer

Animal studies with bisphosphonates are essential to understanding the effects of these compounds on both bone and tumor cells *in vivo*. Four distinct models of osteolytic bone metastases (MDA-MB-231, 4T1, ENU-1564, and Walker carcinosarcoma 256B) and one model of osteoblastic metastases (MCF-7) in breast cancer have been used to test the efficacy and dosing of bisphosphonates. Table 1 summarizes the effects of bisphosphonates on the development and progression of bone metastases in these models.

MDA-MB-231 experimental model

Yoneda *et al*. have utilized the human estrogen receptor-alpha negative breast cancer cell line, MDA-MB-231, in a nude mouse model of metastatic breast cancer to bone. Mice develop osteolytic bone metastases 3–4 weeks post tumor inoculation into the left cardiac ventricle [20]. Bone metastases are a prominent feature of this model and closely resemble the osteolytic metastases frequently seen in breast cancer patients.

Risedronate blocked osteoclastic bone resorption in this model, resulting in fewer new bone metastases and delayed progression of existing metastases [21]. Histo-

Table 1

Effects of bisphosphonates in animal models of breast cancer

Animal model	Bisphosphonate	Type of protocol (preventative/therapeutic)	Effects on skeletal metastases	Effects on extraskeletal metastases	Reference
MDA-MB-231 breast cancer (intracardiac)	Risedronate	Preventative	↓ new skeletal metastases	No effect	Sasaki *et al.* (1995) [21]
		Therapeutic	↓ progression of existing skeletal metastases	No effect	
MDA-MB-231 breast cancer (intracardiac)	YH529	Preventative	↓ skeletal metastases	↑ at low doses; ↓ at higher dose	Sasaki *et al.* (1998) [22]
		Therapeutic	No change	No change	
MDA-MB-231 breast cancer (intracardiac)	Ibandronate	Preventative	↓ skeletal metastases	↑ adrenal metastases	Yoneda *et al.* (2000) [23]
		Therapeutic	↓ progression of established skeletal metastases	No effect	
4T1, mouse mammary tumor cell line (orthotopic)	Zoledronate	Preventative	↓ skeletal metastases	No effect on primary tumor or visceral metastases	Mundy *et al.* (2001) [26]
		Therapeutic	↓ progression of established skeletal metastases	No effect	
ENU-1564 mammary adenocarcinoma cell line (intracardiac, rats)	Risedronate	Therapeutic	↓ skeletal metastases	No effect	Hall and Stoico (1994) [27]
Walker carcinosarcoma 256B cells (intra-aortic)	Clodronate	Preventative	↓ osteolysis; no change in number of skeletal metastases	No effect	Krempien and Manegold (1993) [29]
Walker carcinosarcoma 256B cells (intra-aortic)	Pamidronate	Preventative	↓ skeletal metastases	No effect	Krempien *et al.* (1998) [30]
MCF-7* breast cancer (intracardiac)	Ibandronate	Preventative	↓ skeletal metastases (osteoblastic)	No effect	Yoneda *et al.* (2000) [23]
		Therapeutic	No effect	No effect	

* MCF-7 causes osteoblatic or mixed osteoblastic and osteolytic lesions. ↑, increase; ↓, decrease.

morphometric analysis of the bones showed a decrease in tumor volume in the bone in mice treated with risedronate [21]. Risedronate also increased the survival of the animals compared with untreated mice [21]. Sasaki *et al*. tested an experimental bisphosphonate, YH529, for the ability to decrease bone metastases, and observed a dose-dependent decrease in both osteolytic lesion number and area when the drug was given in a preventative manner (from the time of tumor inoculation until the end of the experiment) [22]. When YH529 was administered to treat established bone metastases, however, it had little effect [22].

Yoneda *et al*. also studied the effects of the bisphosphonate ibandronate on a subclone of MDA-MB-231 cells that is reported to more reliably form metastases to the skeleton and adrenal glands [23]. Ibandronate was administered in a 'preventative' protocol and a 'therapeutic' protocol. In groups treated according to the preventative protocol, in which mice received daily injections of ibandronate beginning at the time of tumor inoculation and continued for the duration of the experiment, a decrease in osteolytic skeletal metastases was observed [23]. However, adrenal metastases were increased in mice treated with ibandronate; an observation that is consistent with the data of other studies [21–24]. In the therapeutic protocol, mice received ibandronate daily only after the development of osteolytic bone metastases. In this case, ibandronate (unlike YH529 in earlier experiments) decreased the progression of the established bone metastases compared with the control group, with no effect on adrenal metastases [23].

Hiraga *et al*. have more recently provided evidence that ibandronate acts by reducing osteoclastic bone resorption and by increasing the apoptosis of osteoclasts [25]. In addition, ibandronate was shown to increase the apoptosis of tumor cells in the experimental bone metastasis model but not in orthotopic mammary fat pad tumors, indicating that the primary effect is in bone and tumors in bone [25].

4T1 experimental model

The second model, also used by Yoneda and colleagues, involves a mouse mammary tumor cell line, 4T1. This model is clinically relevant because syngeneic, immunocompetent mice are inoculated orthotopically into the mammary fat pad, and metastases occur in bone and soft tissue. This is one of the few models in which the cells must go through the multiple steps of the metastatic cascade to develop bone metastases. A primary tumor is usually evident in these mice about 1 week post tumor inoculation. Metastases are identified in the lungs and liver around 2 weeks post tumor inoculation. By week 3 the mice have distant metastases to the skeleton, the kidney, the adrenal glands, the heart and the spleen, and they do not usually survive beyond week 4 or 5 post tumor inoculation. The pattern of metastases and the histologic appearance are similar to those seen in human patients. This model allows for the simultaneous study of the effects of bisphosphonates on bone and soft tissue metastases.

This model was used to examine the therapeutic value of zoledronate, the most potent of the approved bisphosphonates. Administration of the bisphosphonate was begun as soon as the orthotopic tumor was apparent (approximately 7 days post tumor inoculation) [23]. Analysis of X-rays of both the treated and the untreated mice revealed a decrease in lesion area in the long bones of the mice receiving zoledronate. In addition, zoledronate-treated mice exhibited a decrease in osteolytic tumor lesion area in the lumbar spine by histomorphometric analysis [26]. The compound prevented the marked bone destruction seen in the trabecular bone of the tibial growth plates of control mice [26]. Daily treatment of mice bearing 4T1 tumors with zoledronate increased both osteoclast and tumor cell apoptosis within the bone metastases [26]. Finally, zoledronate also resulted in a decrease in the lesion area by X-ray analysis of existing bone metastases by 4T1 cells, while ibandronate and alendronate had no effect on established bone metastases [26]. No effect was observed on visceral metastases or on the primary tumor, however, indicating that the actions of zoledronate as used in this study are limited to bone.

The 4T1 bone metastases model has also been utilized to look at combinations of bisphosphonates with anticancer agents, a situation that more closely resembles the clinical scenario. Yoneda *et al*. [23] examined the effects of incadronate and zoledronate with the anticancer agent, UTF. The combination therapy inhibited metastasis to bone, the liver and the lung. UTF alone resulted in a decrease in tumor burden in the mammary fat pad, as well as in decreased metastases to the lung, the liver and the skeleton [23]. The decrease in bone metastases by UTF alone is probably due to the initial decrease in the size of the primary tumor.

ENU-1564 experimental model

A third model used to study the effects of bisphosphonates is a rat model using the ENU1564 mammary adenocarcinoma cell line. Rats are administered the mammary adenocarcinoma cells via intracardiac inoculation and are monitored for tumor development and the subsequent development of metastases.

This model was used to examine the effects of risedronate on bone metastases. Consistent with the results observed with the MDA-MB-231 model, risedronate resulted in a reduction in the number of skeletal metastases and in the size of the lesions in the skeleton [27]. No difference in visceral metastases was observed [27].

Walker carcinosarcoma 256B experimental model

The fourth model used to assess the use of bisphosphonates in the treatment of skeletal metastases secondary to breast cancer is the Walker carcinosarcoma 256B model. This is a rat model of skeletal metastases in which the cells are implanted directly into the bone. The growth of the Walker carcinosarcoma 256B cells in bone also leads to hypercalcemia, a common complication of bone metastases. Krempien *et al*. [28] have also found that intra-aortic administration of Walker carcinosarcoma 256B cells results in both bone and adrenal metastases in rats. Prophylactic treatment with clodronate, both short-term (5-day treatment) and long-term (21-day treatment), inhibited osteolysis is this model [28]. However, Krempien and Manegold also found that the longer the treatment-free intervals after short-term therapy, the less effective the inhibition [29]. The Walker carcinosarcoma 256B models have also been used to examine the effect of pamidronate on skeletal metastases [24,30]. Krempien *et al*. reported a reduction in skeletal metastases in rats treated with pamidronate [30]. Surprisingly, a second group reported that the tumor burden in bone increased following treatment with pamidronate [30]. The latter result, however, is not consistent with the clinical data that pamidronate reduced skeletal complications in patients with multiple myeloma and breast cancer [31–33].

Kurth *et al*. more recently examined the effects of daily treatment with ibandronate on the bone quality of rats inoculated with Walker carcinosarcoma 256B cells [34]. Treatment with ibandronate was shown to increase the bone density and the bone stability compared with controls [34].

Bisphosphonates and osteoblastic bone metastases

The estrogen receptor positive human breast cancer cell line, MCF-7, has been shown to produce osteoblastic or mixed osteolytic and osteoblastic bone lesions following intracardiac inoculation in a nude mouse model. It has long been hypothesized that bone resorption precedes the new bone formation of osteoblastic metastases, since biochemical markers of osteoclastic bone resorption are markedly increased in patients with osteoblastic metastases. Yoneda *et al*. tested the hypothesis that blocking bone resorption with bisphosphonates may affect the ability of MCF-7 cells to cause osteoblastic metastases [23]. Using the MCF-7 model, they looked at the effects of both early (prior to tumor inoculation) and late (post tumor inoculation, osteoblastic metastases established) treatment with ibandronate [23]. Only the early treatment with ibandronate resulted in inhibition of osteoblastic metastases. This supports the hypothesis that a bone resorptive phase precedes the development of osteoblastic metastases. The use of bisphosphonates to inhibit this resorptive phase may thus significantly reduce the development of osteoblastic metastases.

Discussion

Studies of bisphosphonates in preclinical models of breast cancer metastases to bone illustrate the importance of the bone microenvironment and osteoclastic bone resorption in the development of skeletal metastases of both osteolytic and osteoblastic nature. We have learned from these studies that the primary action of bisphosphonates is on the bone resorbing osteoclasts, and that bisphosphonates may exert secondary effects on the tumor cells in bone. Zoledronate, the newest and most potent bisphosphonate, showed promise in the preservation of bone in the 4T1 model of breast cancer metastasis to bone [23]. It caused a decrease in the osteoclast number and a decrease in the tumor burden in bone [23].

There are multiple explanations for this decrease in tumor burden in bone. First, and most probable, the decrease in osteoclastic bone resorption makes the skeleton a less hospitable environment for the tumor cells, by reducing the release of bone-derived growth factors that may stimulate tumor growth or production of osteolytic factors. Second, the bisphosphonates may have a direct effect on the tumor cells to induce apoptosis. While there are data *in vitro* and in animal models to support this, the concentrations of bisphosphonates at which this occurs *in vitro* are quite high. It is unclear whether such concentrations occur *in vivo*. Such data have yet to be demonstrated in humans with bone metastases. Finally, it is also possible that the decrease in tumor burden in bone is due to an effect of bisphosphonates on angiogenesis [35]. This potential anti-angiogenic effect on tumors remains to be investigated.

The observation in some of the animal models reported here that clearly needs to be clarified in human studies is the issue of extraskeletal metastases. That is, soft tissue metastases were increased following treatment with bisphosphonates, an observation indicating that if the tumor cells find the bone microenvironment inhospitable then they may seed to other tissues. However, clinical data with regard to this are inconsistent. Diel *et al*. found a decrease in visceral metastases in patients with breast cancer following treatment with clodronate [36], while Saarto *et al*. found an increase in visceral metastases [37].

Many questions remain regarding the use of bisphosphonates in the treatment of metastatic breast disease to the skeleton. More work is needed to determine whether bisphosphonates truly have antitumor activity in humans, or whether the reduction in tumor burden in bone is due to the reduction of bone-derived growth factors released into the local microenvironment as a consequence of inhibiting osteoclastic bone resorption. Second, bisphosphonate use in osteoblastic metastases needs to be further explored to prove definitively that treatment inhibiting bone resorption does indeed reduce osteoblastic disease. In

addition, it still remains to be determined whether bisphosphonates increase survival in clinically relevant situations. Patients with metastatic breast cancer are treated with anticancer agents in addition to bisphosphonates, and studies in animal models that mimic this situation would provide more realistic evidence with regard to survival. Finally, the use of bisphosphonates in the treatment of bone metastases of other primary tumors is an area that needs to be explored in both animal models and humans. Many of these questions are currently under investigation, and the answers should provide a strong rationale for the use of bisphosphonates in cancer-induced morbidity of the skeleton.

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References

1. Paget S: **The distribution of secondary growths in cancer of the breast.** *Lancet* 1889, **1**:571-572.
2. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR: **Evidence for a causal role of parathyroid hormone-related protein in pathogenesis of human breast cancer-mediated osteolysis.** *J Clin Invest* 1996, **98**:1544-1549.
3. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massagué J, Mundy GR, Guise TA: **TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development.** *J Clin Invest* 1999, **103**:197-206.
4. Shipman CM, Rogers MJ, Apperley JF, Russell RG, Croucher PI: **Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumor activity.** *Br J Haematol* 1997, **98**:665-672.
5. Shipman CM, Croucher PI, Russell RGR, Helfrich MA, Rogers MJ: **The bisphosphonate incadronate (YM 175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway.** *Cancer Res* 1998, **58**:5294-5297.
6. Aparicio A, Gardner A, Tu Y, Savage A, Berenson J, Lichtenstein A: **In vitro cytoreductive effects on multiple myeloma cells induced by bisphosphonates.** *Leukemia* 1998, **12**:220-229.
7. Busch M, Rave-Frank M, Hille A, Duhmke E: **Influence of clodronate on breast cancer cells in vitro.** *Eur J Med Res* 1998, **3**: 427-431.
8. Fromigue D, Siwek B, Body JJ: **Bisphosphonates inhibit breast cancer cell proliferation.** *Calcif Tissue Int* 1999, **64**:P261.
9. Rogers MJ, Frith JC, Luckman SP, Coxon FP, Benford HL, Monkkonene J, Auriola S, Chilton KM, Russell RG: **Molecular mechanism of action of bisphosphonates.** *Bone* 1999, **24**: 73S-79S.
10. Fleisch H: **Development of bisphosphonates.** *Breast Cancer Res* 2002, **4**:30-34.
11. Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH, Rogers MJ: **Structure–activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates.** *J Pharmacol Exp Ther* 2001, **296**:235-242.
12. Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, Frith JC: **Cellular and molecular mechanisms of action of bisphosphonates.** *Cancer* 2000, **88**:2961-2978.
13. Derenne S, Amiot M, Barille S, Bollette M, Robillard N, Berthaud P, Harousseau JL, Bataille R: **Zoledronate is a potent inhibitor of myeloma cell growth and secretion of IL-6 and MMP-1 by the tumoral environment.** *J Bone Miner Res* 1999, **14**:2048-2056.
14. van der Pluijm G, Vloedgraven H, van Beek E, van der Wee-Pals L, Löwik C, Papapoulos S: **Bisphosphonates inhibit the adhesion of breast cancer cells to bone matrices in vitro.** *J Clin Invest* 1996, **98**:698-705.
15. Senaratne SG, Colston KW: **Direct effects of bisphosphonates on breast cancer cells.** *Breast Cancer Res* 2002, **4**:18-23.
16. Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW: **Bisphosphonates induce apoptosis in human breast cancer cell lines.** *Br J Cancer* 2000, **82**:1459-1468.
17. Boissier S, Magnetto S, Frappart L, Cuzin B, Ebetino FH, Delmas PD, Clezardin P: **Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrix.** *Cancer Res* 1997, **57**:3890-3894.
18. Selander KS, Mönkkönen J, Karhukorpi EK, Harkonen P, Hannuniemi R, Vaanenen HK: **Characteristics of clodronate-induced apoptosis in osteoclasts and macrophages.** *Mol Pharmacol* 1996, **50**:1127-1138.
19. Virtanen SS, Väänänen HK, Härkönen PL, Lakkakorpi PT: **Effects of alendronate on adhesion and invasion of breast and prostate cancer cells.** *Calcif Tissue Int* 2001, **66**:S79.
20. Yoneda T, Sasaki A, Mundy GR: **Osteolytic bone disease in breast cancer.** *Breast Cancer Res Treat* 2001, **32**:73-84.
21. Sasaki A, Boyce BF, Wright KR, Chapman M, Boyce R, Mundy GR, Yoneda T: **Bisphosphonate risedronate reduces metastastic human breast cancer burden in nude mice.** *Cancer Res* 1995, **55**:3551-3557.
22. Sasaki A, Kitamura K, Alcalde RE, Tanaka T, Suzuki A, Etoh Y, Matsumura T: **Effect of a newly developed bisphosphonate, YH529, on osteolytic bone metastases in nude mice.** *Int J Cancer* 1998, **77**:279-285.
23. Yoneda T, Michigami T, Yi B, Williams PJ, Niewolna M, Hiragu T: **Actions of bisphosphonates on bone metastases in animal models of breast carcinoma.** *Cancer Suppl* 2000, **88**:2979-2988.
24. Kostenuik, PJ, Ori FW, Suyama K, Singh G: **Increased growth rate and tumor burden of spontaneously metastastatic Walker 256 cancer cells in the skeleton of bisphosphonate-treated rats.** *Cancer Res* 1993, **53**:5452-5457.
25. Hiraga T, Williams PJ, Mundy GR, Yoneda T: **The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases.** *Cancer Res* 2001, **61**: 4418-4424.
26. Mundy GR, Yoneda T, Hiraga T: **Preclinical studies with zoledronic acid and other bisphosphonates: Impact on the bone microenviroment.** *Semin Oncol* 2001, **28(suppl 6)**:35-44.
27. Hall DG, Stoico G: **Effect of the bisphosphonate Risedronate on bone metastases in rat mammary adenocarcinoma model system.** *J Bone Miner Res* 1994, **9**:221-230.
28. Krempien B, Diel IJ, Jockle-Kretz B, Buchele R, Andre L: **The Walker Carcinosarcoma 256 model of bone metastases, Influence of skeletal metabolism on the development of bone metastases.** *Verh Dtsch Ges Pathol* 1984, **68**:211-216.
29. Krempien B, Manegold C: **Prophylactic treatment of skeletal metastases, tumor-induced osteolysis, and hypercalcemia in rats with the bisphosphonate Cl_2MBP.** *Cancer* 1993, **72**:91-98.
30. Krempien B, Wingen F, Eichmann T, Muller M, Schmahl, D: **Protective effects of a prophylactic treatment with bisphosphonate 3-amino-1-hydroxypropanel, 1 bisphonic acid on the development of tumor osteopathies in rat: experimental studies with the Walker Carcinosarcoma 256.** *Oncology* 1998, **45**:41-46.
31. Hortobagyi GN, Theriault RL, Porter L, Blayney D, Lipton A, Sinoff C, Wheeler H, Simeone JF, Seaman J, Knight RD: **Efficacy of pamidronate in reducting skeletal complications in patients with breast cancer and lytic bone metastases.** *N Engl J Med* 1996, **335**:1785-1791.
32. van Holten-Verzantvoort ATM, Kroon HM, Bijvoet OLM, Cleton FJ, Beex LVAM, Blijham G, Hermans J, Neijt JP, Papapoulos SE, Sleeboom HP: **Palliative pamidronate treatment in patients with bone metastases from breast cancer.** *J Clin Oncol* 1993, **11**:491-498.
33. Lipton A, Theriault RL, Hortobagyi GN, Simeone J, Knight RD, Mellars K, Reitsma DJ, Heffernan M, Seaman JJ: **Pamidronate prevents skeletal complications and is effective palliative treatment in women with breast carcinoma and osteolytic bone metastases — Long term followup of two randomized, placebo-controlled trials.** *Cancer* 2000, **88**:1082-1090.
34. Kurth AHA, Kim S-Z, Sedlmeyer I, Hovy L, Bauss F: **Treatment with ibandronate preserves bone in experimental tumor-induced bone loss.** *J Bone Joint Surg* 2000, **82-B**:126-130.
35. Green JR: **Anti-tumor potential of bisphosphonates.** *Med Klin* 2000, **95**:23-28.

36. Diel IJ, Solomayer E-F, Costa SD, Gollan C, Goerner R, Wallwiener D, Kaufmann M, Bastert G: **Reduction in new metastases in breast cancer with adjuvant clodronate treatment.** *N Engl J Med* 1998, **339**:357-363.
37. Saarto T, Blomqvist C, Virkkunen P, Elomaa I. **Adjuvant clodronate treatment does not reduce the frequency of skeletal metastases in node-positive breast cancer patients: 5-year results of a randomized controlled trial.** *J Clin Oncol* 2001, **19**: 10-17.