Synthetic Matrix Metalloproteinase Inhibitors Inhibit Growth of Established Breast Cancer Osteolytic Lesions and Prolong Survival in Mice

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ABSTRACT

Purpose: Breast cancer frequently leads to incurable bone metastasis. Essential requirements for the development of bone metastasis are cell-cell and cell-matrix interactions, release of bioactive growth factors and cytokines, and removal of large amounts of bone matrix. Matrix metalloproteinases (MMPs) play an important role in all of these processes, but the possibility of using synthetic MMP inhibitors to decrease bone metastasis has received little attention.

Experimental Design: In the present study, we tested two general MMP inhibitors, BB-94 and GM6001, in a mouse model of breast cancer-induced bone metastasis.

Results: In a simulation of intervention therapy, mice were inoculated with breast cancer cells, and at the time of diagnosis of osteolytic lesions, the mice were treated for 10 or 15 consecutive days with BB-94 or GM6001, respectively. Both inhibitors reduced the growth of osteolytic lesions by >55% compared with control mice. Next, we simulated prevention therapy by initiating treatment with GM6001 at time of inoculation with cancer cells or 3 days earlier. Assessment of osteolytic lesions 28 days after inoculation showed that, in both cases, the treatment reduced the size of the osteolytic lesions by 60%, compared with that of control mice. Importantly, MMP inhibition also resulted in extension of symptom-free survival in the mice, whether the treatment was initiated at the time of diagnosis of osteolytic lesions or of cancer cell inoculation.

Conclusions: The present study suggests the potential of synthetic MMP inhibitors as intervention or prevention treatments of breast cancer-induced osteolysis.

INTRODUCTION

Breast cancer is the most frequent cancer in the female population of industrialized countries. Metastases to bone occur in >70% of patients with progressive disease, resulting in debilitating symptoms such as severe bone pain, fractures, hypercalcemia, and spinal cord or nerve compression syndromes. The most common treatments are bisphosphonates, hormone-related compounds, chemotherapy, and radiotherapy. Despite these treatments, it is virtually impossible to cure breast cancer-induced osteolysis, and its related complications remain a daily concern for the clinician (1).

A series of cellular events appears associated with all metastatic processes. These include interactions of the cancer cells with the surrounding stromal cells; interactions with the extracellular matrix leading to matrix recognition, cell-attachment, release of bioactive matrix-bound factors and matrix destruction for tumor expansion; formation of tumor vasculature; and escape from immunoprotection and from cell death (2). It has been recognized that many of these events involve MMPs produced by stromal cells (3). Interestingly, when it comes to tumor progression in bone, the importance of proteinases is heightened. Obviously, tumor expansion in bone requires the removal of an extracellular matrix that is particularly abundant and resistant to degradation. The assistance of bone-resorbing osteoclasts appears mandatory for this destructive task because osteoclasts are the primary cells involved in bone matrix solubilization. The capacity of osteoclasts to degrade bone lies in their ability to secrete protons and specialized collagenolytic proteinases at the bone surface. These proteinases include cathepsin K, a key agent for matrix solubilization, but also MMPs may well be involved in the cathepsin K-independent bone matrix solubilization (4, 5). This view is directly supported by the fact that treating bone with MMP inhibitors leads to an accumulation of demineralized collagen in the subosteoclastic resorption zone (6). Several MMPs are expressed in osteoclasts, the most established being MMP-9 (7–12). Another peculiarity of the bone environment is that bone matrix is a storehouse for cytokines and growth factors (13). Again, the release and activation of these matrix-bound growth factors is greatly determined by MMPs, and a variety of MMPs are present in bone tissue. These include MMP-2, -3, -8, -10, -11, -12, -13, and -14 (10–12). For instance, MMP-2, -3, and -9 are able to

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3 The abbreviations used are: MMP, matrix metalloproteinase; TGF, tumor growth factor; PTHrP, parathyroid hormone related protein; E-MEM, Eagles minimal essential medium; TIMP, tissue inhibitor of metalloproteinase; TRAP, tartrate-resistant acid phosphatase.
release and activate TGF-β (14, 15), a very abundant bone matrix-bound factor. TGF-β stimulates both proliferation of the breast cancer cells and their production of PTHrP, which in turn acts on osteoblasts to induce recruitment of osteoclasts via receptor activator of nuclear factor κB ligand production by the osteoblasts (16). In addition, TGF-β induces the retraction of the osteoblast-like cells lining the bone surface, thereby rendering the bone surface more accessible to osteoclasts; this retraction also involves MMP activity (17). The overall result is a vicious cycle with stimulation of both tumor growth and bone resorption (18). MMP-9 also controls the release of bioactive vascular endothelial growth factor, thereby triggering angiogenesis and tumor growth in pancreatic islets (19) and inducing angiogenesis and osteoclast recruitment in developing bone (20). In addition, factors such as TGF-β, PTHrP, and vascular endothelial growth factor regulate the production of MMPs such as MMP-2, -3, -9, and -13 by cells present in the bone environment (21–24). Therefore, tumor development in bone is a unique situation where the role of MMPs is likely to be more stringent when compared with other situations of tumor progression.

Inhibition of MMP activity may thus be an efficient way to prevent tumor growth when it occurs in bone (25). More specifically, MMP inhibition is expected to have a direct inhibitory activity against both osteolysis and tumor growth and thus appears a promising approach to interrupt the vicious cycle involving mutual stimulation of the latter two processes. Synthetic inhibitors of MMP activity have been developed in recent years. The possibility of using synthetic MMP inhibitors to reduce cancer-induced osteolysis has received little attention; however, two recent reports on the effect of synthetic MMP inhibitors on in vivo breast cancer metastasis to bone show promise when given as a preventative treatment to mice (26, 27). The present study investigates the effect of two broad-spectrum synthetic MMP inhibitors on cancer-induced osteolysis in nude mice and shows that these inhibitors reduce the size of the osteolytic lesions and significantly prolonged symptom-free survival in the mice when given either as an intervention or prevention therapy.

**MATERIALS AND METHODS**

Materials. E-MEM, EDTA tetrasodium (Versene), FCS, and trypsin were purchased from Life Technologies, Inc. (Copenhagen, Denmark). BB-94 and GM6001 were obtained from Dr. P. D. Brown (British Biotech Pharmaceuticals, Ltd., Oxford, United Kingdom) and AMS Scientific (Pleasant Hill, Ca), respectively, and are MMP inhibitors with broad specificity (28, 29). 17β-Estradiol slow-releasing pellets (0.72 mg/pellet) were purchased from Innovative Research of America (Sarasota, FL). Bovine parathyroid hormone 1–34 (PTH) was from Sigma (St. Louis, MO). 45 Calcium chloride was from Amersham (Buckinghamshire, United Kingdom). Rat antimouse CD34 antibody was MCA 1825 from Serotec (Oxford, United Kingdom). Rabbit antihuman MMP-9 antibody cross-reacting with mouse MMP-9 (20) was a gift from Dr. N. Borregaard (Rigshospitalet, Copenhagen, Denmark). Mouse antihuman pan-cytokeratin antibody A45-B/B3 was purchased from ChromaVision Medical Systems (Walldorf, Germany). Other reagents were from suppliers mentioned previously (20, 30, 31).

**In Vitro Bone Resorption Models.** Two culture models were used (20): (a) tibiae of 17-day-old mouse embryos, rich in mature resorbing osteoclasts and therefore allowing a direct evaluation of osteoclast resorptive activity; and (b) metatarsals of 17-day-old mouse embryos, still devoid of marrow cavity and of mature osteoclasts and therefore allowing an evaluation of the recruitment of osteoclasts. Briefly, pregnant NMRI mice (M&B, Ry, Denmark) were injected with 100 μCi of 45Ca s.c. at day 16 of gestation. Twenty-four h later, the three middle metatarsals (trig) and tibiae were isolated. One trig or tibia from the individual fetus was cultured with MMP inhibitor, and the corresponding trig or tibia from the same animal was used as paired control. Isolated trigs and tibiae were cultured for 6 days in 0.4 ml of BGJb medium, supplemented with NaHCO3 (2.2 g/l), NaCl (0.9 g/l), ascorbic acid (50 μg/ml), and Albumax (1 g/l) in the presence or absence of MMP inhibitor. Conditioned medium from bones were collected daily for measurements of 45Ca release and replaced by fresh medium. At the end of the culture, metatarsals and tibiae were demineralized in 5% formic acid to recover the remaining 45Ca. 45Ca release in medium is calculated as the percentage of total recovered 45Ca. The results are shown as ratio of 45Ca release in the treated culture (T) to 45Ca release in the paired control culture (C).

**Cancer Cells.** The estrogen receptor-negative human breast cancer cell line MDA-MB-231 (MDA-231/P), characterized by a high metastatic potential (32), was maintained in E-MEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% (v/v) FCS. An in vivo selected bone-seeking subclone of the MDA-231/P cells was isolated in our laboratory from osteolytic lesions in a nude mouse 28 days after cardiac inoculation (31). This subclone is hereafter referred to as MDA-231/B and used in all subsequent experiments. MDA-231/B cells were maintained for up to six passages under the same conditions as MDA-231/P.

**Induction of Bone Metastasis in Female Nude Mice.** We used the animal model originally described by Arguello et al. (33) and later modified by Yoneda et al. (18) for the study of breast cancer metastasizing to bone. MDA-231/B cells were cultured in 175-cm2 culture flasks (Nunc, DK) to 80–90% confluency, enzymatically detached from the flask by trypsin/ Versene, washed repeatedly in PBS, and resuspended in basal E-MEM at a cell number of 106 cells/ml. Cell viability was checked by trypan blue exclusion, and only cell suspensions with >98% viable cells were used for inoculations. 0.1 ml of the single cell suspension was injected in the left cardiac ventricle of 4-week-old female Balb/C nu/nu mice (M&B). Estrogen pellets were placed s.c. in the mice 3 days before cancer cell inoculation and removed 7 days after cell inoculation. Although the MDA-231 cells are not dependent on estrogen for growth, the MDA-231/B cells appear more aggressive at developing osteolytic lesions in the presence of estrogen.4 MMP inhibitors were administered i.p. at the indicated times. BB-94 was dis-

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solved in PBS/Tween (0.01%), and each injection was at a dosage of 60 mg/kg. GM6001 was dissolved in 4% carboxymethyl cellulose/0.9% saline, and each injection was at a dosage of 100 mg/kg. Mice were killed if they showed any adverse signs or symptoms of disease including weight loss, paralysis, or general discomfort.

**Visualization and Scoring of Osteolytic Metastases.**

To monitor development of osteolytic lesions after cancer cell inoculation, animals were anesthetized and placed in a prone position against a single coated HCM film (Kodak, Glostrup, Denmark) and exposed to X-rays at 30 kVp, 5 mA, for 18 s using a Biotron radiographic inspection unit (Simonsen & Weel, Taastrup, Denmark). Films were developed with the use of a 3M Model XP-2000 (Imation Medical Imaging). Radiographs of mice were captured by a video camera and projected to a computer monitor, where areas of osteolytic metastases in hind limbs were measured by a blinded observer using the stereological program CastGrid (Olympus A/S, Albertslund, Denmark).

**Bone Histology in Nude Mice.**

Tibiae and femurs from animals in each treatment group were isolated immediately after sacrifice and immersed in 4% formaldehyde and kept at 4°C for 48 h. Bones were decalcified in 15% EDTA solution (pH 7.4) for 3 weeks. Five-μm sections of paraffin-embedded tibiae or femurs placed on Superfrost PLUS slides (Menzel-Gläser, Braunschweig, Germany) were air-dried overnight at 37°C. Slides were deparaffinized in two changes of toluene and rehydrated in decreasing concentrations of methanol.

The demonstration of TRAP-positive cells (i.e., osteoclasts) in midsections of bones was performed as described previously with some minor modifications (34). Briefly, slides were stained by incubation for 2 h in 0.7% (w/v) sodium acetate, 0.05% naphthol-AS-BI-phosphate, 0.74% (w/v) 5,5-diethylbarbituric acid, 0.23% (w/v) disodium tartrate, 0.16% (w/v) NaNO₂, and 0.16% (w/v) pararosaniline. Sections were subsequently rinsed in running tap water for 10 min and counterstained with Mayer’s hematoxylin.

**RESULTS**

**Characterization of MDA Cell-induced Osteolytic Lesions.**

Nineteen days after injection of MDA cells, mice showed clear osteolytic lesions, with a preference for the distal femur and the proximal tibia (Fig. 1A). Smaller lesions could be detected before day 19 but were more difficult to accurately quantify (Fig. 1B). The lesions further increased in size between days 19 and 28 (Figs. 1B and 3). Histology revealed very high numbers of osteoclasts at the interface between the tumor and the bone matrix. Their density was much higher compared with bones of non-inoculated mice or to bone areas that did not have tumor (Fig. 1). These observations are consistent with other reports on bone metastasis in humans and mice and suggest that osteoclasts contribute significantly to breast cancer-induced osteolysis (1, 31). As tumors grew beyond a certain size, the area of bone to tumor was decreased, the number of osteoclasts surrounding the tumor also decreased (not shown). A variety of MMPs were induced at specific times and sites during tumor growth; for instance, MMP-9, a well-established MMP of osteoclasts, shows high immunoreactivity in osteoclasts, and more specifically around the nuclei and in the resorption zone between their ruffled border and the bone surface (Fig. 1F). These MMP-9 signals were very high in the osteoclasts in tumor areas while being much weaker in osteoclasts in areas without tumor. Immunoreactivity of MMP-9 was also detected in a few cells proximal to blood vessels within the tumor tissue (Fig. 1F). This has also been reported for pancreatic and breast cancer and ascribed to pericytes in the latter case (19, 35). It is worth noting that MMP-9 was not detected in tumor cells (although cultured MDA-231 cells produce MMP-9; Refs. 25, 26, 27).

**Effect of MMP Inhibitors on Resorption in Different Model Systems.**

Before investigating the effect of the synthetic MMP inhibitors BB-94 and GM6001 on tumor cell-induced osteolysis, we investigated their potency as inhibitors of bone resorption in different bone culture models: E17 tibiae, rich in mature resorbing osteoclasts, and E17 metatarsals, where resorption depends on the recruitment of osteoclasts (Fig. 2). Both MMP inhibitors reduced resorption. Inhibition of resorption in metatarsals reached almost 100% at a daily concentration of 0.3 μM, whereas inhibition in tibiae reached a plateau of 60–70% at concentrations of 0.3–3 μM. BB-94 and GM6001 are thus potent inhibitors of proteinases that significantly contribute to the resorptive activity of mature osteoclasts and of proteinases that are mandatory for osteoclast recruitment. These observations are consistent with data obtained with other MMP inhibitors (6, 7, 30, 36).

**Effect of Administration of MMP Inhibitors at Time of Diagnosis of Osteolytic Lesions: An Intervention Study.**

BB-94 and GM6001 were first tested in a study simulating an intervention treatment of cancer-induced osteolysis, with treatment beginning at time of diagnosis of osteolytic lesions. Thus, mice were inoculated with breast cancer cells, and at day 19, osteolytic lesions were quantified. Mice were then randomly distributed into two groups receiving either vehicle or BB-94 for...
10 consecutive days (Fig. 3A). At day 19, no significant difference in area of osteolytic lesions was found between the vehicle and BB-94-treated groups, thereby demonstrating successful randomization of mice. Between days 19 and 28, the sizes of the lesions increased almost three times in the vehicle group, but in mice treated with BB-94, the increase of the lesions was 1.35 times, which corresponds to a 55% reduction in growth compared with vehicle-treated mice (P < 0.01). In another study, treatment with GM6001 began at an earlier diagnosis of osteolytic lesions (day 14; Fig. 3B). At this time, small lesions are apparent on average in 60% of the mice. The mice were randomized and placed in two groups based on size of osteolysis at diagnosis, and treatment with GM6001 was begun. The area of osteolytic lesions was quantified at days 19 and 28. Between days 19 and 28, growth of osteolytic lesions in the GM6001-treated mice was 30% of that seen in vehicle-treated animals (P < 0.0005). Symptom-free survival in these mice was also prolonged, as determined at day 50 (P < 0.001; Fig. 3C). In addition, a reduction in tumor cell area was observed in osteolytic lesions of mice treated with GM6001 (Fig. 3D). These data thus suggest that MMP inhibitors have therapeutic potential when given at the time of diagnosis.

**Effect of Early Administration of MMP Inhibitor: a Prevention Study.** The possibility that MMP activity contributes to early phases of development of metastatic processes is often considered, but it is unlikely that such early phases are affected in the intervention treatment described above. Therefore, we performed an experiment where daily treatment of GM6001 began either 3 days before inoculation with cancer cells (day 3) or 1 day after inoculation with cancer cells (day 1) because the clinical relevance of beginning therapy before tumor inoculation is debatable. The treatment was either stopped at day 7 (days 3 to 7) or maintained until day 28 (days 3 to 28 or days 1 to 28), and the extent of osteolytic lesions was determined in each group at day 28 (Fig. 4A). In mice treated from days 3 to 7 only, the osteolytic lesions had a tendency to be less extended (30% reduction) when compared with untreated mice, but statistically, the difference was not significant. When the treatment was maintained over the whole period (days 3 to 28 or days 1 to 28), however, the size of osteolytic lesions was only 40% of the area measured in untreated mice (P < 0.0005). According to daily inspections and weight curves, the MMP inhibitor-treated mice were indistinguishable from the vehicle-treated mice from days 3 to 20 of the experiment.
After day 20, MMP inhibitor-treated mice continued to gain weight, whereas the weight of the vehicle-treated mice plateaued or declined. Most importantly, the symptom-free survival of mice treated with MMP inhibitor was significantly prolonged \( (P < 0.0005; \text{Fig. 4B}) \). Three of nine mice treated with MMP inhibitor survived until planned sacrifice at day 49, whereas all vehicle-treated animals \( (n = 9) \) died before day 36.

**DISCUSSION**

Several characteristics of cancer-induced osteolysis make MMPs attractive targets for therapies directed against osteolytic cancer lesions. There is, however, only limited experimental support for this concept. The initial evidence came from a study showing that breast cancer cells transfected with the natural MMP inhibitor, TIMP-2, and inoculated into mice led to reduced bone damage and prolonged survival compared with mice inoculated with nontransfected cells \( (25) \). Further support was given by recent reports showing that daily treatment of mice with the synthetic MMP inhibitor BB-94 or Neovastat, initiated 2 days before inoculation of breast cancer cells, resulted in higher bone content in the femoral metaphysis 3 weeks after tumor cell inoculation \( (26, 27) \). In the present study, we confirm these findings by using another MMP inhibitor, GM6001, for 4 weeks. We further demonstrate that this treatment leads to prolonged survival of the mice. Furthermore, even when postponing the treatment with BB-94 or GM6001 up to the time of diagnosis of osteolytic lesions \( (i.e., \text{14 or 19 days after cancer cell inoculation}) \), we found a 55% reduction of the growth of osteolytic lesions, as well as a reduction in tumor burden. There is also an extension of symptom-free survival in the mice treated from the time of established osteolytic disease.

Agents other than MMP inhibitors have proven to efficiently reduce osteolytic lesions in this mouse model. Neutralizing antibodies against PTHrP were used by Guise et al. \( (37) \) to successfully reduce the area of osteolytic lesions and tumor burden in the bone to <10% of the control. Overexpression of the E-cadherin cell adhesion molecule in the MDA-MB-231 cells in the same model also decreased the area and number of osteolytic lesions and tumor burden by 80% \( (38) \). The angiogenesis inhibitor TNP-470 reduced the number and area of osteolytic lesions by >60% \( (39) \). Expression of dominant-negative TGF-\( \beta \) receptor in breast cancer cells also resulted in >60% reduction in the number and area of osteolytic lesions, and a similar reduction was seen in tumor burden in the bones in this model \( (40) \). Treatment with the vitamin D analogue EB1089 produced similar inhibiting effects on the development of osteolytic lesions induced by MDA-MB-231 cells \( (41) \). Treatment with recombinant osteoprotegerin from the time of inoculation of disease also significantly reduced skeletal tumors in the model \( (42) \). Bisphosphonates such as ibandronate \( (25) \), risedronate \( (43) \), YH529 \( (44) \), and zoledronic acid \( (45) \) have shown at least a 60% reduction in tumor burden or area of osteolysis in treated mice.

Thus on average, the efficacy of the MMP inhibitor treatment used in the present study compares with that of the other drugs tested in the same model. Still, MMP inhibitors present potential advantages compared with other drugs in the treatment of cancer-induced bone disease. The cells producing most of the MMPs in tumors \( \text{in vivo} \) are not the cancer cells but the stromal cells \( (35, 46) \), which are less likely to develop drug resistance. Furthermore, MMP inhibitors are likely to affect MMPs at many different levels of the process leading to osteolytic metastasis: bioactivation of the many matrix-anchored growth factors in bone, angiogenesis, tumor growth/invasion, but also osteoclast recruitment and bone resorption. Thus, one may speculate that targeting MMPs improves the chance of disturbing the process of osteolytic tumor growth at multiple levels, which is in contrast with many other drugs that target only one specific level.

In the case of tumor progression in non-bone tissue, it is currently believed that MMPs are primarily involved in the early
stages of tumor progression and may not be required once metastases have been established (46). On the other hand, our present study shows that MMP inhibitors can be as effective against breast cancer-induced osteolysis whether treatment is initiated at the time of diagnosis of established disease or at the time of inoculation. Conversely, our study shows that MMP inhibitors are less effective against osteolysis when the treatment is restricted to the first week after cancer cell inoculation. Our data thus suggests that there are differences in MMP inhibitor sensitivity of tumor development in bone compared with other tissues. It may be speculated that the reason for this difference is based on peculiarities of bone tissue as explained in the "Introduction," such as the local abundance of MMP-bioactivating growth factors and the need for osteoclastic bone resorption, a process where MMPs play a role, as confirmed in the present study for BB-94 and GM6001 in well-established bone resorption models. Release of growth factors and activity of osteoclasts remain important after initial development of osteolytic tumors and may thus explain why MMP inhibitors are still efficient at treating diagnosed disease in the model.

Thus far, rather disappointing results have been obtained in clinical trials where MMP inhibitors were tested against a series of tumors.
of advanced extraskeletal cancers. Our experimental results suggest that MMP inhibitors may be efficient in clinical situations of cancer-induced osteolysis. The present findings, however, call for further research on the spatial-temporal identification of MMP expression in the bone microenvironment and the changes incurred during establishment of osteolytic tumors. Specific inhibition of these MMPs could potentially improve the treatment of cancer-induced osteolysis. The optimal administration protocol must also be established, including time of administration and determination of optimal biological dosage rather than maximum tolerated dosage. Micrometastases develop in the lungs and visceral organs (not shown) in the experimental model described in this report, and the effect of MMP inhibitors on tumor burden in these organs should also be examined. The importance of such analysis is emphasized when one considers earlier findings of Yoneda et al. (47), demonstrating that bisphosphonates can increase tumor burden in soft tissues while reducing tumor burden in bone. Other reports suggest that these findings can explain the failure of bisphosphonates to increase survival in mice, even when treatment significantly reduced osteolysis (25).

By combining MMP inhibitors with standard chemotherapy, radiotherapy regimens, antiangiogenic agents or bisphosphonates for the treatment of cancer-induced bone osteolytic disease should be considered. Combination experiments based on the use of bisphosphonates and the naturally occurring MMP inhibitor, TIMP-2, already indicate the potential of combining MMP inhibitors with bisphosphonates; the area of osteolysis was reduced by 50% in mice inoculated with the TIMP-2-transfected cell line compared with the parental cell line; additional treatment with the bisphosphonate ibandronate reduced the area of osteolysis to <5% of that produced by the parental cell line (25).

The present data show that a critical involvement of MMPs in clinical situations of cancer-induced osteolysis may have been overlooked thus far. It may thus be of interest to consider clinical trials to evaluate the efficiency of MMP inhibitors for the treatment of early and more advanced cancer-induced osteolysis. On the basis of the present results, evaluations should take into account not only survival but also levels of bone resorption markers, size of osteolytic lesions, and reduction in bone pain.

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