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A matrix metalloproteinase inhibitor, batimastat, retards the development of osteolytic bone metastases by MDA-MB-231 human breast cancer cells in Balb C *nu/nu* mice

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Abstract

Bone resorption is a dominant feature of many bone metastases and releases factors from the bone matrix that can promote the expression of the metastatic phenotype in cancer cells. Since proteolytic enzymes, including matrix metalloproteinases (MMPs) contribute to bone destruction by metastatic tumour cells and host cells, we have examined the effect of a MMP inhibitor, batimastat, on the ability of MDA-MB-231 cells to degrade bone *in vitro* and to form bone metastases in BalbC *nu/nu* mice. *In vitro*, the neoplastic cells produced MMP-2 and MMP-9, degraded [^{3}H]-proline-labelled osteoblast matrices, and formed resorption pits in cortical bone. These phenomena were inhibited by $\leqslant 20$ μM batimastat. To induce vertebral and long bone metastases *in vivo*, 1×10^5 MDA-MB-231 cells were injected into the arterial circulation of BalbC *nu/nu* mice. Test groups were also given 30 mg/kg batimastat intraperitoneally (i.p.). After 21 days, the long bone metastases were characterised by a 67% reduction of metaphyseal medullary bone and complete replacement of marrow by tumour. In tumour-bearing mice that had been treated with 30 mg/kg batimastat i.p., the tumour volume decreased 8-fold, osteolysis was inhibited by 35%, and replacement of the bone marrow by tumour was inhibited by 65%. Similar effects were observed in the vertebral metastases. These data provide evidence that MDA-MB-231 cells can degrade osteoblast matrices and mineralised bone *in vitro* and support the hypothesis that MMPs are involved in the pathogenesis of osteolytic bone metastases *in vivo*. They demonstrate that an agent which inhibits proteolysis can retard the development of osteolytic bone metastases in this model.

Keywords: Neoplasm metastasis; Bone, matrix metalloproteinase; Proteinase inhibitor

1. Introduction

Skeletal metastases are common in patients with advanced cancers of the breast, prostate, lung, thyroid and kidney. Bone resorption is a dominant feature of most bone metastases, mediated by osteoclasts [1], tumour-associated macrophages [2], or metastatic cancer cells [3]. Osteolysis [4] contributes to the pathophysiological progression of bone metastases since the local growth factors that are generated and/or released as part of the bone remodelling process can promote the expression of the metastatic phenotype in osteotropic cancer cells [5]. Moreover, by weakening the structural integrity of bone, osteolysis contributes to the clinical features of bone metastases which include pain, pathological fractures, spinal cord compression, and hypercalcaemia [6].

The matrix metalloproteinases are mediators of homeostatic bone growth and remodelling [7] and are likely to contribute to the invasion and metastasis of malignant tumours in bone [8,9]. In experimental models of bone metastasis, there is evidence that osteolysis and colonisation of the bone marrow by the tumour can be reduced by strategies that inhibit the release or production of proteases [10,11] or by overexpressing tissue inhibitors of matrix metalloproteinases in metastatic cells [12]. On this basis, we have postulated and show here that a synthetic inhibitor of matrix metalloproteinases, batimastat (BB-94), inhibits the activity of matrix metalloproteinases expressed by MDA-MB-231 human breast carcinoma cells, and blocks the ability of these cells to degrade osteoblast-like matrices or to form

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resorption pits in cortical bone. *In vivo*, where more than one cell type may be involved in bone destruction, treatment of tumour-bearing animals with batimastat inhibited tumour-associated osteolysis, tumour growth, and the replacement of marrow by tumour.

2. Materials and methods

2.1. Materials

Dulbecco's Minimal Essential Medium (DMEM) was purchased from GibcoBRL (Edmonton, Alberta, Canada). The MDA-MB-231 cells were a generous gift from G.R. Mundy, University of Texas, San Antonio, TX, USA. The SaOS-2 osteosarcoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA). BalbC *nu*/*nu* mice weighing 18–20 g were purchased from Charles River (Montreal, Quebec, Canada) and housed according to standards established by the University of Manitoba. Batimastat was supplied by British Biotech Pharmaceuticals Ltd, Oxford, UK.

2.2. *Tumour cell growth* in vitro

4×10^2 MDA-MB-231 human breast cancer cells were seeded into 24 well tissue culture plates and allowed to attach for 2 h. Four replicate wells were then cultured in DMEM with 10% fetal bovine serum, in the presence of batimastat at concentrations ranging from 0 to 50 μM. Daily counts of viable cells were obtained by a haemocytometer.

2.3. Enzymography

Enzymography was performed as previously described [4]. Briefly, serum-free conditioned medium was collected over 48 h from confluent cultures of MDA-MB-231 cells. Samples of medium were loaded into 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels containing 0.3% gelatine and resolved by electrophoresis at 4°C at 100 V overnight. The gel was washed in 2.5% Triton X-100 for 2 h at room temperature and incubated for 48 h at 37°C in substrate buffer containing 50 mM Tris–HCl pH 8.9, and 5 mM $CaCl_2$ with varying concentrations of batimastat. The gel was visualised by staining with Coomassie Blue.

2.4. Degradation of extracellular matrix

SaOS-2 (human osteosarcoma) cells were cultured for 5 days in 96-well plates in the presence of 5 μCi/ml [^{3}H]-proline and 25 μg/ml ascorbic acid to form a radiolabelled osteoid-like matrix monolayer on the surface of the tissue culture dish. The osteosarcoma cells were lysed with 20 mM NH_4OH at 37°C for 20 min. The radiolabelled matrices were washed with serum-free DMEM and incubated for 24 h with 1×10^5 MDA-MB-231 tumour cells in the presence of 0–20 μM batimastat. Replicates of 5–6 wells were included for each condition. 100 μl aliquots of medium were collected, added to 6 ml of Beckman ReadySafe™ scintillation fluid, and counted using a Beckman beta counter [4].

2.5. Resorption of mineralised cortical bone

Fresh bovine long bone was obtained commercially, the marrow removed, and the bones were washed in methanol [4]. The bone was cut into 2-mm slices using an Isomet low-speed diamond-edged saw. The slices were then polished using fine quality sandpaper, 20 μm grit, and then with 10 μm grit accompanied by intermittent washing in water, using an ultrasonicator for 5-min periods. The slices were then dehydrated, and sterilised with ethylene oxide. Before each experiment, the slices were incubated for 24 h in serum-free medium, then incubated with 1×10^5 MDA-MB-231 cells with or without 20 μM batimastat for 60 min. The non-adhering cells were washed off with serum-free medium. The bone slices were then incubated for 30 days at 37°C in the presence or absence of batimastat. The cancer cells were removed by incubating the slices with 0.1% Triton X-100 for 6 h with 5-min washing in the ultrasonicator and the slices fixed in 100% ethanol. The slices were sputter-coated with gold and mounted for examination in a Scanning Electron Microscope. Bones were cultured in quadruplicate for each experimental condition. Between 30 to 50 random fields on the surface of the bone slices were analysed for each condition. Shallow pits with a diameter of 20–30 μm were marked. The mean number of pits per 0.62 mm^2 field was calculated.

2.6. Experimental bone metastasis

Four groups of 3–4 week old BalbC *nu*/*nu* mice were established from two sets of littermates, as summarised in Table 1. A normal control group consisted of animals that were neither treated with batimastat nor injected with MDA-MB-231 cells. Two groups of animals were

Table 1
Animal groups used to examine the effects of batimastat on the metastasis of MDA-MB-231 cells to bone

Groups	*n*	Batimastat	Tumour	Batimastat
(Time line)		2 days		21 days
Normal control	5	–		–
Batimastat control[a]	6	+	–	+
Tumour only[b]	4	–	+	–
Tumour and batimastat[a,b]	9	+	+	+

[a] 30 mg/kg batimastat injected intraperitoneally (i.p.) daily.
[b] 1×10^5 MDA-MB-231 cells injected.

given daily batimastat injections (30 mg/kg intraperitoneally (i.p.)) for a period of 23 days. Two groups of mice were given a single intracardiac left ventricular injection of 1×10^5 MDA-MB-231 cells 21 days before terminating the experiment. One group received both batimastat (beginning 2 days before injection of the tumour cells) and tumour cells. Intracardiac injections of MDA-MB-231 cells were employed to induce the formation of metastatic bone tumours as described by Yoneda and colleagues [12]. Briefly, under anaesthesia, a left parasternal longitudinal incision was made and the second intercostal space located. A needle was inserted until pulsatile blood was observed. 1×10^5 MDA-MB-231 human breast cancer cells in a total volume of 0.1 ml were injected within 20 s. The animals were allowed to recover and housed for 21 days, receiving a daily injection of batimastat or vehicle. The mice were killed by exposure to atmospheric CO_2. The left femur and the vertebral column were removed and fixed in 10% buffered formalin for 24 h. The vertebrae and femur were decalcified in 10% formic acid for 24 h and then further fixed in formalin for 24 h. The tissues were embedded in paraffin, following standard protocols. A 7-μM thick section of bone was prepared from the centre of the left femur and from the centre of the vertebral column and stained with haematoxylin and eosin. The metaphyseal medullary bone at the distal end of the left femur and the vertebral bodies were systematically scanned with a 40× objective lens. Five microscopic fields were analysed in each vertebral body with metastases. A Merz graticule was used to perform a morphometric analysis of the percentage area of bone, tumour, and marrow for each section [4,13].

2.7. Batimastat administration

Batimastat stock solution was prepared for use *in vitro* in absolute ethanol at a concentration of 10 mM and diluted in ethanol before each experiment. For experiments *in vivo*, the solution was prepared at a concentration of 3.0 mg/ml in pyrogen-free phosphate buffered saline (GibcoBRL, Edmonton, Alberta, Canada) with 0.01% Tween 80 (Fisher Scientific) and administered by i.p. injection at a daily dose of 30 mg/kg.

3. Results

3.1. Experiments in vitro

Growth curves were obtained to determine if batimastat has an effect on the proliferation of MDA-MB-231

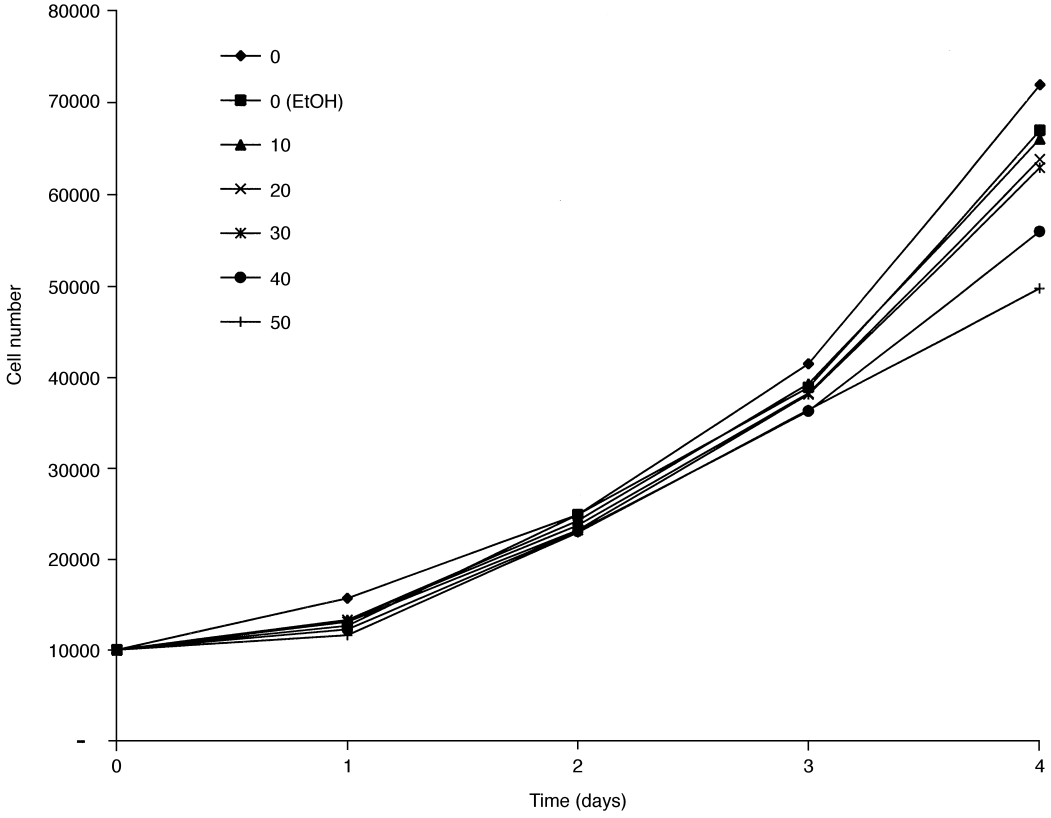

Fig. 1. Effect of batimastat on the growth of MDA-MB-231 human breast cancer cells *in vitro*. 4×10^2 cells were seeded into 24-well tissue culture plates and cultured in the presence of 0–50 μM batimastat. Daily cell counts were obtained by haemocytometer.

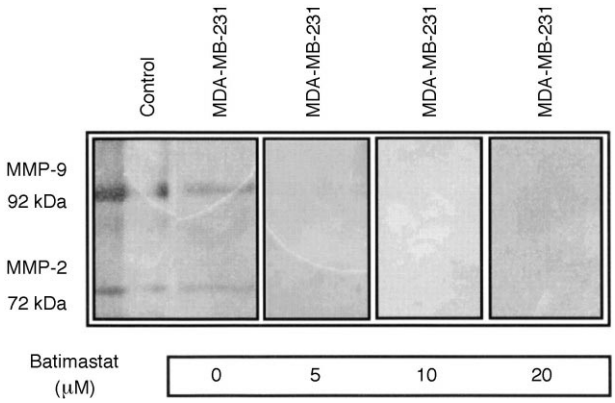

Fig. 2. Effect of batimastat on the expression of MMP-2 and MMP-9 in serum free medium conditioned by MDA-MB-231 cells. Electrophoresis was performed in 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) containing 0.3% gelatine. Zymograms were developed over 48 h in substrate buffer containing 0–20 μM batimastat.

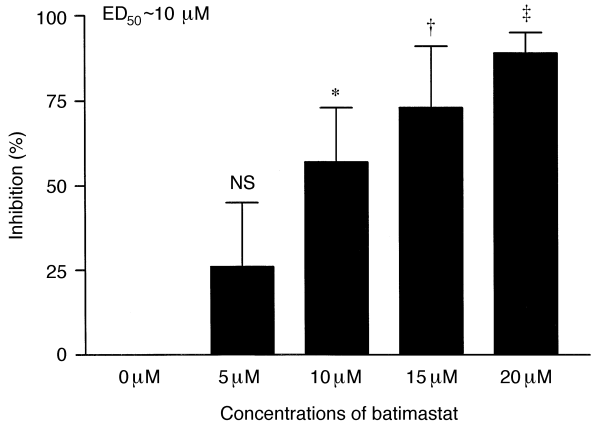

Fig. 3. Effect of batimastat on the degradation of osteoblast-like matrix by MDA-MB-231 cells. MDA-MB-231 cells were cultured on preformed [^{3}H]-proline labelled SaOS-2 osteoblast-like matrix in the presence of 0–20 μM batimastat. Degradation was determined by counting radioactivity released into the culture medium after 24 h. Not statistically significant by standard Student's *t*-test = NS; Statistically significant = *$P \leqslant 0.05$; †$P \leqslant 0.01$; ‡$P \leqslant 0.001$. ED, effective dose.

breast cancer cells *in vitro*. After 4 days culture, the proliferation of the MDA-MB-231 cells was inhibited by approximately 4% in the presence of 10 μM batimastat or the ethanol vehicle and was inhibited up to approximately 30% at concentrations of 50 μM (Fig. 1).

Using enzymography, the serum-free medium of MDA-MB-231 cells, conditioned for 48 h, exhibited enzymatic activities at molecular weights of 92 kDA and 72 kDa, corresponding to the expression of matrix metalloproteinase (MMP)-9 and MMP-2 respectively. These activities were blocked when batimastat was added to the zymographic substrate buffer at concentrations $\geqslant 5$ μM (Fig. 2). Metalloproteinase expression was not inhibited in serum-free conditioned medium, collected from cells that had been cultured to confluence in the presence of 5–20 μM batimastat (data not shown). These results indicated that exposure to batimastat did not alter the expression of MMPs by the neoplastic cells.

Assays to examine the ability of batimastat to inhibit the degradation of non-mineralised bone matrix *in vitro* were performed to model its putative effects on the degradation of non-mineralised osteoid *in vivo*. In three independent experiments, batimastat inhibited the degradation of osteoblast-like matrix by MDA-MB-231 cells in a dose-dependent manner. In all three experiments, matrix degradation was inhibited completely by batimastat at concentrations of 20 μM with an effective dose $(ED)_{50} \cong 10$ μM (Fig. 3).

Assays to examine the effects of batimastat on the ability of MDA-MB-231 cells to directly degrade mineralised bone were performed as previously described [4,14]. MDA-MB-231 cells cultured on devitalised polished slices of bovine cortical bone generated 1.7±0.2 well-defined surface excavations per 0.62 mm^2 field after 30 days. In the presence of 20 μM batimastat, the number of pits decreased by 24% to 1.3±0.2 pits per field ($P \leqslant 0.076$) (Fig. 4). The areas of these pits were not significantly different ($P \leqslant 0.32$) between bones cultured in medium (169±15 μm^2) or in the presence of 20 μM batimastat (148±15 μm^2).

3.2. *Experiments* in vivo

Since metastatic osteolysis can be effected by host-derived osteoclasts and macrophages, as well as by tumour cells, it was important to examine the effects of batimastat *in vivo*. Groups of BalbC *nu/nu* mice were treated according to the protocols described in Table 1. Mice in the tumour-bearing groups were given intracardiac injections of 1×10^5 MDA-MB-231 cells and killed 21 days after tumour cell inoculation. The data obtained by histomorphometric analysis of the distal femoral metaphyses in the two independent experiments are combined in Fig. 5. Compared with non-tumour bearing controls, the non-tumour bearing animals treated with batimastat exhibited a 20% ($P \leqslant 0.3377$) increase in the area of medullary bone. In the tumour-bearing animals, there was a 67% ($P \leqslant 0.0001$) reduction of metaphyseal medullary bone at the distal end of the left femur, compared with normal controls. In tumour-bearing mice treated with batimastat, osteolysis was inhibited by 35% ($P \leqslant 0.02$), compared with untreated tumour-bearing mice. The area of the metaphysis occupied by tumour (tumour burden) was reduced by 68% in tumour-bearing animals treated with batimastat ($P \leqslant 0.0001$). The distance to which the tumour extended into the metaphysis beyond the epiphyseal growth plate was reduced by 75% ($P \leqslant 0.0001$). Overall, the total tumour volume decreased from 5.0±0.3 ($\times 10^{-3}$)

mm^3 in the untreated tumour-bearing animals to 0.6 ± 0.2 ($\times10^{-3}$) mm^3 in the tumour-bearing batimastat-treated mice ($P\leqslant0.0001$). Whereas in normal animals, marrow occupied $85\pm2\%$ of the metaphyseal area, this was reduced to 0% ($P\leqslant0.0001$) in the tumour-bearing animals. Marrow loss was inhibited by 65% ($P\leqslant0.0001$) in tumour-bearing animals treated with batimastat.

In the two experiments, similar results were obtained following analysis of vertebral bone (Table 2). Metastatic tumour was detected in 74% of the vertebral bodies of tumour-bearing animals, but this was reduced to 24% of the vertebral bodies in the tumour-bearing animals treated with batimastat ($P\leqslant0.0001$, data not shown). Batimastat treatment was associated with a 96% inhibition of vertebral bone tumour burden ($P\leqslant0.0001$), complete inhibition of tumour-associated osteolysis ($P\leqslant0.001$), and 53% inhibition of marrow replacement ($P\leqslant0.0001$).

4. Discussion

Bone metastases occur in approximately 80% of patients with late stage cancer. They are characterised by cancer cell growth and bone destruction which contribute to their pathophysiological development and clinical presentation. Since bone metastases are not usually detectable until they have become advanced lesions, they are often incurable. The early stages in their formation are asymptomatic and begin as single micrometastatic cells from the bloodstream. As pre-

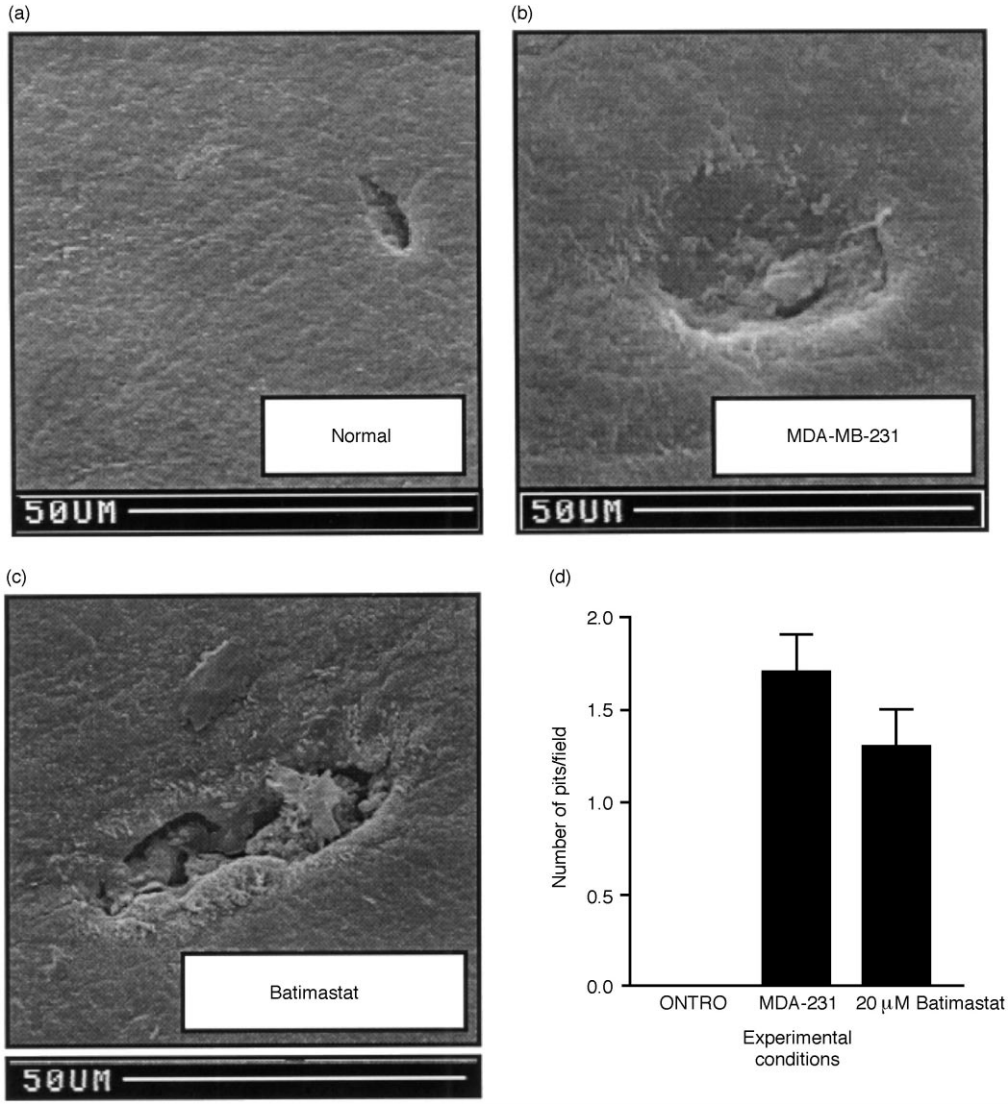

Fig. 4. Effect of batimastat on the formation of cortical bone resorption pits by MDA-MB-231 cells *in vitro*. (a) Appearance of untreated cortical bone after 30 days culture shows an osteocytic lacuna. (b) Resorption pit formed by MDA-MB-231 cells following 30 days coculture with MDA-MB-231 cells. (c) Resorption pit formed by an MDA-MB-231 cell cultured in the presence of batimastat. Scanning electron micrographs ×600. (d) Quantification of pit formation by MDA-MB-231 cells after 30 days incubation in the absence or presence of 20 μM batimastat. Field = 0.62 mm^2.

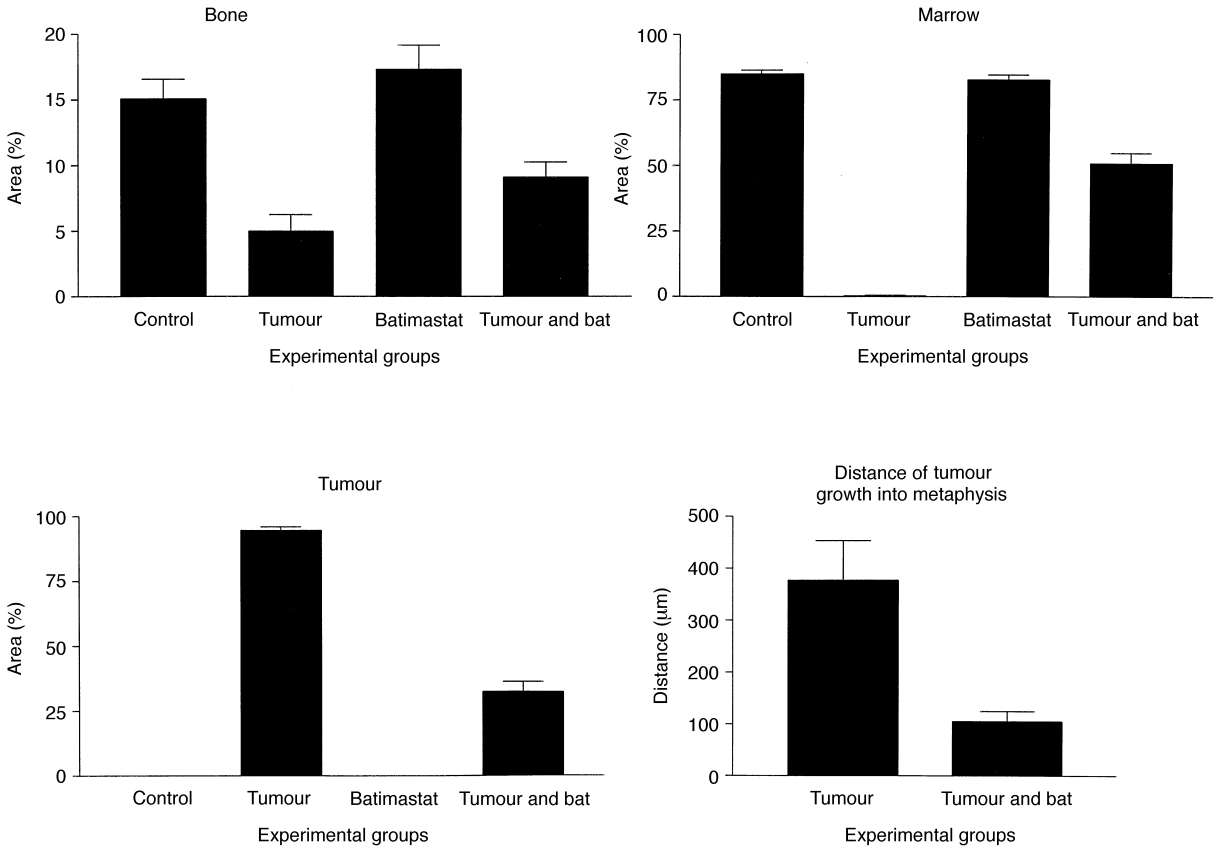

Fig. 5. Effect of batimastat on the development of experimental bone metastases by MDA-MB-231 breast cancer cells in BalbC *nu/nu* mice. Morphometric analysis was performed on histological sections of decalcified left distal femoral metaphysis 21 days after intracardiac injection of tumour cells. The experiment was duplicated to ensure reproducibility. The data presented here are combined from the two independent experiments. Not statistically significant by standard Student's *t*-test = NS; statistically significant by standard Student's *t*-test = $*P \leqslant 0.05$; $\dagger P \leqslant 0.01$; $\ddagger P \leqslant 0.001$. BAT, batimastat.

dicted by Paget [15], it has been established that there is a synergistic relationship between the micrometastatic cells and the bone environment, which creates a favourable condition for the development and growth of disseminated tumour cells [5]. Bone resorption mediates the progression of bone metastases by releasing local matrix-derived factors that can promote the expression of metastatic phenotypes in cancer cells including cancer cell chemotaxis [16], tumour growth [17,18], the expression of cell-surface adhesion molecules for bone matrix [19], and expression of MMPs [20,21]. As proteolytic enzymes, including MMPs, contribute to the mechanisms of osteolysis, we postulated that an MMP inhibitor would block osteolysis and interfere with the formation of lesions in established *in vitro* and *in vivo* models of bone metastasis.

Batimastat, an inhibitor of matrix metalloproteinases, acts by binding the zinc ion in the catalytic site, common to all matrix metalloproteinases. In animal models, batimastat has been reported to inhibit the formation of metastases through its inhibitory effects on tumour growth [22,23], tumour cell invasion of extracellular matrix [23–25] or angiogenesis [23,25–27] and by promoting stromal encapsulation [28]. Batimastat has been shown to reduce the activities of the 72-kDa and 92-kDa MMPs expressed by the human breast cancer cell line MDA-MB-435, to inhibit the local regrowth of resected MDA-MB-435 tumours implanted into athymic nude mice, and to inhibit the formation of lung metastases in these animals [29]. The effects of batimastat on bone metastasis do not appear to have been examined previously.

Table 2
Effect of batimastat on the formation of vertebral metastases by MDA-MB-231 breast cancer cells in BalbC *nu/nu* mice[a]

Groups	Bone (%)	Marrow (%)	Tumour (%)
Normal control	15±2	85±2	0±0
Tumour only	10±2	34±8	56±6
Batimastat control	16±1	84±1	0±0
Tumour and batimastat	20±2†	78±3*	2±1*

[a] Morphometric analysis of decalcified vertebral medullary bone (5 fields/vertebrae, ×200 magnification) was performed 21 days after intracardiac injection of tumour cells. $*P \leqslant 0.0001$ by Student's *t*-test when compared with the untreated (tumour only) group. †($P \leqslant 0.0013$).

In our experiments, batimastat did not inhibit the expression of MMP-2 and MMP-9 by the MDA-MB-231 cells but at concentrations $\geqslant 5$ μM completely inhibited the gelatinolytic activities of secreted MMPs in serum-free medium and concentrations $\geqslant 10$ μM blocked cell-mediated degradation of osteoblast-like matrices. While it is recognised that osteoclastic bone resorption contributes significantly to the osteolytic features of most bone metastases [30], the present experiments confirmed the ability of an osteolytic cancer cell line to induce pit formation in devitalised cortical bone slices in the absence of osteoclasts [4,14]. The ability of a human cancer cell line to cause degradation of mineralised bone was also reconfirmed [14] and was also partially inhibited by batimastat.

The concentrations of batimastat employed in the *in vivo* experiments were based upon precedence from other studies in the literature although they may have exceeded doses required to achieve optimal therapeutic effects. By using histomorphometry to analyse the metastatic lesions, we were able to distinguish between osteolysis, tumour burden, and marrow replacement which can not be accomplished by more conventional radiological measurements. The effects of batimastat treatment on the formation of bone metastases by MDA-MB-231 cells were striking and confirmed in two experiments. The marked osteolytic effects of the tumour were blocked in both femoral and vertebral bone, accompanied by diminished extension of tumour into the medullary long bone, decreased tumour volume, sparing of haematopoietic tissue, and a decrease in the number of metastases found in the vertebral bodies. We were unable to accurately quantify the effects of batimastat on tumour angiogenesis [27,31] as the decalcification procedures we employed appeared to block the ability of established histological markers of angiogenesis to identify blood vessels in our sections. Histomorphometric analysis of the long bones and vertebral bones of non tumour-bearing mice treated with batimastat demonstrated an unexpected increase in the volume of medullary bone, compared with normal untreated controls. An analysis of this phenomenon will be undertaken.

The colonisation of bone marrow by micrometastatic cells is a documented early event in the clinical course of human cancer and is being increasingly regarded as a prognostic factor of clinical significance [32]. Thus, batimastat was administered prior to the injection of tumour cells in order to optimise its potential effects on developing bone metastases rather than to study its actions on established lesions. Given the important contribution of bone resorption to the pathophysiological development of bone metastases, we predict that therapeutic strategies will be most effective if directed at inhibiting the interactions between micrometastatic cells and the bone microcompartment, as opposed to treatment of established lesions.

Acknowledgement

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