Hydroxamate-Type Matrix Metalloproteinase Inhibitor Batimastat Promotes Liver Metastasis

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Abstract

Overexpression of matrix metalloproteinases (MMPs) facilitates tumor cell invasion. Synthetic MMP inhibitors such as batimastat have been designed to treat cancer. We report that because of batimastat treatment, human breast carcinoma cells metastasized to the liver in nude mice and that an increase of liver metastases of murine T-cell lymphoma cells was observed in syngeneic mice. Batimastat treatment also caused liver-specific overexpression of MMPs-2, -9, and mRNA up-regulation of angiogenesis factors and caspase-1, even in tumor-free animals. Induction of organ-specific side effects need to be taken into account regarding further development and clinical use of synthetic MMP inhibitors.

Introduction

Tumor invasion and metastasis requires proteolytic degradation of components of the extracellular matrix such as collagen, proteoglycans, laminin, elastin, and fibronectin. MMPs are the only enzymes known to degrade fibrillar collagens and are able to degrade essentially all of the extracellular matrix components. MMPs also substantially contribute to other steps in the metastatic cascade, such as angiogenesis, differentiation, proliferation, and apoptosis. Therefore, MMPs are considered to be important regulators of tumor growth, both at the primary site and in distant metastases. In the clinical setting, increase of some MMPs is associated with poor prognosis of cancer patients. Consequently, synthetic MMP inhibitors have been developed and administered to patients to reduce cancer spread.

The synthetic hydroxamate-type inhibitor batimastat inhibits tumor growth and lung metastasis in different mouse tumor models (2) and has been tested in clinical therapy trials (3, 4). Still, it remains to be shown that hydroxamate-type of MMP inhibitors have statistically significant advantages over placebo regarding either primary or secondary end points of the respective therapy trials in human cancer.

In the present study, we investigated the effect of batimastat treatment on distant metastasis in two different mouse tumor models. Applying cDNA-array technology, we identified batimastat-induced changes of expression of several tumor-promoting genes.

Materials and Methods

Experimental Metastasis Assay. Pathogen-free, female athymic (nu/nu, CD1) mice and female DBA/2 mice (both 4–6 weeks of age; Charles River, Sulzfeld, Germany) were inoculated with either 10⁶ lacZ-tagged human MDA-MB-231 BAG cells (5) or 10⁶ DBA2-syngeneic lacZ-tagged murine L-CL5 cells (6) into the tail vein of each mouse, respectively. Fifty-two days after inoculation of MDA-MB-231 BAG cells or 6 days after inoculation of L-CL5s cells when macrometastases (>0.2 mm) were already formed in this model (7), mice were sacrificed, livers and liver were removed, and these organs were stained with X-Gal (Ref. 6; Roche Diagnostics, Penzberg, Germany). After the respective time points, blue macrometastatic foci on the surface of the organs were counted. This method allows assessment of the metastatic pattern even of lymphoma cells (7). [4-((N-Hydroxyamino)-2R-isobutyl-3S-(thiopen-2-ylthio-methyl)-succinyl]-l-phenylalanine-N-methylamide, which is identical to batimastat and will be referred to as batimastat in this study (kindly provided by Dr. H.-W. Krell, Roche Diagnostics; synthesized according to the PCT Patent Application WO 90/05719), was suspended in sterile PBS/0.01% Tween 80 (Sigma, Munich, Germany) and administered i.p. at a daily dose of 30 mg/kg (previously shown to be tolerable and effective; Ref. 8), from the day after tumor cell inoculation until the day before organ removal.

Zymography. Frozen tissue (0.5 cm³ of a liver lobe or half of a lung) was homogenized for 10 s in a Mini-beadbeater in the presence of zirconium beads (1.0-mm diameter; Biospec Products, Inc., Bartlesville, Oklahoma) and 300 μl of extraction buffer [50 mM Tris/HCl, 5 mM CaCl₂, 200 mM NaCl, and 1% Triton X-100 (pH 7.5)]. After centrifugation of the tissue homogenate (4°C; 12,000 × g; 10 min), the supernatant was collected, snap-frozen in liquid nitrogen, and stored in aliquots at −80°C. Zymographic detection of gelatinolytic activity in 15% SDS-polyacrylamide gels was performed according to Edwards et al. (9). Recombinant MMP-2 and MMP-9 (Roche Diagnostics) served as standards and were used after a 2-h activation with amino-phenyl mercuric acetate (Sigma).

RNA Isolation, Atlas-Array, and Northern Blot Analysis. Mice were treated with batimastat (30 mg/kg/day in PBS/0.01% Tween 80) for 12 consecutive days; the control mice received PBS/Tween only. On day 13, mice were sacrificed, and livers and lungs were collected, snap frozen in liquid nitrogen, and stored at −80°C. Isolation of total tissue RNA and subsequent Northern blot analysis were performed as described (10). Differentially expressed mRNA in organs of batimastat-treated mice versus control mice were identified by Atlas Mouse cDNA Expression Arrays (Clontech, Heidelberg, Germany). For this purpose, we isolated mRNA from the lung (as an internal control) and liver tissue of tumor-free mice, which had received daily i.p. injections of batimastat or, as a control, vehicle only. cDNA was synthesized and hybridized to the arrays according to the manufacturer’s protocol. For the generation of specific cDNA probes (HGF, bFGF, angiogenin, and caspase-1), primers designed by Clontech and synthesized by MWG-Biotech AG (Ebersbach, Germany) were used for PCR amplification, and the products were used in the subsequent Northern blot analysis.

Statistical Analysis. Kruskal-Wallis One Way ANOVA on Ranks was performed to assess differences between groups. In case of a statistically significant difference (P < 0.05), pairwise comparisons of groups were done by Dunn’s method.

Results and Discussion

The resulting metastatic pattern in lungs and liver of mice as an effect of treatment with the synthetic hydroxamate-type MMP inhibitor batimastat is presented, using lacZ-tagged tumor cells (7). Experimental metastasis was generated by injection of lacZ-tagged MDA-
MB-231 BAG breast cancer cells into the tail veins of nude mice, and, as a result, metastases were formed in the lung but not in the liver. After tumor cell administration, one group of mice (n = 6) was subjected to daily i.p. injections with batimastat, whereas the group of control mice (n = 18) was treated with the vehicle only. Mice were sacrificed, lungs and livers collected, X-Gal stained, and macrometastases quantified. In the batimastat-treated group, a slight trend in reduction of lung metastases was observed as compared with the control group (Fig. 1A). A different metastatic pattern of the breast cancer cells was observed in the batimastat-treated animals; in addition to the lung metastases, all but one animal of the batimastat-treated group developed liver metastases, whereas no liver metastases were seen in the control group (Fig. 1A).

L.5s murine T-cell lymphoma cells form liver metastases and, therefore, allow examination of the effect of batimastat on preexisting liver metastases. These cells directly colonize the liver upon i.v. injection without forming lung metastases. The day after tumor cell injection, one group of mice (n = 6) was treated with daily i.p. injections of batimastat, whereas the control group (n = 9) received the vehicle only. Six days after tumor cell inoculation, the mice were sacrificed, and the livers were removed and examined for number of metastases. Compared with the control group, a significant increase of liver metastases in the batimastat-treated group (Fig. 1B). Even a 3-day batimastat treatment before tumor cell inoculation did not prevent the significant increase of liver metastases (data not shown).

In DBA/2 mice that did not receive tumor cells, liver tissue revealed a baseline expression of active MMP-2 and no MMP-9 (Fig. 2, Lane 2). Interestingly, in mice that were never inoculated with tumor cells, batimastat treatment led to expression of pro-MMP-9 and increased the level of MMP-2 (Fig. 2, Lane 3). In mice inoculated with LCL.5s T-cell lymphoma cells, expression of pro-MMP-9 but no elevation of the baseline level of MMP-2 was detected in liver tissue afflicted with metastases (Fig. 2, Lane 4). Both liver cells and tumor cells could be responsible for this increase in pro-MMP-9. Batimastat treatment for 6 days after lymphoma cell inoculation led to further elevation of pro-MMP-9 and also of MMP-2 expression in the liver (Fig. 2, Lane 5).

To reveal what further changes in organ-specific gene expression are induced by batimastat, cDNAs of livers and lungs from control nude mice or mice that were treated with batimastat for 12 consecutive days were subjected to microarrays. To exclude interference with the gene expression associated with the presence of tumor cells, the mice were not inoculated with tumor cells. Assessment of differentially expressed genes in the liver, compared with the lungs, allowed detection of those genes that revealed liver-specific alteration of transcription (at least a 2-fold difference in expression). In addition to the increased MMP expression because of batimastat treatment, we observed that liver-specific mRNA expression of HGF/SF, bFGF, angiogenin, and caspase-1 was significantly elevated, as revealed by Mouse-Atlas™ Arrays and confirmed by Northern blot analysis (Fig. 3). HGF/SF, an

![Fig. 2. Zymographic detection of MMP-2 and -9 protein expression in livers of batimastat-treated DBA/2 mice.](image-url)
in severe combined immunodeficient mice found no reduction of metastasis in the liver (22). We found previously that batimastat treatment inhibits primary tumor growth of i.p. inoculated human esophagus and ovarian carcinoma cells in nude mice but at the same time leads to formation of secondary tumors in the liver (23). These findings provide further evidence that batimastat is able to induce liver-specific changes, which could lead to promotion of tumor progression.

In cancer patients, marimastat, an p.o. administrated derivative of batimastat, has been tested in Phase II and Phase III clinical studies. Three of these studies have recently been closed without reaching their primary end point, i.e., a reduction in mortality, attributable to marimastat treatment (24). Our data on the liver-specific batimastat-induced metastasis, together with the evidence for induction of gelsatines and angiogenesis-promoting genes during treatment with batimastat in tumor-free mice, indicate the need for further research before synthetic MMP inhibitors are used for treatment of cancer patients.

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