Inhibition of Organ Invasion by the Matrix Metalloproteinase Inhibitor Batimastat (BB-94) in Two Human Colon Carcinoma Metastasis Models

Susan A. Watson, Teresa M. Morris, Graham Robinson, Michael J. Crimmin, Peter D. Brown, and Jack D. Hardcastle

ABSTRACT

The effect of the matrix metalloproteinase inhibitor batimastat was evaluated in two human colorectal cancer metastasis models involving: (a) the liver-invasive tumor C170HM2 and (b) the lung-invasive tumor AP5LV, both of which have been shown to express the M, 72,000 type IV collagenase. Batimastat at concentrations between 0.01 and 3.0 µg/ml had no direct cytotoxic effects on the in vitro growth of the cell lines. In the liver-invasive tumor model, batimastat administered i.p. from day 10 to termination of the therapy (day 39) at 40 mg/kg reduced both the mean number of liver tumors (35% of vehicle-treated control; P < 0.05) and the cross-sectional area of the tumors (43% of vehicle-treated control; P < 0.05). In the lung-invasive tumor model, batimastat administered daily (40 mg/kg i.p.) significantly reduced tumor weight within the lung (72% of vehicle-treated control; P < 0.05) but did not significantly affect nodule number. In the latter model, in which the take rate was unaffected, tumor cells were introduced into the lateral tail vein, and lung localization may have been a physical phenomenon not involving invasion. In the former model, tumor cells were introduced directly into the peritoneal cavity, and from there the cells adhered to and invaded the liver capsule. Because the take rate is significantly reduced, it may be that the matrix metalloproteinases are involved in this process. Batimastat may be a therapeutic modality for the treatment of colorectal cancer metastasis.

INTRODUCTION

Metastatic spread occurs in more than 60% of patients with colorectal cancer; the major target organ is the liver (1, 2). Therefore, there is an urgent need to develop methods of treating advanced colorectal cancer, and one approach may be to modify the action of proteolytic enzymes produced by the tumor during secondary spread.

Enzymes are thought to play an important role in conferring invasive and metastatic potential on tumors. Under normal physiological conditions, such enzymes are tightly regulated both by secretion in an inactive form and by the presence of specific inhibitors. In tumors that metastasize, this control is lost, resulting in the breakdown of both the basement membrane and the interstitial stroma, which in turn allows invasion and widespread dissemination in lymph and blood (3).

The role of MMPs3 in tumor progression has become better understood in recent years as these proteinases have been purified and cloned. The MMP group consists of three classes of enzymes: (a) the type I collagenases, which break down type I, II, and III fibrillar collagens; (b) the type IV collagenases or gelatinases, which break down type IV and V collagens, which are important components of the basement membrane; and (c) the stromelysins and matrilysins, which break down fibronectin, proteoglycans, and laminin (4). Collectively, the members of this family of enzymes degrade most of the components of extracellular matrix. A large body of correlative data that implicates the involvement of MMPs in the malignant progression of many common tumors now exists (4). In colorectal cancer, Newell et al. (5) have identified, by in situ hybridization, the presence of mRNA for many members of the MMP family. In addition, members of the MMP group have been evaluated, and these have been shown to have a role in the processes of invasion and metastasis. Fibrillar collagenolytic activity has been shown to correlate with histological grade (6). The expression of the M, 72,000 type IV collagenase has been related to tumor progression (7), and in situ hybridization studies have shown that the tissue stroma adjacent to the invasive front of the tumor is the source of the enzyme (8). Matrilysin is associated with the majority of colorectal tumors and has been shown to correlate with lymph node metastasis (9).

These correlative studies have provided important clues as to the function of MMP in human colorectal neoplasms, but they do not indicate whether the expression of MMP activity is an important element of the invasive phenotype. In an attempt to answer this question, the effect of a low-molecular weight synthetic MMP inhibitor, batimastat, was examined in two different xenograft models of human colorectal carcinoma. In a previous study (10), batimastat was shown to inhibit the metastatic spread of the B16 murine melanoma. The colorectal cancer models in this study do not allow the role of human stromal tissue to be studied, but they do permit a characterization of the invasive behavior of human tumors to be made.

C170HM2 is a liver-invasive human colorectal tumor line that was derived originally by repeated passage in vivo and was selected for its ability to invade and grow within the liver after i.p. administration (11). Immunohistochemical studies have revealed the presence of interstitial collagenase at the invading edge of the C170HM2 liver tumors, and zymography has revealed the presence of low amounts of M, 72,000 gelatinase within the tumors (11). Therefore, C170HM2 models the invasion of the liver capsule by peritoneally seeded colorectal tumor cells. It is also possible that liver tumors may arise internally by lymphatic and hematogenous spread.

AP5LV is a human colorectal tumor line, recently established from a primary colorectal tumor specimen, that was selected from a lung colony by a single passage in vivo after i.v. administration. Therefore, AP5LV models the lung colonization by blood-borne colorectal tumor cells. These two models were used to study different aspects of the invasive behavior of human colorectal carcinoma.

MATERIALS AND METHODS

Description of Batimastat (BB-94)

The characteristics of batimastat are: chemical name, [4-(N-hydroxyamino)-2R-isobutyl-3-S-(thiethylthiomethyl)-succinyl]-l-phenylalanine-N-methylamide; M, 478; charge at neutral pH, M, 18,500; and solubility, 6 nm in water.

Biochemistry. The enzyme-inhibition profile of batimastat has been evaluated and is expressed in terms of the IC50. For collagenase, the IC50 is 5 nm; for stromelysin, 20 nm, for the M, 72,000 and 92,000 gelatinases, 4 and 1.5 nm, respectively; and for matrilysin, 6 nm. Batimastat was shown to have no activity on angiotensin-converting enzyme and enkephalinase at concentrations greater than 1000 nm.
Initiation of in Vivo Models

C170HM<sub>2</sub> Hepatic Metastasis Model. Male 6–8-week-old MF<sub>1</sub> nu/nu nude mice were used for the C170HM<sub>2</sub> model (Harlan Olac, Oxford, United Kingdom). The animals were maintained in sterile isolation at 26°C and received food and water ad libitum. C170HM<sub>2</sub> cells were maintained in vitro in RPMI 1640 (GIBCO, Paisley, United Kingdom) containing 10% fetal calf serum (FCS) at 37°C in a humidified incubator gassed with CO<sub>2</sub>. Cells were seeded into 75-cm<sup>2</sup> tissue culture flasks (Costar, Cambridge, MA) 3 days before harvesting with 0.025% EDTA for in vivo use to ensure the cell population was in the exponential phase of growth (i.e., approximately 75% confluency). C170HM<sub>2</sub> cells were injected into the peritoneal cavity in a 1-ml volume containing 10<sup>6</sup> cells in 0.9% NaCl solution, pH 7.3 (Oxford, Basingstoke, United Kingdom). The experiment was terminated on day 40, at which stage it is known that the mean weight of a liver-associated tumor is approximately 0.6 g, and the take rate is approximately 80%. A small number of peritoneal nodules, generally found at the injection site, were shown to develop in some of the animals, but these were usually discrete and easily dissected for quantification purposes. At the termination of the experiment, livers were dissected carefully and weighed, as were any peritoneal tumors. The exposed cross-sectional areas of the liver-invading tumors and the peritoneal nodules were assessed by calliper measurement by an independent observer. The weights of the tumor-containing livers were compared with livers from identical age- and sex-matched non-tumor-bearing animals, and the weight of tumor was calculated by subtraction. Administration of Batimastat in the C170HM<sub>2</sub> Model. Batimastat was suspended at a concentration of 2.5 mg/ml in 0.9% NaCl solution containing 0.01% (v/v) heat-inactivated FCS (GIBCO) in a 1:1 combination with RPMI 1640 containing 0.5% BSA (Sigma). Batimastat or saline/Tween was then added to the cells in a 100-μl volume at concentrations ranging from 0.01 to 3.0 μg/ml. After 4 days of incubation, 50 μl of MTT (Sigma) were added to each well at a final concentration of 1 mg/ml and incubated with the cells for 4 h at 37°C according to the method of Mosmann (14). The MTT and residual medium were removed by aspiration, and crystals were solubilized by the addition of 75 μl of DMSO (Sigma). The plates were agitated for 5 min before the absorbance was read at 550 nm. The effect of batimastat on cell growth was calculated as a percentage of the appropriate saline/Tween control. MTT is converted to an insoluble product by the cellular reductive capacity and has been shown to correlate with direct cell counts (15). This was evaluated in this study when direct cell counts, as assessed by a hemocytometer, were compared with MTT absorbance with the use of initial seeding concentrations from 10<sup>3</sup> to 10<sup>5</sup> cells/well.

Histological Analysis of Tumor Tissue

The tumors were fixed in 10% formal calcium and embedded in paraffin wax. Sections were cut at 5 μm and stained with hematoxylin and eosin.

Statistical Analysis

In vivo and in vitro data were analyzed by a one-way ANOVA with Tukey’s test of multiple comparisons or a Mann-Whitney nonparametric analysis with the use of the SPSS program for the IBM personal computer.

RESULTS

Effect of Batimastat on in Vitro Growth of C170HM<sub>2</sub> and APSLV. In vitro proliferation in the presence of batimastat was assessed by the MTT absorbance assay. This method was validated before its use to ensure it was an accurate assessment of cell proliferation. C170HM<sub>2</sub> cells were plated in duplicate into 96-well plates at concentrations ranging from 10<sup>3</sup> to 10<sup>5</sup> cells/well, and cell numbers were assessed by both the MTT assay and direct cell counts. Regression analysis revealed a significant correlation between the two methods (P < 0.01). The most accurate correlation was between 10<sup>4</sup> and 10<sup>5</sup> cells/well, and this cell density range was used in the in vitro assay.

The effect of batimastat on the in vitro growth of C170HM<sub>2</sub> and APSLV in both serum-free and serum-containing culture media was determined three times. Batimastat had no significant effect on the growth of either cell line (data not shown). Effect of Batimastat on in Vivo Growth of C170HM<sub>2</sub>. Treatment of nude mice bearing C170HM<sub>2</sub> xenografts with batimastat resulted in a significant decrease in both the number and cross-sectional area of liver tumors when compared with both vehicle-treated controls and mice treated with the poorly active diastereoisomer BB-1268 (Figs. 1 and 2). The mean number of liver tumors in the batimastat-treated group was reduced to 35% of the vehicle-treated controls (P < 0.05),
At day 39, the experiment was terminated, and the number of liver tumors was measured. Batimastat, BB-1268, and vehicle were administered i.p. once daily from day 10 onward. At day 39, the experiment was terminated, and the number of liver tumors was measured. Columns, mean; bars, SD. * P < 0.05 from the vehicle-treated control, as evaluated by a one-way ANOVA.

Whereas BB-1268 treatment increased mean number of liver tumors by 52% of the vehicle-treated control (P < 0.05; Fig. 1). Fig. 2 shows the distribution of visible liver tumors (0–4 or more tumors/mouse). In the batimastat-treated group, 50% of animals had no visible tumors compared with only 10% in the vehicle-treated and 5% in the BB-1268-treated groups. In addition, in the batimastat-treated group, only 5% of animals had four or more tumors, whereas 15 and 30% of vehicle- and BB-1268-treated groups, respectively, had four or more tumors.

With regard to tumor size, Fig. 3 shows the mean cross-sectional area of the liver and peritoneal tumors. The batimastat-treated group had a significantly reduced mean area of C170HM2 liver tumors (43% of vehicle-treated controls; P < 0.05), whereas BB-1268-treated tumors had a significantly elevated mean area when compared with both vehicle-treated controls and the batimastat-treated group (139% of the vehicle-treated controls; P < 0.05).

When the mean liver weight is expressed as a proportion of the mean animal weight the resultant ratio for the BB-1268-treated group was significantly greater than those for the batimastat- and vehicle-treated groups, which were not significantly different from one another (Table 1). The ratios of liver:whole-body weight showed a tendency to be smaller in the tumor-free animals, as expected. Twice weekly animal weight measurements showed that batimastat and BB-1268 had no significant effect on the final weights of the animals, despite being administered for 39 days (data not shown).

**Effect of Batimastat on in Vivo Growth of APSLV.** Daily treatment with batimastat resulted in a significant reduction in APSLV tumor weight within the lung (72% of vehicle-treated control) when compared with vehicle-treated controls (P < 0.05) and 3-day treatment with either batimastat-treated mice or vehicle-treated controls (Fig. 4). The latter two were not significantly different from one another. No significant difference in visible nodule number was observed among all four groups (Table 2). Batimastat had no effect on the lung weight of non-tumor-bearing mice (data not shown).

**Histological Analysis of Tumor Tissue.** In the vehicle-treated animals, the liver is massively infiltrated by a poorly differentiated carcinoma. The tumor cells are arranged in nests; the larger ones show central coagulative necrosis. In these, there is a peripheral zone of

---

**Table 1. Effect of batimastat on the mean liver weight:animal weight ratio of nude mice bearing C170HM2 xenografts on day 39 after cell administration**

<table>
<thead>
<tr>
<th>Tumor bearing</th>
<th>Mean liver weight:animal weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.066</td>
</tr>
<tr>
<td>Batimastat</td>
<td>0.061</td>
</tr>
<tr>
<td>BB-1268</td>
<td>0.106</td>
</tr>
</tbody>
</table>

---

**Table 2. Effect of batimastat on the lung weight and tumor nodule number of SCID mice after an i.v. injection of APSLV cells**

<table>
<thead>
<tr>
<th>Group/length of administration of agents</th>
<th>Visible nodules/lung</th>
<th>Percentage of vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle/3 days</td>
<td>212.0 (30.7)</td>
<td>100</td>
</tr>
<tr>
<td>Batimastat/3 days</td>
<td>196.0 (30.9)</td>
<td>92</td>
</tr>
<tr>
<td>Vehicle/26 days</td>
<td>219.0 (18.7)</td>
<td>100</td>
</tr>
<tr>
<td>Batimastat/26 days</td>
<td>216.0 (65.0)</td>
<td>99</td>
</tr>
</tbody>
</table>

---

The APSLV xenograft model was initiated in SCID mice by i.v. injection of $4 \times 10^6$ cells. At day 28, the experiment was terminated, and visible nodules per lung were counted. The AP5LV xenograft model was initiated in nude mice by injection of $10^6$ cells into the peritoneal cavity. The majority of tumor growth is within the liver; however, discrete peritoneal tumors may also develop. Batimastat, BB-1268 (40 mg/kg), and the vehicle (saline/Tween) were administered to the animals once daily. At day 39, the experiment was terminated, and the liver weights of both tumor-bearing and non-tumor-bearing animals were measured. *P < 0.025 from vehicle-treated control (long dosage), as analyzed by a Mann-Whitney nonparametric test.
viable cells and an underlying zone of cells showing evidence of pyknosis and karyorrhexis (Fig. 5a).

In the batimastat-treated animals, the overall appearance is similar to that in the vehicle-treated animals. However, in the nests showing central necrosis, the peripheral zone of viable cells is thinner and the necrosis is more advanced, inasmuch as there is little evidence of pyknosis and karyorrhexis (Fig. 5b).

DISCUSSION

MMPs are one of the main groups of enzymes involved in colorectal tumor progression. In a study by van der Stappen et al. (6), it was shown that fibrillar collagenase activity correlated with grade of histological differentiation. Type IV collagenase also has been shown to have a role in colorectal cancer progression. Detection of the enzyme by in situ hybridization revealed it to be present in the stroma surrounding the tumor (8). Matrilysin, a member of the stromelysin family, has been shown, again by mRNA in situ hybridization, to be located within the tumor cells of colorectal cancers (9).

Batimastat is known to inhibit a wide range of MMP, including interstitial collagenase, \( M_{72,000} \), and \( M_{92,000} \) collagenases, matrilysin, and matrilysin (4), and has been shown to inhibit metastatic spread of the B16 murine melanoma (10). C170HM\(_2\), during invasion within the liver, has been shown to be associated with \( M_{72,000} \) collagenase and interstitial collagenase, indicating that, during invasive growth, members of the MMP family are active. Treatment with batimastat reduced the number of liver tumors forming, i.e., affected take rate, and also inhibited the hepatic growth of the tumors that did form, indicating that, for C170HM\(_2\), metalloproteinases may have been necessary for the initial attachment and subsequent invasive growth through the liver capsule and for the invasive growth within the parenchyma. Histological analysis confirmed that growth of the solid tumors within the liver appeared to be affected by batimastat because the degree of necrosis within the tumors was greater and at a more advanced stage. The effect of the agent was not evaluated directly on angiogenesis because the poor angiogenic profiles of human xenografts make quantification difficult. However, the enhancement of necrosis was indicative of a reduction in vascularization, and angiogenesis has been shown to be inhibited by batimastat in an in vivo matrigel invasion model (16).

It is of interest that the inactive isomer BB-1268 increased both the take rate and growth of C170HM\(_2\) tumors. The poorly active diastereoisomer was included as a control because batimastat is administered in the form of a particulate suspension. BB-1268 has similar physicochemical properties to batimastat, and it is possible that the drug, while being cleared by the RES, may have partially blocked the action of this system, allowing more tumor cells to remain in the peritoneal cavity for enhanced time periods. This may have resulted in greater attachment to the liver when compared with the vehicle-treated control with a fully functional RES. Batimastat may have had the same effect on the RES as did BB-1268 because it was also present as a particulate suspension, and, therefore, its effect on the take rate and growth of C170HM\(_2\) should, perhaps, be compared directly with those of BB-1268.

Batimastat affected the growth but not the take rate within the lungs. The lung colonization model involves i.v. administration of cells and may not involve an invasive step (cells may just lodge within capillary beds). Subsequent outgrowth would appear, in part, to involve invasive enzymes, because the tumors in the batimastat-treated group were of a smaller size than were those of the vehicle-treated control group. This result correlates with results obtained from a study by Koop et al. (17), in which B16 melanoma cells genetically manipulated to overexpress tissue inhibitor of metalloproteinase-1 were compared with nonexpressed parietal cells for their ability to extravasate with the use of intravital microscopy. They found that there was no difference in the time course of extravasation in the two lines, but there was a reduction in size and number of tumors, possibly as a result of a reduced contact phenomenon. The lack of an effect on extravasation has also been shown with batimastat, indicating that MMP regulation achieves importance at the postextravasation stage of metastatic growth.

The MMP inhibitor batimastat significantly reduced tumor growth within both lung and liver tissue in two experimental human colorectal cancer metastasis models. In vitro assessment of the direct effect of batimastat on tumor growth revealed that it did not induce nonspecific cytotoxic effects on the cells. Although zymography identified both \( M_{72,000} \) and \( M_{92,000} \) gelatinases (18, 11), the contribution of other metalloproteinase family members known to be expressed by colorectal tumors (5) is not known. A thorough characterization of the MMP profiles of both cell lines will be established through the detection of mRNA for the enzymes by in situ hybridization. This will then allow the enzyme types affected by batimastat to be identified.

An agent such as batimastat may have a therapeutic role in inhib-
iting invasion and secondary growth of colorectal cancer, for which, at the present time, there are limited therapeutic options.

ACKNOWLEDGMENTS

We acknowledge Dr. B. Gallimore (CRC Laboratories, University of Birmingham, Birmingham, United Kingdom) for providing the original SCID mouse breeding nucleus, British Biotech for providing the funding for the studies and for supplying the inhibitors, and D. Milanowska for typing the manuscript.

REFERENCES

Inhibition of Organ Invasion by the Matrix Metalloproteinase Inhibitor Batimastat (BB-94) in Two Human Colon Carcinoma Metastasis Models

Susan A. Watson, Teresa M. Morris, Graham Robinson, et al.