

Matrix Metalloproteinase Inhibitor BB-94 (Batimastat) Inhibits Human Colon Tumor Growth and Spread in a Patient-like Orthotopic Model in Nude Mice

X. Wang, X. Fu, P. D. Brown, M. J. Crimmin, and R. M. Hoffman

AntiCancer, Inc., San Diego, California 92111 [X. W., X. F., R. M. H.], and British Bio-technology, Ltd., Watlington Road, Cowley, Oxford, OX4 5LY, United Kingdom [P. D. B., M. J. C.]

ABSTRACT

Matrix metalloproteinases have been implicated in the growth and spread of metastatic tumors. This role was investigated in an orthotopic transplant model of human colon cancer in nude mice using the matrix metalloproteinase inhibitor BB-94 (batimastat). Fragments of human colon carcinoma (1-1.5 mm) were surgically implanted orthotopically on the colon in 40 athymic *nu/nu* mice. Administration of BB-94 or vehicle (phosphate buffered saline, pH 7.4, containing 0.01% Tween 80) commenced 7 days after tumor implantation (20 animals/group). Animals received 30 mg/kg BB-94 i.p. once daily for the first 60 days and then 3 times weekly. Treatment with BB-94 caused a reduction in the median weight of the primary tumor from 293 mg in the control group to 144 mg in the BB-94 treated group ($P < 0.001$). BB-94 treatment also reduced the incidence of local and regional invasion, from 12 of 18 mice in the control group (67%) to 7 of 20 mice in the treated group (35%). Six mice in the control group were also found to have metastases in the liver, lung, peritoneum, abdominal wall, or local lymph nodes. Only two mice in the BB-94 group had evidence of metastatic disease, in both cases confined to the abdominal wall. The reduction in tumor progression observed in the BB-94-treated group translated into an improvement in the survival of this group, from a median survival time of 110 days in the control group to a median survival time of 140 days in the treated group ($P < 0.01$). Treatment with BB-94 was not associated with any obvious toxic effect, and these results suggest that such agents may be effective as adjunctive cancer therapies.

INTRODUCTION

Previous studies with the native matrix metalloproteinase inhibitors, TIMP-1¹ and TIMP-2, have demonstrated the potential of these inhibitors to block specific steps in the processes of tumor growth and metastatic progression. In a study of B16 mouse melanoma in mice TIMP-1 was shown to inhibit lung colonization by blood-borne melanoma cells, one of the final steps in the metastatic process (1). Results from a separate study of malignant 4R rat embryo fibroblasts, transfected with the natural matrix metalloproteinase inhibitor TIMP-2, suggested that TIMP-2 can inhibit solid tumor growth (2). Recently, transfection of B16-F10 cells with TIMP-1 has been shown to be associated with inhibition of both the primary tumor growth and metastatic potential of these cells (3).

Collectively, these studies support the hypothesis that matrix metalloproteinase inhibitors can restrict malignant progression by blocking the breakdown of matrix structure that is required for metastasis and tumor expression. However, these studies have examined tumor growth in syngeneic models with mouse and rat tumors. The current study was designed to allow the process of malignant progression to be examined as a whole, in a model that resembled as closely as possible the clinical situation. In this model fragments of metastatic human colorectal carcinoma are surgically implanted on the colon of nude mice. These fragments grow rapidly in the colon environment to form large primary colon tumors. The tumors invade locally and

subsequently metastasize to sites common in metastatic human colorectal cancer, namely, the lymph nodes, liver, peritoneum, and lung (4, 5). The objective of the study was to test the synthetic low molecular weight matrix metalloproteinase inhibitor, BB-94 (batimastat), for its ability to inhibit primary tumor growth, local invasion, and metastatic spread. The effect of BB-94 treatment on the survival of the tumor bearing mice was also examined.

BB-94 (batimastat) ([4-*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(thienylthiomethyl)succinyl]-L-phenylalanine-*N*-methylamide, M_r , 478) is a peptide-like analogue of the collagen substrate. IC_{50} s were determined for BB-94 *in vitro* against the following metalloproteinases: interstitial collagenase, 3 nM; stromelysin, 20 nM; M_r 72,000 type IV collagenase, 4 nM; M_r 92,000 type IV collagenase, 4 nM; and matrilysin, 6 nM. The inhibitor shows little detectable inhibitory activity against other metalloproteinases such as angiotensin converting enzyme (0% inhibition at 1000 nM) (6).

MATERIALS AND METHODS

Animals. Male and female athymic *nu/nu* mice 4-6 weeks of age were used for the study. The animals were maintained in a sterile environment and cages, food, and bedding were autoclaved. The animal diets were obtained from Harlan Teklad (Madison, WI) and 0.15% (v/v) HCl was added to the drinking water. Forty mice were used for the study, 20 each in the treatment and control groups.

Colon Carcinoma Xenograft. The human colon mucinous adenocarcinoma was obtained from colon cancer patient AC1935. The tumor was in the 5th passage when used in the study. Before implantation the tumor was harvested from the colon of a nude mouse, cut into 1-1.5-mm fragments, and placed in Earle's minimal essential medium.

Surgical Orthotopic Implantation. The animals were anesthetized with isoflurane and the abdomens were sterilized with iodine and alcohol. A small midline incision was made in the abdomen and the colocecal portion of the colon was exposed. The serosa was removed from the site where the tumor fragments were to be implanted and 8-10 fragments were implanted on the top of the animal intestine. An 8-0 surgical suture was used to penetrate these small tumor fragments and suture them on the intestine wall. The intestine was returned to the abdominal cavity and the abdominal wall was closed with 7-0 surgical sutures. The animals were maintained in the sterile environment after surgery.

Pharmacokinetics of BB-94. The dosing regimen used in the current study was based on a pharmacokinetic analysis performed prior to the study in BALB/c mice (6-8 weeks of age). The concentration of BB-94 in the blood was determined by *ex vivo* bioassay. Briefly, 0.5 ml of blood was taken by cardiac puncture at appropriate times and placed in a polypropylene tube containing 3 ml of methanol. The precipitated proteins were separated by centrifugation and 2.5-ml portions of the methanol extract were dried under vacuum. The extracted BB-94 was resolubilized in 200 μ l of dimethyl sulfoxide and added to tubes containing radiolabeled type I collagen and collagenase. The concentration of inhibitor in the blood extracts was determined by comparison with the degree of inhibition of collagenase in parallel incubations that contained known concentrations of BB-94. Separate experiments in which BB-94 was added directly to whole blood revealed that the extraction efficiency of the methanol process was approximately 50%.

The blood levels of BB-94 following a single 30-mg/kg dose ranged between 30 and 12 ng/ml over 24 h. These values, when corrected for extraction efficiency (~50%), are over 10-fold higher than the IC_{50} of this inhibitor for collagenase (1.5 ng/ml) and the M_r 92,000 and M_r 72,000 type IV

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¹ The abbreviation used is: TIMP, tissue inhibitor of metalloproteinases.

collagenases (2 ng/ml). On the basis of these results a dose of 30 mg/kg i.p. once daily was selected. However, in view of the length of the experiment this was reduced to 30 mg/kg three times weekly after 60 days in order to reduce any morbidity associated with the i.p. injection. Administration of the metalloproteinase inhibitor BB-94 or vehicle (phosphate buffered saline, pH 7.4, containing 0.01% Tween 80) began 1 week after surgery.

Evaluation of Response. During the course of the study, both primary tumor size and the performance status of each animal were followed. On autopsy the weight of the primary tumor was calculated as

$$\text{Weight} = \frac{\text{width}^2 \times \text{length}}{4}$$

The extent of both local and distant tumor spread was also assessed. Tissue samples were also processed for histology by fixation in 10% formalin followed by paraffin embedding and sectioning. The sections were stained with hematoxylin and eosin.

The sizes of primary tumors in the two groups were compared using the Mann-Whitney two-tailed test and the differences in survival were compared using the log rank test.

RESULTS

Effect of BB-94 on Primary Tumor Growth. Tumors developed on the colon in all of the animals, although two animals in the control group were lost following death and post-mortem data were not obtained. Treatment with BB-94 caused a significant reduction in the median weight of the primary tumor from 293 mg (range, 1141 to 124 mg) in the control group to 144 mg (range, 424 to 38 mg) in the BB-94 treated group ($P < 0.001$).

Effect of BB-94 on Local/Regional Invasion. Treatment with BB-94 caused a marked reduction in the incidence of tumor invasion of adjacent tissue, from 12 of 18 mice in the control group (67%) to 7 of 20 mice in the BB-94 treated group (35%) (Table 1). In the control group there were 8 cases of invasion of adjacent abdominal wall and 4 cases of invasion of the peritoneal surface. In the BB-94 treated group there were 6 cases of abdominal wall invasion and 1 case of peritoneal invasion.

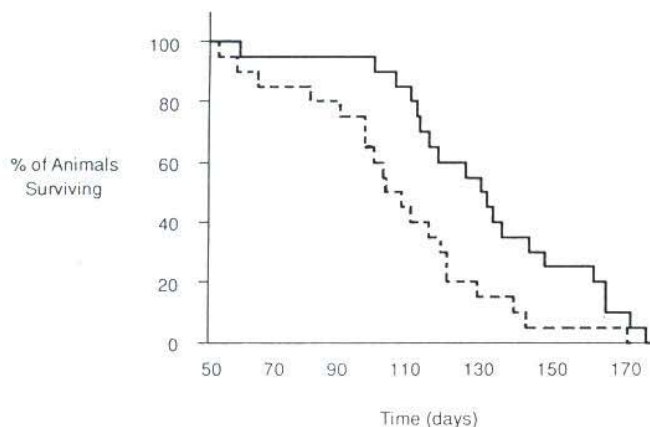


Fig 1. Survival curve for mice bearing orthotopically transplanted human colon carcinoma. Animals were treated with BB-94 (—) or vehicle (---) once daily from day 7 to day 60 and three times weekly thereafter.

Table 1 Effect of BB94 on tumor progression

Group	Primary Tumor size (mg)		Survival time (days)		Incidence of*	
	Mean ± SD	Median	Mean ± SD	Median	Invasion	Metastasis
Control	362 ± 246	293	112 ± 33	110	12/18	6/18
BB-94	147 ± 91	144	140 ± 33	140	7/20	2/20

Effect of BB-94 on Metastasis. Treatment with BB-94 caused a reduction in the incidence of metastasis. In the control group 3 mice had metastases at a distant site on the abdominal wall, 1 mouse had metastases on the parietal peritoneum; 1 mouse had malignant ascites; and 1 mouse had extensive metastatic disease with metastases in the cecum, liver, and mesentery lymph nodes. In addition, another mouse in this group had a possible lung metastasis. In contrast, only 2 mice in the BB-94 treated group showed signs of distant tumor spread, in both cases to the abdominal wall (Table 1).

$P < 0.01$ for total invasion and metastases comparing the treated and control group.

Effect of BB-94 on Survival. Treatment with BB-94 resulted in a modest improvement in survival. On day 138, 50% of the BB-94 treated animals remained alive compared with only 15% of the control animals. The median survival time of the control group was 110 days (range, 187–59 days) compared to 140 days (range, 193–60 days) for the BB-94 treated group ($P < 0.01$) (Fig. 1).

$P < 0.025$ for survival probability difference between the treated and control group at day 138 using the Mantel-Haenszel test (7).

Effect of BB-94 on Tumor Histology. Although it has not been possible to quantitate differences in histological appearance, the tumors in the control group showed high degrees of viability and cellularity, whereas those from the BB-94 treated animals appeared less viable, with lower cell densities and often increased amounts of mucin (Fig. 2).

Effect of BB-94 on Animal Weight. Treatment with BB-94 caused no significant changes in animal weight when compared to the control group, indicating that BB-94 was not overtly toxic (data not shown).

DISCUSSION

The current model of orthotopic implantation of a human colon carcinoma provides a unique opportunity to study a human malignancy in a context that is as close as possible to the clinical condition. In this study, treatment with BB-94 resulted in inhibition of primary tumor growth, local/regional spread, and distant metastasis. Since the implanted tumor was in direct contact with BB-94 it is possible that some of the antitumor activity might be the result of direct toxicity. However, studies with two other human colorectal carcinoma cell lines, AP-5 and C170HM2, and the mouse melanoma cell line B16-BL6 have shown that BB-94 does not inhibit proliferation of these cells, even at concentrations approaching saturation ($6 \mu\text{M}$).² It has not been possible to examine *in vitro* the effect of BB-94 on the colorectal carcinoma used in the current study. However, direct cytotoxic effect seems unlikely, particularly in view of the lack of overt toxicity during the 193-day study.

A large body of correlative data now exists supporting the involvement of matrix metalloproteinases in the malignant progression of many common tumors (for review see Ref. 8). In the case of human colorectal carcinoma, fibrillar collagenolytic activity has been shown to correlate with histological grade (9) and expression of the M_r 72,000 gelatinase has been correlated with tumor progression (10). *In situ* RNA hybridization studies have shown the source of the M_r 72,000 gelatinase in colorectal carcinoma to be the tissue stroma adjacent to the invasive front of the tumour (11). The antitumor effects observed in the current study are consistent with the established action of a matrix metalloproteinase inhibitor such as BB-94. The model has allowed the action of this inhibitor to be studied on the process of malignant progression as a whole. By blocking matrix degradation

* S. Watson and R. Giavazzi, personal communication.

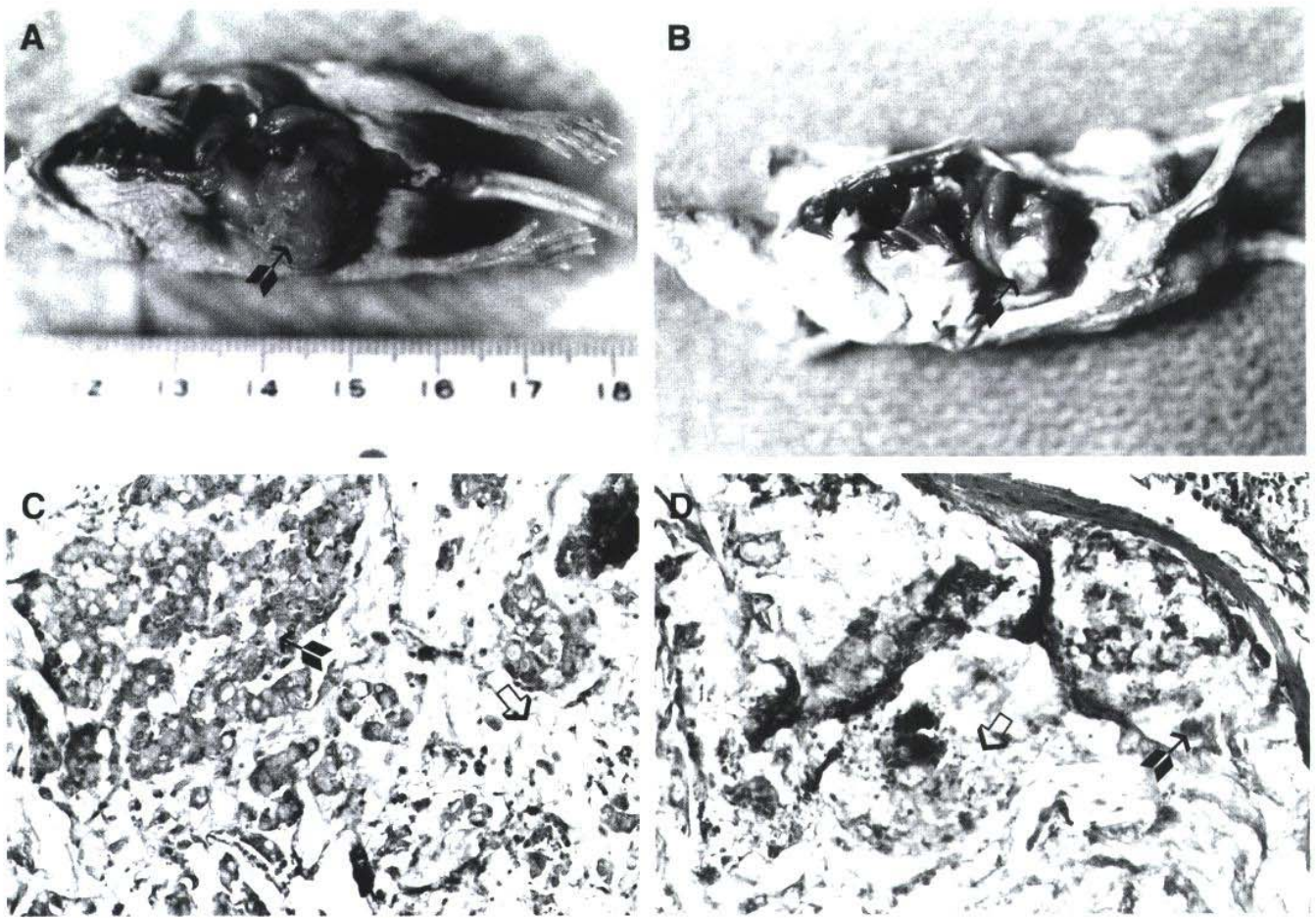


Fig 2. A, gross picture of the orthotopically implanted human colon tumor in an untreated mouse. The implanted tumor is 10 x 13.4 mm at day 126 (arrow). B, gross picture of the orthotopically implanted human colon tumor in a BB-94 treated mouse. The implanted tumor is 5.2 x 5.6 mm at day 180 (arrow). The tumor appears more firm than the tumor in the untreated mice. C, histopathology of primary human colon tumor of an untreated mouse shown in A. Solid arrow, tumor cells; open arrow, mucin. D, histopathology of primary human colon tumor of a BB-94 treated mouse shown in B. The tumor seems to have more mucin (open arrow) and less tumor cells (solid arrow) than the untreated mice.

BB-94 was able to inhibit not only metastatic spread but also "primary" tumor growth.

This effect on the growth of a solid tumor has important implications for the possible therapeutic use of matrix metalloproteinases. The prevention of metastasis has always been of more interest to academic scientists than to practicing oncologists. The latter are acutely aware of the fact that the patients in need of more effective therapy are those whose tumors have already metastasized. Following surgery, chemotherapy remains the most effective way to check the relentless growth and spread of metastatic tumors. Provided agents such as BB-94 show in the clinic the low toxicity profile seen in preclinical studies, it would be possible to envision their use as adjunctive agents to complement more toxic chemotherapies. Used in this way it is possible that matrix metalloproteinase inhibitors could contain both tumor growth and spread between cycles of cytotoxic agents. The results from this study indicate that i.p. BB-94 should be investigated in the clinic as a therapy for inhibiting malignant progression in colon carcinoma.

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