Addition of Matrix Metalloproteinase Inhibition to Conventional Cytotoxic Therapy Reduces Tumor Implantation and Prolongs Survival in a Murine Model of Human Pancreatic Cancer

Mahmudul Haq, Alexis Shafii, Emmanuel E. Zervos, and Alexander S. Rosemurgy

Department of Surgery, University of South Florida, Tampa, Florida 33612 [M. H., A. S. R.]; Department of Surgical Oncology, Ohio State University, Columbus, Ohio 43210 [E. E. Z.]; and James A. Haley Veterans Affairs, Tampa, Florida 33612 [A. S.]

ABSTRACT
Matrix metalloproteinases (MMPs) participate in basement membrane degradation, a critical step in invasion of cancer cells. We have previously shown that MMP inhibition of pancreatic cancers improves survival and decreases MMP production in vivo. The purpose of this study was to determine whether BB-94 was better than cytotoxic therapy and would increase the efficacy of cytotoxic therapy (gemcitabine) in a murine model of human pancreatic cancer. A human pancreatic adenocarcinoma cell line (HPAC) was injected into the pancreata of BALB/c nu/nu mice. The mice were randomized 7 days after cancer cell injection to receive vehicle control, BB-94, gemcitabine, or gemcitabine and BB-94 until death or sacrifice at 84 days. At necropsy, tumors were harvested, and the relative enzyme activities of MMP-2 and MMP-9 were determined by gelatin zymography. Active MMP-2 levels in serum were determined using an ELISA technique. Combination treatment with gemcitabine and BB-94 significantly reduced implantation rates and improved survival in mice with documented orthotopic tumors compared with either therapy alone or control. Tumor levels of active and latent MMP-2 were higher than those of MMP-9 in both treated and control mice. There was a significant reduction of tumor MMP-2 activity in mice treated with BB-94, gemcitabine, or gemcitabine and BB-94. Serum levels of active MMP-2 were reduced in all treated groups, with the greatest reduction occurring in mice treated with gemcitabine and BB-94. Combination therapy with gemcitabine and BB-94 reduces cancer implantation and improves survival compared to treatment with BB-94, gemcitabine, or vehicle control alone. MMP production was reduced in all treated groups, reflecting reduced tumor progression, which was particularly seen with combination therapy with gemcitabine and BB-94. This study supports combining MMP inhibition with cytotoxic therapy (gemcitabine) for patients with pancreatic cancer.

INTRODUCTION
Pancreatic cancer is a deadly cancer. It is one of the few cancers whose incidence equals its death rate (1). Despite advances in diagnostic and operative techniques, only a small percentage of patients with this cancer survive more than 1 year (1).

The impact of conventional chemotherapy, such as 5-fluorouracil, on pancreatic cancer has been minimal (2). Approved by the United States Food and Drug Administration only several years ago, gemcitabine has begun to play a fundamental role in the chemotherapeutic approach to pancreatic cancer (3). Nonetheless, results with chemotherapy, including gemcitabine, are not significantly remarkable (4).

Pancreatic cancers express a relatively high concentration of MMPs (5). These enzymes participate in basement membrane and peritumoral stromal degradation and angiogenesis, critical aspects of cancer growth and invasion (6–8). Trial studies with the MMP inhibitor have suggested that MMP inhibition prolongs survival in a murine model of human pancreatic cancer, and these inhibitors are well tolerated by humans with pancreatic cancer (9, 10). Additional studies have shed light on the pathways and implications of MMP inhibition in pancreatic cancer (11) and further promote this treatment approach.

This study was undertaken in an established murine orthotopic model of human pancreatic cancer to confirm that MMP inhibition prolongs survival. This study was also designed to compare MMP inhibition with gemcitabine therapy and determine the impact of combination therapy involving both gemcitabine and MMP inhibition. Our hypotheses in undertaking this study were that MMP inhibition using BB-94 in a murine model of human pancreatic cancer would improve survival, would be superior to gemcitabine therapy, and would augment gemcitabine therapy.

MATERIALS AND METHODS

Tested Drugs. BB-94 (batimastat) was provided by British Bio-Technology Ltd. (Cowley, Oxford, United Kingdom). BB-94 is a synthetic compound of low molecular weight (M, 478,000) that inhibits a broad spectrum of MMPs in the low nanomolar range. BB-94 was brought into suspension at 7.5 mg/ml (5 mM) in PBS plus 0.01% (v/v) Tween 80 (pH 7.2; Sigma, St. Louis, MO) by sonication. In vivo, BB-94 (30 mg/kg) or vehicle alone was injected i.p. into mice beginning 7 days after celiotomy and orthotopic tumor injection and continuing every other day until death or sacrifice. This dosing regimen has been noted to generate serum levels between 12 and 30 ng/ml over 24 h, and, when corrected for extraction efficiency (50%), is 10-fold higher than the IC50 of MMP-2 and MMP-9 (12).

Gemcitabine (chemically, difluorodeoxycytidine) is a nucleoside analogue with a wide antimetabolite activity in murine models of solid tumors (13). After intracellular phosphorylation, the drug competes with deoxycytidine triphosphate for incorporation into DNA (14, 15), which in turn inhibits DNA synthesis (16). The drug also appears to inhibit DNA synthesis by inhibition of ribonucleotide reductase (16). Gemcitabine (Genzor; Eli Lilly & Co., Indianapolis, IN) came in a suspension (1 g of gemcitabine hydrochloride, 1 g of manitol, and 62.5 mg of sodium acetate). Hydrochloric acid and/or sodium hydroxide may have been used to adjust the pH value. Gemcitabine was administered at 120 mg/kg after dilution with 0.9% sodium chloride (2.4 mg/mouse/injection i.p.) every third day from 7 days after orthotopic injection until death or sacrifice.

Pancreatic Cancer Cell Line. HPAC (ATCC CRL-2119) is a moderately differentiated human adenocarcinoma of ductal origin (17). HPAC cells were grown in DMEM:F12–4F5, which is a mixture of DMEM and Ham’s F-12 nutrient medium (1:1) containing 1.2 g/liter NaHCO3 and 15 mM HEPES (pH 7.3; Life Technologies, Inc., Gaithersburg, MD). This culture medium is supplemented with six factors (5% fetal bovine serum, 2 μg/ml insulin, 5 μg/ml transferrin, 40 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, and 1× antibiotic/antimycotic mixture). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2-enriched air, and medium was exchanged every 3 days according to the protocol.

 Xenografts. Ten million HPAC cells were injected at celiotomy into the head of pancreata of BALB/c nu/nu mice (Life Sciences, Inc., St. Petersburg, FL) as described previously (9). All mice were maintained under identical conditions. Seven days after celiotomy and intrapancreatic tumor injection, the mice were randomly assigned to one of four treatment groups: (a) vehicle control (n = 26); (b) BB-94 (n = 27); (c) gemcitabine (n = 28); or (d) 

Received 9/20/99; accepted 4/10/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Department of Surgery, University of South Florida, P. O. Box 1289, Room F145, Tampa General Hospital, Tampa, Florida 33601. Phone: (813) 251-7393; Fax: (813) 253-1920.
2 The abbreviation used is: MMP, matrix metalloproteinase.
combination therapy involving gemcitabine and BB-94 (n = 24). Preterminal mice and mice alive at 84 days were sacrificed, and their tumors were harvested, weighed, and snap-frozen for MMP analysis.

### Protein Extraction
Tumor tissue (100 mg) from each mouse was individually homogenized at 4°C in 0.1 M Tris buffer (pH 8.1) containing protease inhibitors (100 μg/ml aprotinin, 200 μm phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin; Sigma). The homogenate was sonicated for 1 min at 4°C and then spun in an ultracentrifuge at 100,000 X g for 45 min at 4°C. The supernatant was collected, and protein concentrations were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA).

### Affinity Chromatography
Gelatin-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) was washed three times with equilibration buffer [50 mM Tris, 150 mM NaCl, 0.02% Tween 20, 0.7% Brij 35, and 10 mM EDTA (pH 7.5)]. Twenty μl of equilibrated gelatin-Sepharose 4B were added to 1600 μg of tumor protein extracts and diluted to a final volume of 1 ml with PBS. Samples were then placed on an end-over-end shaker overnight at 4°C to allow the binding of gelatinases to the gelatin-Sepharose. After overnight shaking, nonspecific bound proteins were washed from gelatin-Sepharose with salted equilibrium buffer (50 mM Tris, 200 mM NaCl, 5 mM CaCl₂, 0.02% Tween 20, 0.07% Brij 35, and 10 mM EDTA). MMPs were eluted from the washed gelatin-Sepharose by adding 15 μl of nonreducing Laemmli solution.

### Gelatin Zymography
The affinity-purified samples (diluted 1:4) were electrophoretically separated on an 8% SDS-PAGE gel impregnated with gelatin (1 mg/ml). After incubation, the gels were rinsed twice in 2.5% Triton X-100 and three times in double-distilled H₂O. The gels were then incubated at 37°C for 4 h in buffer [200 mM NaCl, 10 mM CaCl₂, 0.07% Brij 35, and 50 mM Tris-HCl (pH 7.4)]. The gels were stained with 0.05% Coomassie Brilliant Blue and destained in 10% acetic acid in H₂O. Gelatinolytic enzymes were detected as transparent bands on the background of the Coomassie Brilliant-stained gel. Relative enzyme activity was quantified by densitometric analysis of the negatively stained bands. Latent MMP-2 (M₉ 72,000), activated MMP-2 (M₉ 62,000), latent MMP-9 (M₉ 92,000), and activated MMP-9 (M₉ 86,000) were identified by comparing them with known gelatinolytic activities from conditioned media of HT-1080 cells (18).

### ELISA
Active levels of serum MMP-2 were determined by using an ELISA technique (Amersham Pharmacia Biotech). With aseptic precaution, blood was collected by cardiac puncture at the time of nude mice necropsy. Clear serum was collected after centrifugation at 3000 rpm for 10 min at 4°C. The experiment was done according to the recommended procedure suggested by the company. One hundred μl of standards and serum samples from the mice were incubated in 96-well microtiter plates precoated with anti-MMP-2 antibody. Any MMP-2 present would be bound to the wells, with other components of the sample being removed by washing and aspiration. Endogenous free active MMP-2 was detected through activation of the modified prodetection enzyme and the subsequent cleavage of its chromogenic peptide substrate. The resultant color was read at 405 nm wavelength for absorbance by a microtiter plate spectrophotometer (Dynatech Laboratories Inc.). The concentration of active MMP-2 in a given sample was determined by interpolation from a standard curve.

### Data and Statistical Analyses
Relative band densities were determined using the UVP GDS 8000 gel documentation system (UV Products, Upland, CA). Peak areas were determined using GDS Image Analysis Software (UV Products) and compared using the ANOVA test. Implantation rates and survival curves in mice were compared using contingency tables, the log likelihood ratio test, and the Wilcoxon test. Animal weights and tumor weights were averaged for each group and compared using the Mann-Whitney U test. MMP levels in tumors and serum were compared by using an ANOVA test of repeated measures. Statistical analysis was undertaken using TRUE-EPISTAT (Epistat Services, Richardson, TX). Data are reported as the mean ± STD when appropriate. Significance was accepted with 95% confidence.

### RESULTS

#### Implantation Rates
Therapy with BB-94, gemcitabine, or both was initiated randomly 7 days after celiotomy and orthotopic injection of HPAC cells. Based on autopsy findings, mice receiving vehicle control had an implantation rate of 81% (21 of 26 mice). Implantation was decreased to 71% in mice receiving BB-94 (19 of 27 mice; P = 0.53 compared with control) and 75% in mice receiving gemcitabine (21 of 28 mice; P = 0.75 compared with control, log likelihood ratio test; Fig. 1). Implantation of HPAC occurred in only 5 of 24 (21%) mice receiving combination therapy of BB-94 and gemcitabine, significantly below the implantation rates seen with either therapy alone (P < 0.0001) or the implantation rates seen with the other therapy groups combined (P < 0.0001, log likelihood ratio test; Fig. 1).

#### Mice Weights
Whereas subjective measures of the quality of murine life are difficult to record and convey, mice receiving combination therapy seemed more active and healthier than mice receiving either therapy alone or vehicle control. Consistent with this observation, mice receiving combination therapy maintained their weight significantly better than mice receiving either therapy alone or control (P < 0.05; Fig. 2).

#### Tumor Weights
Orthotopic tumors were carefully dissected free of all surrounding normal tissue and weighed. Compared with tumor weights in mice receiving vehicle control (n = 21), tumor weights...
were significantly reduced in mice receiving gemcitabine \((n = 21)\) and in mice receiving the combination of gemcitabine and BB-94 \((n = 5)\) therapy \((P < 0.05; \text{Fig. 3})\).

**Tumor MMP Levels.** Tumor MMP-2 levels were higher than MMP-9 levels, in both active and latent forms, in all groups of mice (Fig. 4, A and B). Active MMP-9 levels were low, and changes with therapy were small, although therapy did reduce the levels (Fig. 4, A and B). There was a significant reduction in active form MMP-2 and latent form MMP-2 with therapy \((P < 0.05)\). Among mice receiving therapy, differences in MMP levels were not significant (Fig. 4, A and B). Similar previous results were confirmed by Western blot from our laboratory (19).

**Serum MMP-2 Levels.** Active form MMP-2 levels in the serum were highest in mice receiving vehicle control. Levels were reduced in mice receiving BB-94, gemcitabine, or combination therapy (Fig. 5). Active form MMP-2 levels were lowest in mice receiving combination therapy \((\text{Gem} + \text{BB-94})\).

**Survival.** Survival data involved only mice with documented cancers at autopsy. BB-94 and gemcitabine each improved survival compared with vehicle control, but without statistical significance. Mice receiving vehicle control \((n = 21)\) survived, on average, 62.5 \(\pm\) 12.6 days, whereas those receiving BB-94 \((n = 19)\) survived, on average, 67.5 \(\pm\) 13.0 days \((P = 0.47\) compared with control, Student’s \(t\) test). Mice receiving gemcitabine \((n = 21)\) alone survived for an average of 74.5 \(\pm\) 11.0 days \((P = 0.07\) compared to control). The combination of gemcitabine and BB-94 \((n = 5)\) improved average survival to 82.0 \(\pm\) 0.00 days, which was superior to survival with vehicle control \((P < 0.001)\), BB-94 alone \((P < 0.0001)\), gemcitabine alone \((P < 0.0001)\), and all other therapy groups combined \((P < 0.001, \text{Wilcoxon test})\). Survival curves documented the superiority of combination therapy \((P < 0.0001, \text{Wilcoxon test}; \text{Fig. 6})\).

**DISCUSSION**

With reduced operative morbidity and prospects of new anticancer therapies, there is renewed interest in impacting pancreatic cancer. The introduction of gemcitabine in 1996 improved the medicinal armamentarium against pancreatic cancer, particularly for quality of life issues. Although gemcitabine was believed to impact survival, improvement over existing therapies, including 5-fluorouracil, did not seem meaningful because mean survival for patients with unresectable disease was only increased by a few weeks (4). The development of MMP inhibitors added a novel approach to the treatment of pancreatic cancer, with promising results in preclinical (9) and clinical studies (10). Until this study, the relative efficacies of MMP inhibition and gemcitabine had not been determined in any format, and nothing was known of their potential additive efficacy. This preclinical study documents that combination therapy of MMP inhibition and cytotoxic therapy with gemcitabine is superior to either therapy alone in the treatment of human pancreatic cancer.
of therapy 7 days after orthotopic injection of HPAC cells removed potential treatment bias.

Both gemcitabine and BB-94 reduced rates of cancer implantation after injection at celiotomy, although neither reduction was significant. Combined therapy of BB-94 and gemcitabine dramatically reduced implantation, although therapy was initiated 7 days after tumor cell injection. This reduction was so profound that only a small number of mice receiving combination therapy were noted to have pancreatic cancer at necropsy after sacrifice. The implications of this seem profound, suggesting a role for combination therapy as an adjuvant therapy after pancreatic resections of curative intent, when the potential tumor burden should be very low.

The impact of therapy on survival was determined by analyzing the impact of therapy on survival curves, but not significantly. Conversely, combination therapy of gemcitabine and BB-94 significantly and dramatically impacted survival, regardless of how it was quantified or described. Consistent with improved survival, mice receiving combination therapy enjoyed an improved quality of life as evidenced by subjective measures such as shinier coats, increased energy and activity, and an overall healthier appearance and objective measures such as more optimal body weights and reduced tumor weights.

Of the 15 recognized MMPs, MMP-2 and MMP-9 are felt to be the most important in determining the aggressiveness of pancreatic cancer. We have shown that inhibition of MMP-2 seems to be most important in prolonging survival (20). With this specific cancer cell line in this model, tumor MMP-2 levels were greater than MMP-9 levels. Changes in active form MMP-9 levels with therapy were small, although all three therapies reduced tumor MMP-9 levels compared with the vehicle control. This reduction was more evident with latent form MMP-9. Tumor MMP-2 levels were relatively higher than tumor MMP-9 levels, and there were significant reductions in both active and latent forms of MMP-2 with all therapies, but combination therapy seemed to be the best at reducing active MMP-2 levels.

Because tumor MMP levels were determined per standard weight of tumor, the smallest tumors had the least amount of MMP. Because combination therapy resulted in the smallest tumors, a broad assimilation of the data conveys that combination therapy with gemcitabine and BB-94 unquestionably produced the most notable reduction in total MMP levels. Consistent with this observation are the serum levels of active form MMP-2 among the four treatment groups. Combination therapy with gemcitabine and BB-94 resulted in the lowest serum level of active form MMP-2.

Compared with the vehicle control, gemcitabine reduces MMP levels by decreasing tumor growth kinetics and tumor size and thereby indirectly reduces tumor and serum MMP levels and activity commensurately. BB-94 directly inhibits MMP activity and zymogen activation (19) and thereby reduces tumor growth, size, and kinetic activity. Combined therapy with gemcitabine and BB-94 inhibits MMP activity and reduces tumor growth kinetics, thereby limiting tumor growth through two diverse mechanisms, resulting in the lowest MMP levels and the best outcomes. Moreover, similar results have been reported previously in an ovarian carcinoma xenograft when BB-94 was administered in adjuvant therapy with a different cytotoxic agent, namely, cisplatin (22).

Gemcitabine has become well established in the treatment of pancreatic cancer (4). MMP inhibition, however, remains to be established, although early trials suggest a clinical role (23, 24) that remains to be defined. From this preclinical study, it seems that combination therapy of MMP inhibition and cytotoxic therapy holds promise in the treatment of pancreatic cancer. This seems especially true under the circumstance of relatively minimal tumor burden. A clinical trial using marimastat to compare MMP inhibition with gemcitabine in patients with unresectable pancreatic cancer has completed enrollment, and the results will soon be forthcoming. A trial comparing gemcitabine and marimastat with gemcitabine alone in patients with unresectable pancreatic cancer has completed enrollment, and the results will be known within 1 year.
This preclinical study gives us hope that MMP inhibition will significantly prolong the survival of pancreatic cancer patients as compared with that seen in patients treated with cytotoxic therapy alone and begins to elucidate mechanisms that may result in prolonged survival.

REFERENCES

Addition of Matrix Metalloproteinase Inhibition to Conventional Cytotoxic Therapy Reduces Tumor Implantation and Prolongs Survival in a Murine Model of Human Pancreatic Cancer

Mahmudul Haq, Alexis Shafii, Emmanuel E. Zervos, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/12/3207

Cited articles
This article cites 22 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/12/3207.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/12/3207.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/60/12/3207.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.