

# Metronomic Gemcitabine in Combination with Sunitinib Inhibits Multisite Metastasis and Increases Survival in an Orthotopic Model of Pancreatic Cancer

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## Abstract

Metronomic chemotherapy suppresses growth of primary tumors and established metastases. However, its effect on metastatic progression is essentially unknown. We report the treatment of a metastatically competent model of pancreatic cancer with metronomic gemcitabine and sunitinib. Mice with orthotopic, red fluorescent protein-expressing, pancreatic cancer tumorgrafts were treated with gemcitabine on a metronomic (1 mg/kg daily, METG) or maximum tolerated dose (150 mg/kg twice weekly, MTDG) schedule with or without sunitinib (SU). Rates of primary tumor growth, metastasis, ascites, and survival were calculated. Gemcitabine at a daily dose of 2 mg or greater led to toxicity within 1 month in mice without tumors but METG at 1 mg/kg/d was well tolerated. Mice with pancreatic cancer tumorgrafts died with metastatic disease at a median of 25 days. METG/SU significantly prolonged median overall survival (44 days) compared with control or either regimen alone ( $P < 0.05$ ). Primary tumor growth was inhibited by METG/SU ( $P = 0.03$ ) but neither METG nor sunitinib alone. In contrast, treatment with METG suppressed metastasis at multiple sites, an effect enhanced by sunitinib. MTDG with or without sunitinib had the most favorable effect on primary tumor growth and survival, but its antimetastatic efficacy was similar to that of METG/SU. von Willebrand factor expression was inhibited by METG. Antimetastatic activity approaching that of MTDG is achieved with a total dose reduced 42 times using METG and is further enhanced by sunitinib. Our results suggest the potential of this therapeutic paradigm against pancreatic cancer in the adjuvant and maintenance settings. *Mol Cancer Ther*; 9(7); 2068–78. ©2010 AACR.

## Introduction

Pancreatic adenocarcinoma has an approximate annual incidence in the United States of 42,000 patients (1). Unfortunately, despite advances in treatment, it remains the fourth leading cause of cancer death in this country. The virulence of pancreatic cancer is due in large part to its aggressive tumor biology and high metastatic potential. Indeed, less than 15% of patients who are diagnosed with pancreatic cancer have localized, surgically resectable disease at presentation (2). Among patients with operable disease who undergo apparently complete surgical resection, most recur at locoregional or distant sites within 7 years—even those who undergo a margin-negative resection with negative lymph nodes (3). Because pancreatic cancer is relatively resistant to chemotherapy,

prognosis following systemic treatment of recurrent or advanced disease is even poorer. In such patients, rapid distant progression typically leads to death within 1 year despite conventional therapies (4). Thus, regardless of the initial stage at presentation, essentially all patients with pancreatic cancer ultimately die of extrapancreatic disease. Effective new strategies targeting both local progression and distant metastasis are urgently needed.

Cytotoxic chemotherapeutics have typically been administered in short cycles, separated by treatment breaks, at the maximum tolerated dose (MTD). Unfortunately, the high doses used in these schedules are often associated with significant toxicity. In addition, the obligatory treatment breaks between cycles designed to minimize toxicity present the opportunity for tumor regrowth and the development of chemoresistance. To minimize these confounding factors, investigators have turned toward alternate therapeutic strategies. One such approach is “metronomic” drug dosing, in which standard chemotherapeutics are administered at doses well below their MTD over long periods without treatment breaks (5). The anticancer effects of metronomic regimens may be due in part to inhibition of endothelial cell proliferation and loss of the supporting tumor microvasculature (6, 7).

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Metronomic dosing of various agents has been shown to inhibit primary tumor growth of several experimental human cancers (8–10). Subsequent studies have shown that the anticancer activity of metronomically dosed drugs may be further enhanced by combination therapy with antiangiogenic or antistromal agents (11, 12). Although the beneficial effects of metronomic chemotherapy on primary tumor growth and established metastatic disease (13, 14) have been described, little knowledge exists regarding the impact of this strategy on the development and progression of solid tumor metastases. Moreover, the effect of metronomic chemotherapy on pancreatic cancer progression and dissemination is completely unknown.

Gemcitabine represents the primary systemic agent used for patients with pancreatic cancer. On standard MTD schedules, the drug is associated with manageable toxicity, and its administration has led to a survival benefit both in the primary and adjuvant settings (4, 15). We have previously shown that the clinical effects of gemcitabine derive, in part, from its significant antiangiogenic and antimetastatic properties (16, 17). In a recent report, gemcitabine was shown to have activity against orthotopic human pancreatic cancer primary tumors when dosed on a metronomic schedule (18). However, the model used in that study did not disseminate, and therefore no conclusions regarding the antimetastatic efficacy of metronomic gemcitabine (METG) were possible.

Given the critical influence of metastasis on the clinical history of pancreatic cancer, we sought to characterize the effects of METG and combination therapy on primary tumor growth and the metastatic progression of a clinically relevant, *in vivo* model. To this end, we used our well-described, highly metastatic, red fluorescent protein (RFP)-expressing, orthotopic tumorgraft model of human pancreatic cancer (19–22). We evaluated gemcitabine, delivered on a metronomic schedule, with or without sunitinib, a receptor tyrosine kinase inhibitor previously shown to be active against experimental pancreatic cancer in combinations with radiation and high-dose gemcitabine (21–24).

## Materials and Methods

### Human cell lines, animals, and drugs

The human pancreatic cancer cell lines MIA PaCa-2, BxPC-3, and PANC-1 and human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection. Cancer cells were grown and maintained in Dulbecco's modified Eagle's medium (MIA PaCa2 and PANC-1) or RPMI medium (BxPC-3) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin (Invitrogen). HUVECs were grown on endothelial cell attachment factor (Sigma)-coated plates in human endothelial growth medium-2 (PromoCell, Inc.) supplemented with 10 ng/mL fibroblast growth factor, 5 ng/mL epidermal growth factor, 0.5 ng/mL vascular endothelial growth factor, 0.2 µg/mL hydrocortisone, 22.5 µg/mL heparin, and

2% fetal calf serum. Cells were maintained with humidity at 37°C with 5% CO<sub>2</sub>.

Female nude mice (NCR-nu) ~5 weeks of age were maintained in a barrier facility on high-efficiency particulate air-filtered racks. The animals were fed with autoclaved laboratory rodent diet (Teckland LM-485; Western Research Products). Animal experiments were done in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85-23) under NIH assurance no. A3873-01.

Gemcitabine (Gemzar, Eli Lilly) was reconstituted in PBS for both *in vitro* and *in vivo* experiments and was prepared weekly. For animals treated with MTD gemcitabine (MTDG), 150 mg/kg was delivered twice weekly by intraperitoneal injection. At this dose, we previously showed significant activity against RFP-expressing pancreatic cancer tumorgrafts (21). Animals treated with gemcitabine on a low-dose, metronomic schedule (METG) received 1 mg/kg daily. This dose was chosen based on prior evidence (18) and was confirmed in our laboratory by the toxicity study detailed below. Sunitinib (Sutent, Pfizer) was reconstituted with DMSO and diluted with PBS for *in vitro* experiments. For *in vivo* experiments, sunitinib was administered in PBS by oral gavage at a dose of 40 mg/kg daily, a dose selected based on its activity against primary cancer tumorgrafts in prior reports (11, 24, 25).

### Cytotoxicity assay and assessment of synergism

Cancer cells and HUVECs were seeded on 96-well plates in 100 µL of growth medium at a density of 2,000 cells per well. After 24 hours of incubation, monolayer cultures were exposed to drugs in treatment medium consisting of Dulbecco's modified Eagle's medium/RPMI + 1% fetal bovine serum (for cancer cells) or human endothelial growth medium-2 supplemented with 2% fetal bovine serum, 0.05% epidermal growth factor, 0.025% basic fibroblast growth factor, 20 ng/mL insulin-like growth factor, 1 µg/mL ascorbic acid, 22.5 µg/mL heparin, and 0.2 µg/mL hydrocortisone (for HUVECs). Sunitinib doses were standardized at a 0.1% DMSO concentration.

After 72 hours, cell viability was assessed using an 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]H-tetrazolium-5-carboxanilide inner salt (XTT) assay. Briefly, phenazine methosulfate and XTT reagent were added to each well containing 150 µL of medium. The number of viable cells was quantified by a colorimetric assay using a plate reader at an absorbance wavelength of 492 nm and a reference of 630 nm. The percentage of viable cells was normalized to medium background and control, and expressed as relative fluorescence units. Quadruplicate wells of each condition were analyzed, and each experiment was done three times. The IC<sub>50</sub> for each treatment for each cell line was determined by nonlinear regression of the dose-response data using Prism 5.0 for Mac OSX (GraphPad).

The presence or absence of synergism between gemcitabine and sunitinib was determined using the method of Chou using CalcuSyn software v. 2.1 (Biosoft, Inc.;

**Table 1.** *In vitro* efficacy of gemcitabine and sunitinib on pancreatic and endothelial cells

Cell line	Gemcitabine (nmol/L)	Sunitinib ( $\mu\text{mol/L}$ )
Mia PaCa-2	13.9 (5.3–37.2)	8.0 (4.1–15.6)
Panc-1	29.0 (18.5–46.0)	19.5 (11.5–32.9)
BxPC-3	15.1 (10.0–22.9)	5.6 (3.2–9.9)
HUVEC	2.5 (1.3–4.7)	17.0 (11.8–24.8)

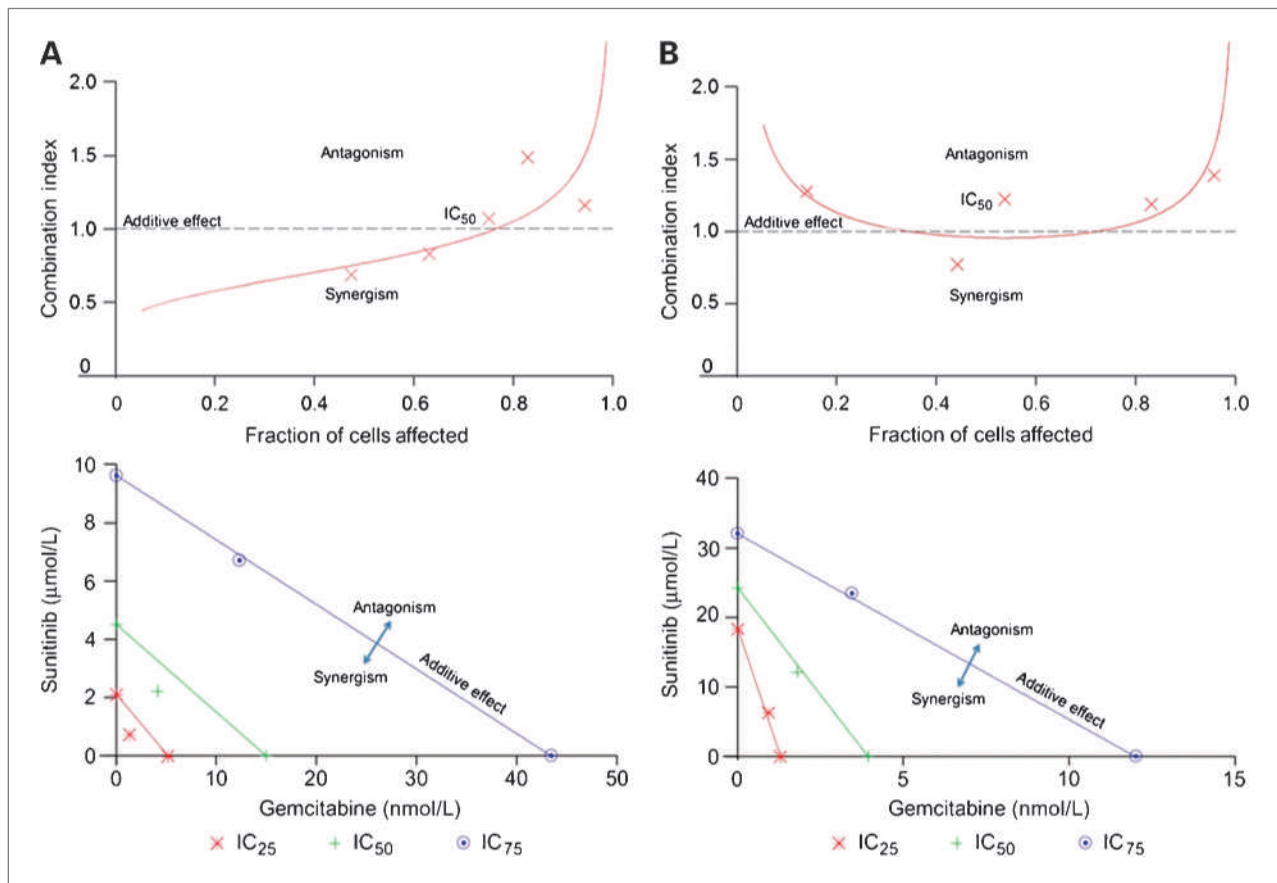
NOTE: Data are  $\text{IC}_{50}$  and 95% confidence intervals for gemcitabine and sunitinib on pancreatic cancer cells and HUVECs.

refs. 26, 27). Briefly, pancreatic cancer cells and HUVECs were exposed to 1:1 ratios of the respective  $\text{IC}_{50}$  values for sunitinib and gemcitabine at  $1/4 \times \text{IC}_{50}$ ,  $1/2 \times \text{IC}_{50}$ ,  $\text{IC}_{50}$ ,  $2 \times \text{IC}_{50}$ , and  $4 \times \text{IC}_{50}$ . Cell viability was determined after treatment for 72 hours with the XTT assay as previously described. The combination index (CI) was calcu-

lated to determine the presence of synergism ( $\text{CI} < 1$ ), an additive effect ( $\text{CI} = 1$ ), or antagonism ( $\text{CI} > 1$ ) between gemcitabine and sunitinib (26, 27).

### Subcutaneous tumor growth and surgical orthotopic implantation of MIA PaCa-2-RFP tumorgrafts

A highly fluorescent, RFP-expressing orthotopic tumor model was constructed as previously described (21, 22). Briefly, the pDsRed-2 vector (Clontech Laboratories, Inc.) was used to engineer clones of MIA PaCa-2 cells that stably express RFP. MIA PaCa-2-RFP cells were harvested by trypsinization and washed three times with PBS. Cells were injected subcutaneously into mice in a total volume of 0.2 mL within 30 minutes of harvesting. The subcutaneous tumors were used as the source of tissue for subsequent surgical orthotopic implantation (SOI) onto the pancreas (28). Subcutaneously grown MIA PaCa-2-RFP tumors in the exponential growth phase were resected aseptically. Necrotic tissues were cut away, and the remaining tumor was minced into 1-mm<sup>3</sup> pieces in RPMI. Mice were then anesthetized, and their abdomens were



**Figure 1.** Analysis of synergy between gemcitabine and sunitinib *in vitro* against (A) human pancreatic cancer MIA PaCa-2 cells and (B) HUVECs using the method of Chou (26, 27). Cells were exposed to fixed  $\text{IC}_{50}$  ratios of gemcitabine and sunitinib, and cell viability was assessed using the XTT assay. The CI plots (top panels) show that gemcitabine and sunitinib exert a synergistic inhibitory effect on MIA PaCa-2 cells at low doses, as denoted by a  $\text{CI} < 1$ . An additive inhibitory effect was shown on HUVECs ( $\text{CI} = 1$ ). These results are further reflected in the multiple effect-level isobolograms (bottom panels). Combination data points below, at, and above the isobologram line for a given effect level indicate synergy, additive effect, and antagonism, respectively.

sterilized. An incision was created through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed, and one tumor fragment was transplanted onto the middle of the gland using an 8-0 surgical suture (Davis-Geck, Inc.). The pancreas was then returned into the peritoneal cavity, and the abdominal wall was closed in two layers using a 6-0 surgical suture. All procedures were done under 2.5× optical magnification.

### ***In vivo* assessment of treatment response on pancreatic cancer growth and metastasis**

To determine the effects of treatment with gemcitabine, sunitinib, or combinations thereof on primary tumor growth and metastatic progression, mice were randomized into cohorts of five after SOI. Treatment groups included (a) METG, (b) MTDG, (c) sunitinib, (d) METG plus sunitinib (METG/SU), (e) MTDG plus sunitinib (MTDG/SU), or (f) control. To determine the effect of these regimens on the development of metastases, treatment was initiated on post-SOI day 5, at which time whole-body fluorescence imaging revealed tiny primary tumors and an absence of distant metastases. Therapy was continued for 14 days. Animals were weighed weekly and monitored daily for ascites and cachexia.

Euthanasia was done 24 hours following the last treatment. Abdominal and thoracic organs were exposed, and ascites were aspirated. Whole-body brightfield and fluorescence images were obtained with an Olympus OV100 fluorescence imaging system (Olympus Corp.). The primary tumor was excised and weighed. The location and number of metastases and micrometastases were assessed by a single observer at individual anatomic sites distant from the pancreas, including the liver, portal

lymph nodes, lung, small bowel mesentery, diaphragm, contralateral peritoneum, and genitourinary system. The following semiquantitative scale was used: 1+, few microscopic lesions identified with fluorescence imaging only; 2+, extensive lesions visible using fluorescence imaging and/or few macroscopic lesions visible in bright light; 3+, moderate macroscopically visible lesions; 4+, extensive macroscopically visible lesions.

### ***In vivo* assessment of chronic effects of therapy**

To assess the effect of chronic therapy with gemcitabine, sunitinib, or combinations thereof on both cancer and host, mice with orthotopically implanted fluorescent human pancreatic cancer tumorgrafts were randomized into six cohorts of 10 mice each and were treated on the schedules defined above until premonitory. Animals were weighed twice weekly and monitored daily for visible ascites and cachexia. Whole-body fluorescence imaging was done with the Indec FluorVivo imaging system (Indec Biosystems, Santa Clara, CA). Mice were sacrificed and processed as described above when movement was significantly impaired, weight loss >15% was observed, or tumor ulceration was noted.

### **Histology and immunohistochemistry**

Immediately following explantation, tumorgrafts were processed and nonnecrotic samples from each specimen were fixed in buffered formalin and embedded in paraffin. Tissue slices were stained with hematoxylin and eosin using standard methods. Visualization of intratumoral microvessels was accomplished by immunohistochemical techniques and staining. After deparaffinization and rehydration, slides were pretreated in Target Retrieval (Dako, 1699; pH 6.0) using a pressure cooker. Tissues were stained with a 1:8,000 dilution of anti-rabbit polyclonal von Willebrand factor/factor VIII (Dako, A0082) on the Dako Autostainer Plus. EnVision+ Dual Link System-HRP (Dako, K4061) was used for detection, and DAB+ (Dako, K3468) was used for visualization.

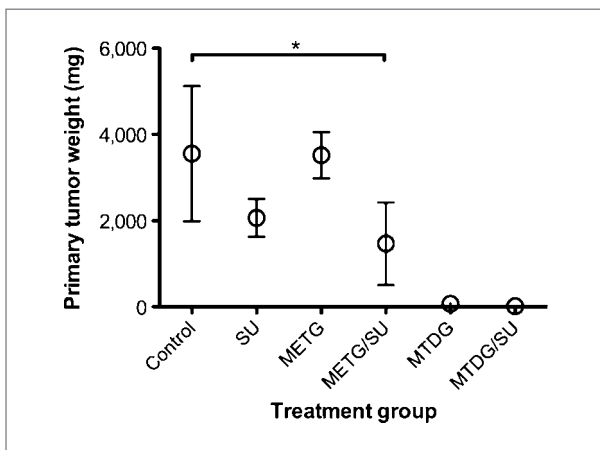
### **Statistical analysis**

SPSS 17.0 (SPSS, Inc.) was used for all statistical analyses. Survival curves were constructed and analyzed using the Kaplan-Meier method. The log-rank test was used to compare survival between groups. Pearson  $\chi^2$  test and Student's *t* test were used to compare discrete and continuous variables, respectively. A *P* value of  $\leq 0.05$  was considered statistically significant for all comparisons.

## **Results**

### **Cytotoxic effects of gemcitabine and sunitinib on pancreatic cancer cells and HUVECs *in vitro***

Both gemcitabine and sunitinib inhibited proliferation of human pancreatic cancer cells and HUVECs in a dose-dependent manner (data not shown). The respective  $IC_{50}$  values of each cell line to gemcitabine and sunitinib are shown in Table 1.



**Figure 2.** Primary tumor weights following 14 days of therapy. METG or sunitinib alone had no significant effect on primary tumor growth relative to control, but the combination therapy did ( $P = 0.03$ ). MTDG gemcitabine with or without sunitinib almost completely inhibited primary tumor growth. Plots represent mean weight (g)  $\pm$  SD of primary tumors resected at autopsy from five mice per treatment group following 14 days of therapy.

**Table 2.** Distribution of metastases following treatment for 2 weeks as described in Materials and Methods

Treatment group	Control					SU					METG				
Mice, <i>N</i>	5					5					5				
Mice with mats, <i>n</i>	5					5					5				
Mouse	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
Liver, <i>n</i>	5					5					4				
Grade*	2+	1+	2+	1+	2+	1+	1+	1+	2+	2+	1+	1+	0	1+	1+
Portal LN, <i>n</i>	5					5					1				
Grade*	4+	2+	4+	1+	4+	1+	3+	2+	4+	2+	0	0	0	0	2+
Lung, <i>n</i>	3					3					1				
Grade*	0	1+	1+	0	1+	0	2+	0	1+	2	+0	0	0	1+	0
Mesentery, <i>n</i>	5					5					3				
Grade*	4+	3+	4+	3+	4+	2	3+	2+	2+	2+	1+	0	2+	0	1+
Diaphragm, <i>n</i>	5					1					1				
Grade*	4+	3+	2+	1+	4+	0	0	0	4+	0	0	0	0	0	1+
Peritoneum**, <i>n</i>	4					1					0				
Grade*	3+	3+	2+	0	4+	0	4+	0	0	0	0	0	0	0	0
GU tract, <i>n</i>	5					4					0				
Grade*	4+	3+	3+	2+	3+	2+	3+	0	4+	2+	0	0	0	0	0

(Continued on the following page)

Simultaneous exposure to doses of gemcitabine and sunitinib had a synergistic inhibitory effect on MIA PaCa-2 proliferation at low-dose combinations, which was lost at higher doses. We were not able to show synergy between gemcitabine and sunitinib on HUVECs, but an additive inhibitory effect on proliferation was documented (Fig. 1).

#### Effect of METG and combination therapy on primary pancreatic cancer tumorgrafts and metastases *in vivo*

A previous study suggested that gemcitabine at a chronic dose of 1 mg/kg/d was associated with little toxicity; however, that toxicity increased rapidly at higher daily doses (18). To confirm this, groups of three nude mice were treated with gemcitabine intraperitoneally at doses from 1 to 5 mg/kg/d. Mice treated with gemcitabine at 2 mg/kg/d or greater exhibited gross signs of toxicity, including weight loss, postural changes, and muscle wasting, which mandated discontinuation of treatment or certain death within 2 weeks (data not shown). In contrast, 1 mg/kg/d gemcitabine was associated with minimal gross toxicity after treatment for 30 days. This dose was therefore selected as the metronomic dose (METG) used in subsequent experiments.

Cohorts of mice bearing fluorescent, orthotopically implanted human pancreatic cancer tumorgrafts were treated on the schedule described in Materials and Methods. Following 14 days of treatment, METG with or without sunitinib had the greatest inhibitory effect on primary tumor growth: 80% of mice so treated were found to have microscopic primary tumors that were

only visible by fluorescence imaging. To determine the relative response of primary cancer growth to each of the remaining treatments, tumors were collected and weighed as shown in Fig. 2. The mean tumor weight of cohorts treated with METG (3.56 g) or sunitinib (2.07 g) alone were similar to that of control (3.55 g,  $P = 0.9$  and  $0.07$ , respectively). In contrast, treatment with METG/SU had a significant inhibitory effect on primary tumor growth relative to control (mean tumor weight 1.4 g,  $P = 0.03$ ).

To determine the relative effect of each treatment on the development and progression of metastases, the distribution of metastases was evaluated using brightfield and fluorescence imaging as described in Materials and Methods (Table 2; Fig. 3). All five control mice had an extensive metastatic burden, with four of five mice having at least moderate macroscopic (grade 3) disease at three or more locations. Although sunitinib alone reduced the metastatic burden at each individual site, all five mice had microscopic or macroscopic disease in at least three locations. In contrast, although all mice treated with METG developed metastases, macroscopic tumor was identified in only one animal. Of the remaining four mice, microscopic lesions were identified in the liver ( $n = 3$ ), lung ( $n = 1$ ), and mesentery ( $n = 2$ ) using fluorescence imaging. Addition of sunitinib to METG further inhibited metastasis: three mice so treated had no extra-pancreatic disease identified by either brightfield or fluorescence imaging, and the two mice that did had at most low volume macroscopic (grade 2) disease at two and five sites, respectively.



**Table 2.** Distribution of metastases following treatment for 2 weeks as described in Materials and Methods (Cont'd)

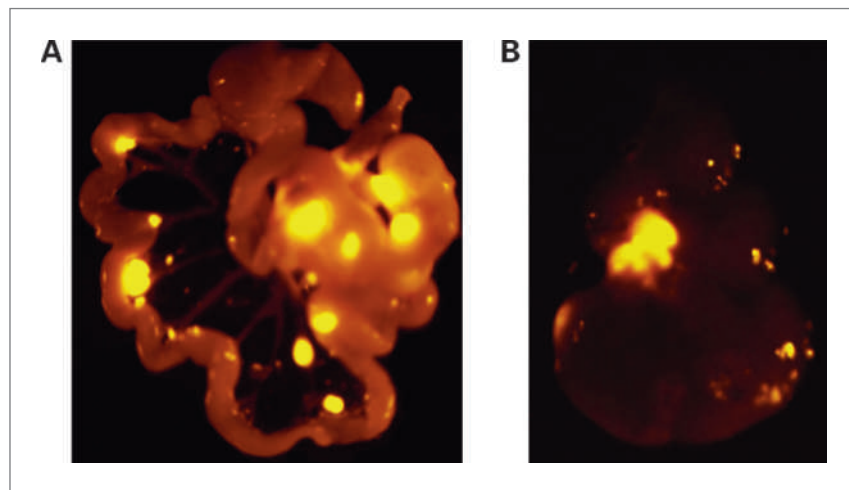
METG/SU					MTDG					MTDG/SU				
				5					5					5
				2					1					1
I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
			2					0					1	
0	1+	0	1+	0	0	0	0	0	0	1+	0	0	0	0
			0					0					0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			1					0					0	
0	0	0	1+	0	0	0	0	0	0	0	0	0	0	0
			1					1					0	
0	0	0	2+	0	0	0	0	0	1+	0	0	0	0	0
			1					1					0	
0	0	0	1+	0	0	0	0	0	1+	0	0	0	0	0
			1					0					0	
0	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
			1					0					0	
0	0	0	1+	0	0	0	0	0	0	0	0	0	0	0

NOTE: METG with or without sunitinib had a strong inhibitory effect on tumor dissemination compared with control or sunitinib alone. MTDG had the largest inhibitory effect on metastasis. The following semiquantitative scale was used at each site: 1+, few microscopic lesions identified with fluorescence imaging only; 2+, extensive lesions visible using fluorescence imaging and/or few macroscopic lesions visible in bright light; 3+, moderate macroscopically visible lesions; 4+, extensive macroscopically visible lesions. The grading scheme is illustrated in Fig. 3.

An additional marker of disease progression was the presence of ascites. Bloody, moderate to large-volume ascites was found in five of five control mice at the time of sacrifice. Thin, low to moderate volume ascites was recovered in four of five mice that received sunitinib, five of five mice

that received METG, and one of five mice that received METG/SU. Only one mouse in the MTDG group and no mice in the MTDG/SU group had ascites on necropsy.

Immunohistochemical staining of explanted tumors following 14 days of therapy revealed minimal von



**Figure 3.** The grading scheme in Table 2 is illustrated using fluorescence images of the small bowel (A) and liver (B) taken from a control animal after 2 weeks of treatment. Both images are typical images of this study. Grade 4 metastases were identified in the porta hepatis and small bowel mesentery. Grade 2 metastases were visualized in the liver parenchyma.

Willebrand factor expression among tumors treated with METG with or without sunitinib relative to control. In contrast, the degree of von Willebrand factor staining among tumors treated with sunitinib appeared identical to controls (data not shown).

### Effects of chronic therapy on ascites, cachexia, and survival

To evaluate the chronic effects of METG with or without sunitinib on both cancer and host, mice with orthotopically implanted, RFP-expressing human pancreatic cancer tumorgrafts were randomized to the six treatment arms (10 mice per group) described above. Animals were evaluated and imaged twice weekly. Levels of ascites and cachexia were documented until preterminal. One mouse in each of the control and MTDG groups died 5 days after SOI following inadvertent injection with a concentrated anesthesia solution. These two mice were excluded from the analysis.

Although chronic treatment until necropsy was planned in all groups as described in Materials and Methods, treatment with MTDG with or without sunitinib was terminated at treatment day 52. In each of these treatment groups, three mice died ~54 days following implantation despite an absence of significant tumor on either physical exam or fluorescence imaging. Necropsy revealed findings of distended bowel and histologic changes similar to those found in mice treated with daily gemcitabine at a dose above 1 mg/kg/d as described above.

The median survival (MS) of control mice was 25 days following implantation. Survival was limited by the development of progressive local and distant disease (Figs. 4 and 5). Both METG (MS = 32 days,  $P = 0.002$ ) and sunitinib (MS = 31 days,  $P = 0.002$ ) alone had a statistically significant, albeit modest, effect on survival relative to control. Combination therapy with METG/SU (MS = 44 days) improved survival compared with control ( $P = 0.002$ ) or either METG or sunitinib alone ( $P = 0.04$  and  $0.0004$ , respectively). MTDG with or without sunitinib had the most significant effect on survival (MS = 87 and 77 days, respectively), although, as noted above, life-limiting toxicity was noted after chronic administration.

The progressive accumulation of ascites and the development of tumor-associated cachexia were virtually constant findings among animals with orthotopic pancreatic cancer tumorgrafts. Ascites was identified in 6 (67%) control animals by day 16; by day 19, all control animals had ascites and 6 (67%) control animals exhibited intrascapular muscle wasting indicative of cachexia. Thick, bloody ascites was recovered from 7 (78%) control animals at necropsy. In contrast, monotherapy with either METG or sunitinib delayed the onset of ascites. Animals treated with METG did not begin to accumulate intraperitoneal fluid until after day 27. Low-volume, thin ascites was identified in 4 (40%) animals at necropsy. Similarly, only 3 (30%) animals treated with sunitinib alone had visible peritoneal fluid by day 23; ascites was confirmed in 2 (20%) animals at necropsy. As expected, METG/SU fur-

ther delayed ascites: only one (10%) animal had fluid at day 37, and only two animals had ascites recovered at death. Mice treated with MTDG rarely developed ascites: only two mice treated with MTDG alone and none of the mice treated with MTDG/SU had ascites at necropsy. Cachexia was a late but constant finding in all treatment groups, except those mice that died from apparent gemcitabine toxicity, as described above.

### Discussion

Malignant solid tumors are composed of neoplastic cancer cells, stromal elements, and a supportive microvasculature. Standard cytotoxic regimens used for patients with such cancers typically target the cancer cell component of the tumor. These regimens use chemotherapeutics delivered at the highest possible dose in several short courses separated by breaks to allow sufficient recovery. Recently, administration of chemotherapeutics on low-dose, metronomic schedules has gained attention as a means to limit the drawbacks of standard treatment regimens—namely, significant toxicity, the development of chemoresistance, and, ultimately, poor long-term efficacy (6). In preclinical studies, metronomic dosing regimens and combination strategies based thereon have inhibited primary tumor growth of malignancies, including those of the breast, brain, lung, prostate, and pancreas, through an antiangiogenic mechanism (8, 14, 18, 29). Further studies have shown that metronomic therapy may also control established metastatic disease (13, 14). Within this context, we sought to evaluate the effect of a combination metronomic strategy on the development and progression of pancreatic cancer metastasis. Using a highly aggressive, metastatically competent model, we show that METG with sunitinib effectively inhibits the development of metastases in, and prolongs the survival of, immunoincompetent mice with orthotopically implanted pancreatic cancer.

For patients with pancreatic cancer, gemcitabine represents the standard cytotoxic agent used both as primary therapy for advanced disease and in the adjuvant setting following resection of localized primary cancers (4, 15). The drug is a deoxycytidine analogue that is incorporated into DNA strands and leads to inhibition of DNA synthesis. In addition to its cytotoxic action against cancer cells, we previously showed that the activity of this and similar agents, when delivered at standard doses, is also due in part to antiangiogenic activity against nascent tumor-supporting microvasculature and an overall antimetastatic effect (16, 17, 19). Administered to patients with advanced disease, the toxicity associated with gemcitabine is not insignificant (4). In the adjuvant setting, the potential for toxicity may have particular clinical relevance: in one third of patients with resected pancreatic cancer in whom adjuvant therapy is planned, treatment is delayed or never delivered due to a depressed performance status that prohibits the timely administration of cytotoxic agents (30).

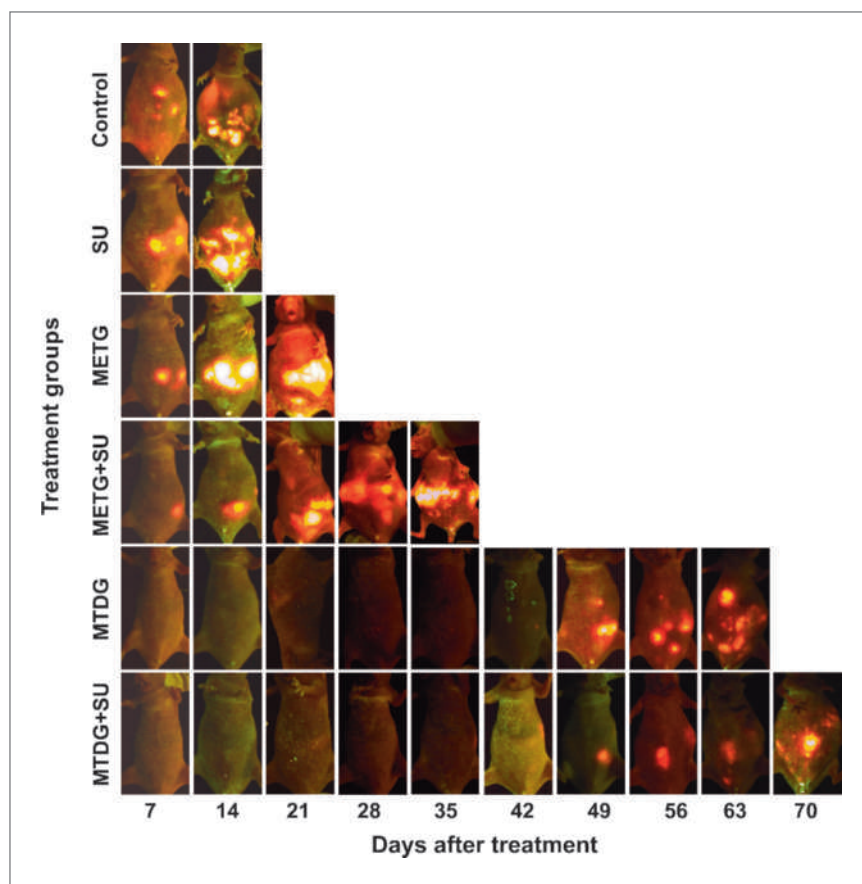
Within this context, Laquente et al. recently suggested a role for low-dose METG in the treatment of patients with pancreatic cancer (18). In a preclinical study that used an orthotopic mouse model based on the human pancreatic cancer cell line NP18, gemcitabine administered for 1 month at a daily dose of 1 mg/kg was compared with 100 mg/kg administered 0, 3, 6, and 9 days following implantation. Both schedules inhibited primary tumor growth relative to control; moreover, the antitumor efficacy of the metronomic schedule was found to be equivalent to that of the standard schedule. METG was associated with increased tumor expression of thrombospondin-1, an active angiogenesis inhibitor thought to mediate the effects of metronomic treatment (7, 31).

In a further effort to improve the modest results observed with standard cytotoxic chemotherapy, several targeted, orally administered agents, including bevacizumab (32), cetuximab (33), atixinib (34), and erlotinib (35), have been clinically evaluated in combination with standard-dose gemcitabine for patients with pancreatic cancer. Of these, only erlotinib has been found to have significant activity in combination with gemcitabine (35). Nonetheless, a strong preclinical rationale supports a thorough evaluation of these agents in combination with metronomic chemotherapy (12). Using a novel protocol of sequential MTD followed by metronomic

cyclophosphamide in combination with imatinib and sunitinib, Pietras observed almost complete primary tumor regression in an aggressive, transgenic mouse model of pancreatic islet cell cancer (9). In another series of experiments, Blansfield showed that combination anti-stromal therapy with metronomic cyclophosphamide, sunitinib, and lenalidomide inhibited growth of multiple cancer cell types and primary cancer xenografts (11). Moreover, combination therapy reduced compensatory upregulation of proangiogenic proteins relative to monotherapy. Similar results have been obtained by other investigators (36, 37).

Based on these studies, we sought to explore the effects of a combination metronomic strategy on primary pancreatic cancer growth and the development and progression of metastasis. To this end, we used an RFP-expressing tumorgraft model based on the aggressive human pancreatic cancer cell line MIA PaCa-2. This well-characterized model allows serial imaging and quantification of cancer growth and dissemination in the live animal, as well as the identification of micrometastases at necropsy in distant organs (21, 22). Moreover, it exhibits a pattern of aggressive growth and early metastatic spread characteristic of human disease, permitting investigations of the systemic cancer response to both primary and adjuvant therapies (19, 20).

**Figure 4.** Serial whole-body fluorescence imaging of live mice during chronic therapy. These are typical images of this study. The survival of mice treated with gemcitabine on the MTD schedule was most favorable, but treatment had to be discontinued on treatment day 52 because of toxicity, after which tumor progression led to death. Each treatment depicts serial whole-body images of one representative animal from each 10-mouse group.





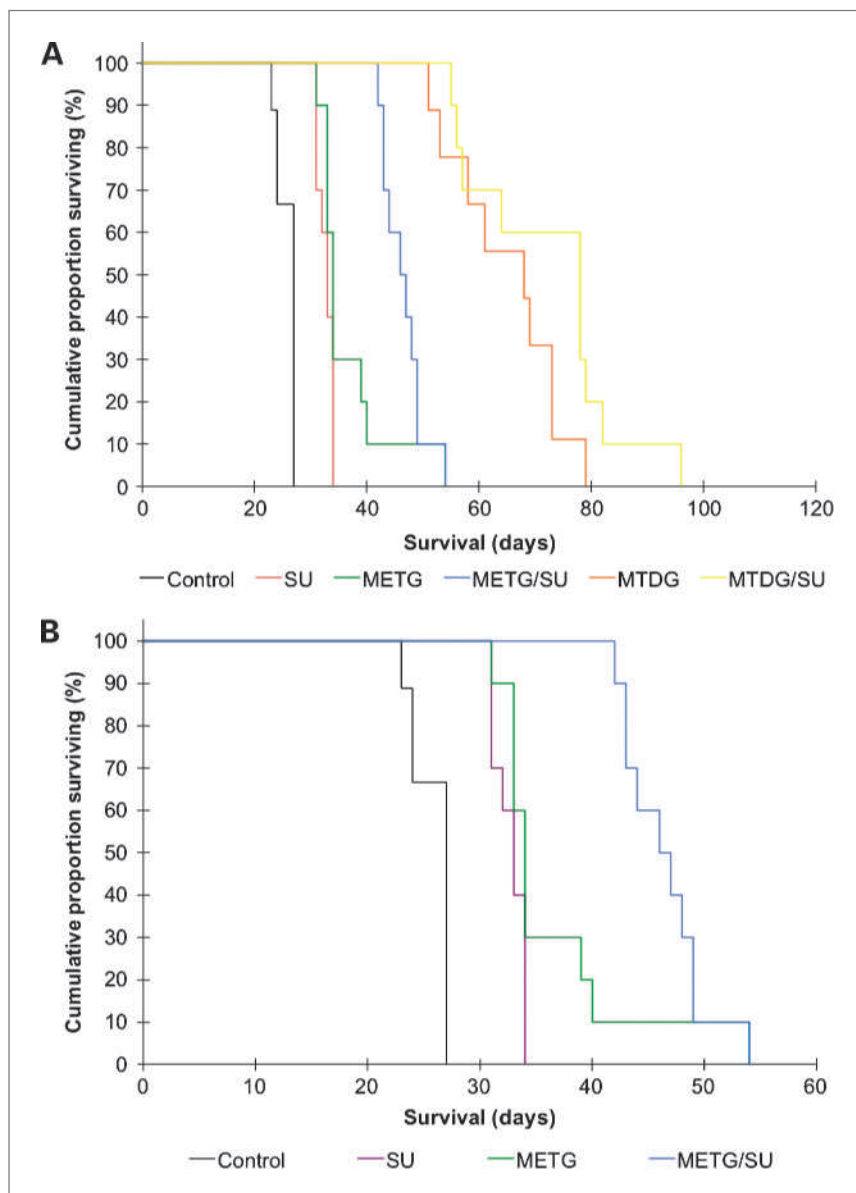


Figure 5. Kaplan-Meier survival curves generated from cohorts of mice chronically treated with gemcitabine, sunitinib, or combinations thereof. METG/SU prolonged survival relative to control ( $P = 0.002$ ), METG alone ( $P = 0.04$ ), and sunitinib alone ( $P = 0.0004$ ). A, all six treatment groups, B, METG with and without sunitinib compared to sunitinib alone and control.

Contradictory to the findings of Laquente et al. (18), we found that metronomic daily dosing of gemcitabine at 1 mg/kg/d had inferior activity against the growth of primary pancreatic cancer tumorgrafts than MTDG. At least two factors likely account for this discrepancy. First, we based our model on the highly aggressive cell line MIA PaCa-2, which is characterized by rapid tumor growth *in vivo*. It is likely that the primary growth of the human pancreatic cancer tumorgrafts in our study outpaced the inhibitory effects on the supporting microvasculature exerted by METG, relative to the more indolently growing tumorgrafts used in the Laquente report. We also used a different MTD schedule than that reported by Laquente—150 mg/kg twice weekly in a 2-week cycle compared with 100 mg/kg twice weekly

for the first 2 weeks of a 1-month cycle used by Laquente. The MTD dose we used was therefore not only higher than that used by Laquente but was also not followed by a 2-week treatment-free period before sacrifice, within which the tumors treated with MTDG could regrow even as the metronomically treated animals still received chemotherapy. Importantly, our findings are consistent with those of recent studies that show that the effects of metronomic therapy may be more pronounced against established metastatic disease than primary tumors themselves (13, 14). It should also be noted that there does not seem to be a correlation between tumor size and metastasis because METG alone had no significant effect on tumor size (Fig. 2) but had a dramatic effect on the incidence and distribution of metastasis (Fig. 3; Table 2).

Although Laquente et al. used an orthotopic tumor-graft model, the indolence of the NP18 cell line selected was also associated with an absence of metastases, even among untreated animals. The effect of gemcitabine on metastatic progression, whether on a metronomic or MTD dosing schedule, could therefore not be assessed. In this study, we show that the antimetastatic effect of gemcitabine we previously reported is retained at ultra-low doses of the drug (16). Indeed, the exquisitely sensitive fluorescence detection system used in this study allowed us to show that antimetastatic activity approaching that of MTDG is achieved with a total dose reduced 42 times using a metronomic dosing schedule.

For combination regimens, we selected sunitinib, which inhibits cellular signaling by targeting multiple receptor tyrosine kinases, including the receptors for platelet derived growth factor and vascular endothelial growth factor. The drug has been approved by the Food and Drug Administration for patients with renal cell carcinoma and gastrointestinal stromal tumors. Intraperitoneal injection of sunitinib has been shown to have efficacy against subcutaneous pancreatic cancer tumorgrafts in combination with gemcitabine or external beam radiation in pre-clinical studies (23, 24). In our study using highly aggressive, orthotopic human tumors, oral sunitinib alone had no effect on primary tumor growth relative to control. However, the agent reduced the frequency of metastasis, delayed the onset of ascites, and was associated with a significant, albeit modest effect on survival. Treatment with METG and sunitinib was well tolerated. Moreover, combination therapy with METG and sunitinib inhibited primary tumor growth relative to control, had a favorable effect on the metastatic profile, and led to a significant survival advantage relative to control or either agent alone.

Importantly, we were unable to detect a difference between MTDG and MTDG/SU, both of which had more activity in this model than the metronomic regimens. However, the remarkable similarity between the antimetastatic activity of ultra-low-dose gemcitabine plus sunitinib and the higher-dose gemcitabine regimens must be emphasized. Moreover, these data also suggest a possible benefit of a "chemoswitch" regimen, in which a short course of MTD therapy is followed by a maintenance phase of metronomic therapy, as previously described both preclinically (9) and clinically (38). Switching the target of therapy from the cancer cell to the supporting microvasculature may be a method to evade the conse-

quences of the chemoresistance that eventually develops to gemcitabine (21). These possibilities represent critical "next steps" for study in our laboratory.

Finally, in this study, we noted that both METG and sunitinib have a favorable effect on the development of spontaneous ascites and cachexia in mice with orthotopic human pancreatic cancer tumorgrafts. The angiogenic basis for malignant ascites, which involves increased vascular permeability secondary to proangiogenic mediators, has been well described (39–42). Antiangiogenic regimens have been shown to reduce the quantity of malignant ascites by blockade of vascular endothelial growth factor activity in several preclinical models, including models of pancreatic cancer (43, 44). Peritoneal cancer dissemination and ascites are a common clinical problem for patients with pancreatic cancer that is associated with a profound negative impact on quality of life. Unfortunately, patients with ascites rarely have a performance status sufficient for high-dose chemotherapy or other standard treatments. Our results suggest that low-dose metronomic therapy and/or sunitinib might be a well-tolerated approach to reduce ascites and improve quality of life among fragile patients with advanced disease.

In summary, we found that therapy with METG with and without sunitinib was well tolerated and was associated with a significant effect on the development of metastases and ascites in our aggressive, orthotopic model. Most importantly, this regimen had a beneficial effect on overall survival. The mechanism of these effects involves cytotoxic activity on both the cancer cells and supporting microvasculature. These results reveal potential uses for this or a similar combination of drugs in patients with resected or advanced pancreatic cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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