

The Combination of Epidermal Growth Factor Receptor Inhibitors with Gemcitabine and Radiation in Pancreatic Cancer

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Abstract Purpose: Gemcitabine-radiotherapy is a standard treatment for locally advanced pancreatic cancer. Clinical data have shown that gemcitabine plus erlotinib is superior to gemcitabine alone for advanced pancreatic cancer. Therefore, we investigated the effects of the combination of epidermal growth factor receptor inhibitors with gemcitabine and radiation on a pancreatic cancer model.

Experimental Design: EGFR signaling was analyzed by measuring phosphorylated EGFR (pEGFR^{(Y845), (Y1173)}) and AKT (pAKT^(S473)) protein levels in pancreatic cancer cell lines and tumors. The effects of scheduling on gemcitabine-mediated cytotoxicity and radiosensitization combined with erlotinib were determined by clonogenic survival. *In vivo*, the effects of cetuximab or erlotinib in combination with gemcitabine-radiation on the growth of BxPC-3 tumor xenografts were measured.

Results: We found *in vitro* that gemcitabine induced phosphorylation of EGFR at Y845 and Y1173 that was blocked by erlotinib. Treatment of BxPC-3 cells with gemcitabine before erlotinib enhanced gemcitabine-mediated cytotoxicity without abrogating radiosensitization. *In vivo*, cetuximab or erlotinib in combination with gemcitabine-radiation inhibited growth compared with gemcitabine-radiation (time to tumor doubling: gemcitabine + radiation, 19 ± 3 days; cetuximab + gemcitabine + radiation, 30 ± 3 days; *P* < 0.05, erlotinib + gemcitabine + radiation 28 ± 3 days; *P* < 0.1). Cetuximab or erlotinib in combination with gemcitabine-radiation resulted in significant inhibition of pEGFR^(Y1173) and pAKT^(S473) early in treatment, and pEGFR^(Y845), pEGFR^(Y1173), and pAKT^(S473) by the end of treatment. This study shows a novel difference pEGFR^(Y845) and pEGFR^(Y1173) in response to EGFR inhibition.

Conclusions: These results show that the EGFR inhibitors cetuximab and erlotinib increase the efficacy of gemcitabine-radiation. This work supports the integration of EGFR inhibitors with gemcitabine-radiation in clinical trials for pancreatic cancer.

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States and has 1- and 5-year survival rates of 26% and 5%, respectively (1). In the past 10 years, gemcitabine has replaced 5-fluorouracil as the standard therapy for metastatic pancreatic cancer. The combination of highly conformal radiation with gemcitabine improves median survival in patients with locally advanced disease (12-13 months; ref. 2) compared with historical controls who received

gemcitabine alone (7-9 months; refs. 3, 4). Although local tumor control is an important issue, the majority of treatment failures are due to systemic disease progression (5). Therefore, our approach in designing clinical trials for pancreatic cancer has emphasized improving systemic disease control while maintaining local tumor control.

A number of studies have been conducted in an effort to improve gemcitabine-radiotherapy by adding other chemotherapeutic agents. Based on clinical data suggesting that cisplatin combined with gemcitabine produced a survival benefit in patients with metastatic pancreatic cancer (6), we designed a preclinical study combining cisplatin with gemcitabine-radiation (7). The finding that cisplatin and gemcitabine produced synergistic cytotoxicity without abrogating gemcitabine-mediated radiosensitization led us to investigate the combination in a phase I/II clinical trial. This study suggested that full systemic doses of gemcitabine and cisplatin could be administered in combination with conformal tumor radiation, and showed a promising median survival of 13 months (8).

More recently, our focus has shifted to the combination of molecularly targeted therapies with gemcitabine-radiation. Epidermal growth factor (EGF) receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. In response to

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stimulation by ligands such as EGF or transforming growth factor α , the receptor homodimerizes or heterodimerizes (with other ErbB family members) and undergoes autophosphorylation at a number of tyrosine residues, including 974, 992, 1045, 1068, 1086, 1148, and 1173 (9). Alternatively, the Src nonreceptor kinase can phosphorylate EGFR at tyrosine residues 845 and 1101 (10, 11). EGFR phosphorylation produces activation of downstream signaling pathways involving STAT, AKT, extracellular signal-regulated kinase, and PKC, which induce cellular responses such as survival, oncogenesis, angiogenesis, cell cycle progression, and transformation. EGFR-targeting monoclonal antibodies, as well as small molecule inhibitors, have been approved by Food and Drug Administration for use in several tumor sites (12, 13), alone and in combination with gemcitabine for pancreatic cancer (14). Although EGFR mutation is rare in pancreatic cancer (2-4%; refs. 15, 16), overexpression of EGFR occurs in at least one-half of all pancreatic cancers (17, 18), and correlates with a poor prognosis (19, 20). A recent phase III clinical trial comparing patients with advanced pancreatic cancer randomized to receive gemcitabine plus the EGFR inhibitor, erlotinib, or gemcitabine alone showed a significant improvement in median survival of 6.2 versus 5.9 months as well as 1-year survival of 23% versus 17%, respectively (21).

Therefore, we designed a study to determine whether the addition of an EGFR antagonist to gemcitabine and radiation could produce radiosensitization greater than or equal to that produced by gemcitabine and radiation. This approach is consistent with our clinical goal of improving systemic therapy while maintaining or improving local control (radiosensitization). We hypothesized that the schedule of gemcitabine, radiation, and EGFR inhibitor as well as the EGFR phosphorylation status and the choice of EGFR antagonist would influence pancreatic cancer cell survival and tumor growth. We first identified a schedule *in vitro* that enhanced cytotoxicity, and radiosensitized at least as much as gemcitabine alone. We then adapted this schedule to a murine pancreatic tumor xenograft model, where we assessed the effect of adding either erlotinib or cetuximab to gemcitabine radiotherapy on tumor growth and EGFR signaling.

Materials and Methods

Cell lines and drug solutions. The human pancreatic adenocarcinoma cell lines BxPC-3, Panc-1, and MPanc-96 were obtained from American Type Culture Collection and maintained in RPMI 1640 (BxPC-3 and MPanc-96) or DMEM medium, with 10% fetal bovine serum and antibiotics at 37°C in 5% CO₂. Gemcitabine (supplied by Eli Lilly) was dissolved in PBS and stored at -20°C (*in vitro*) or 0.9% sodium chloride (*in vivo*). Erlotinib was provided by OSI Pharmaceuticals and dissolved in DMSO (*in vitro*) or 0.1% Tween-80, 0.3% sodium carboxymethylcellulose (Type 7L2P, Aqualon); (*in vitro*). Cetuximab was provided by ImClone as a 2 mg/mL aqueous solution. Cells were routinely screened for *Mycoplasma* contamination.

Clonogenic cell survival assay. Clonogenic assays were done using standard techniques as described previously (22). Drug cytotoxicity was calculated as the ratio of surviving drug-treated cells relative to untreated controls. Radiation survival data from drug-treated cells were corrected for plating efficiency using an unirradiated plate treated with drug under the same conditions. Cell survival curves were fitted using the linear-quadratic equation, and the mean inactivation dose calculated according to the method of Fertil and colleagues (23). The cell survival

enhancement ratio was calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose after drug exposure. A value significantly greater than 1 indicates radiosensitization.

Tumor growth studies. BxPC-3 cells (5×10^6) were transplanted s.c. into the flank of athymic Nude-Foxn1^{nu} mice (Harlan). Treatment was started once a tumor reached 100 mm³. Animals were given gemcitabine on day 0 and 7, erlotinib on days 1 to 5 and 8 to 12, cetuximab on days 1 and 8, radiation on days 1 to 5 and 8 to 12 (4 h post-erlotinib or cetuximab), and no treatment on days 6 and 12. For immunoblot studies, treatment was ended and tumors harvested on day 2. Body weight and tumor size were measured thrice per week. Tumor volume was calculated according to the equation for a prolate spheroid, tumor volume = $\pi/6$ (ab^2), where a and b are the longer and shorter dimensions of the tumor, respectively. Measurements were made until day 90 or until the tumor volume increased by approximately a factor of 10, at which point the animals were sacrificed to avoid potential discomfort. Animals were handled according to the established procedures of the University of Michigan Laboratory Animals Maintenance Manual.

Irradiation. Irradiations were carried out using a Pantak Therapax DXT 300 Model X-ray unit (PANTAK) at a dose rate of ~ 3 Gy/min. Dosimetry was carried out using an ionization chamber connected to an electrometer system that is directly traceable to a National Institute of Standards and Technology calibration. For tumor irradiation, animals were anesthetized with ketamine/xylazine and positioned such that the apex of each flank tumor was at the center of a 2.4-cm aperture in the secondary collimator and irradiated, with the rest of the mouse being shielded from radiation.

Immunoblotting. Cell pellets or pulverized frozen tumors were prepared in buffer containing 10 mmol/L Tris (pH 7.4), 2% SDS, 1 \times Complete Protease Inhibitor Cocktail (Roche), 1 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, 1 mmol/L sodium PPI, and phosphatase inhibitor cocktails 1 and 2 (according to the manufacturer's instructions; Sigma). Protein concentration was determined with the Bicinchoninic Acid Protein Assay Reagent (Pierce). Samples were diluted in loading buffer [0.32 mol/L Tris-HCl, 10% glycerol, 2% SDS, 0.2% bromophenol blue, 4% 2-mercaptoethanol (pH 6.8)] and resolved on 4% to 12% gradient Bis-Tris gels (Invitrogen). Separated proteins were transferred to polyvinylidene fluoride membranes (Millipore) and hybridized overnight at 4°C with antibodies recognizing pEGFR^(Y845), pEGFR^(Y1173), pAKT^(S473), AKT (Cell Signaling Technology), EGFR, or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology). Membranes were then probed with secondary antibodies, incubated with Enhanced Chemiluminescence Plus reagent (Amersham Biosciences), and exposed to film. The ImageJ program (NIH) was used for quantification of the specific protein bands on film.

Statistics. All statistical analyses were done using SAS v9.1 (SAS Institute). The time to doubling was determined for each xenograft by identifying the earliest day on which it was at least twice as large as on the first day of treatment, and then estimating the exact time of doubling by linear interpolation with the previous measurement. Because most animals had two implants, the differential effect of treatments on time to doubling was analyzed by a random effects ANOVA, which accounts for correlation between tumors within the same animal. Estimates of means, differences between means, their SEs and statistical significance are all derived from the ANOVA models. Phosphorylation data were analyzed using a standard ANOVA model.

Results

We began this study by examining the effect of gemcitabine and erlotinib on EGFR signaling in several pancreatic cancer cell lines. Because our previous work in head and neck cancer cells showed that gemcitabine induced phosphorylation of EGFR at the Src-dependent phosphorylation site, Y845, we investigated this site as well as the autophosphorylation site,

Y1173. We found in the BxPC-3, Panc-1, and MPanc-96 cell lines, treated for 2 hours with approximately IC₃₀ (G₁) or IC₇₀ (G₂) concentrations of gemcitabine that phosphorylation of EGFR was induced at Y845 by the 48-hour posttreatment time point (Fig. 1). Phosphorylation of EGFR at Y1173 occurred in BxPC-3 and Panc-1 cells in response to gemcitabine. Furthermore, we found that a 24-hour exposure to 3 μmol/L erlotinib dramatically reduced the basal levels of pEGFR^(Y845) and pEGFR^(Y1173), as well as blocked the induction of pEGFR in response to gemcitabine. We also examined the levels of phosphorylated AKT (pAKT^(S473)). In BxPC-3 cells, treatment with erlotinib, gemcitabine, or the combination of both resulted in reduced levels of pAKT^(S473) (Fig. 1). In contrast,

in Panc-1 and MPanc-96 cells, pAKT^(S473) levels were resistant to erlotinib or gemcitabine alone and were only reduced in response to the combination.

To understand the influence of EGFR inhibitors on gemcitabine-mediated radiosensitization, we first examined cytotoxicity in response to two different schedules of gemcitabine and erlotinib. In the first schedule, a 2-hour gemcitabine treatment was followed 24 hours later by a 72-hour erlotinib exposure (Fig. 2A). Treatment under this condition resulted in an enhancement of gemcitabine-mediated cytotoxicity by erlotinib (Fig. 2B). In the second schedule, erlotinib was given for 72 hours and followed by a 2-hour gemcitabine exposure and a 22-hour gap before plating for clonogenic survival. In contrast to the first schedule, the second schedule produced no enhanced gemcitabine cytotoxicity by erlotinib. To further understand the importance of scheduling EGFR inhibitors with gemcitabine and radiation, we did radiosensitization experiments under these two treatment conditions, in which irradiation was done 24 hours after gemcitabine treatment. For both schedules 1 and 2, gemcitabine alone produced significant radiosensitization (radiation enhancement ratio, 1.9 ± 0.10) that was unaffected by the addition of erlotinib (radiation enhancement ratio, 1.9 ± 0.04 and 1.7 ± 0.05, respectively; Fig. 2C-D). Furthermore, erlotinib alone did not produce radiosensitization under either schedule. Although it would have been optimal if erlotinib had enhanced gemcitabine-mediated radiosensitization, the finding that erlotinib did not abrogate gemcitabine-mediated radiosensitization was an acceptable outcome because our clinical goal for pancreatic cancer is to improve systemic therapy while maintaining or improving local radiosensitization.

EGFR inhibitors have been shown to inhibit angiogenesis, invasion, and proliferation in addition to promote apoptosis (24). Therefore, we wanted to test the effects of EGFR inhibition, gemcitabine, and radiation in a murine pancreatic tumor xenograft model. Because previous data showed that cetuximab could enhance the inhibition of pancreatic tumor growth by gemcitabine-radiation (25), we wished to compare the efficacy of cetuximab and erlotinib. We selected relatively low doses of gemcitabine (120 mg/kg) and radiation (1 Gy × 10) for these studies, which would permit us to detect further inhibition of tumor growth by the addition of cetuximab or erlotinib if it occurred. We administered cetuximab once per week or erlotinib daily (each for 2 cycles and 4 hours post-irradiation; Fig. 3A) based on the substantially longer half-life of cetuximab (95 hours; ref. 26) relative to erlotinib (36 hours; ref. 27). Treatment with gemcitabine, cetuximab, erlotinib, or radiation alone did not produce any effect on tumor growth (Fig. 3B; Table 1). As anticipated, the addition of gemcitabine to radiation extended the time required for tumor volume doubling, although it did not reach statistical significance (P = 0.12). Cetuximab in combination with radiation produced a significant delay in the time to tumor volume doubling compared with either untreated tumors (Δ = 10.4 ± 4.5 days; P < 0.03) or tumors treated with cetuximab alone (Δ = 9.7 ± 4.5 days; P < 0.04). The time to tumor volume doubling was also significantly longer after treatment with gemcitabine and erlotinib compared with control (Δ = 11.7 ± 4.5 days; P < 0.02) or gemcitabine alone (Δ = 12.2 ± 4.5 days; P < 0.01). The most effective regimen for inhibition of tumor growth as evidenced by the significantly increased time to tumor volume doubling

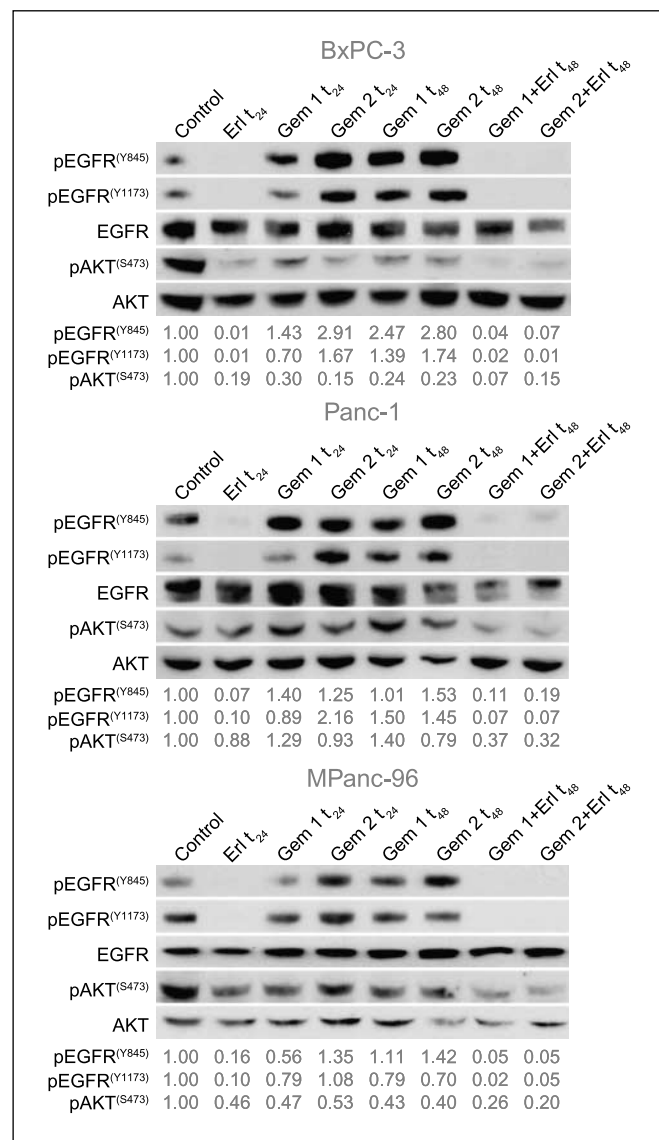
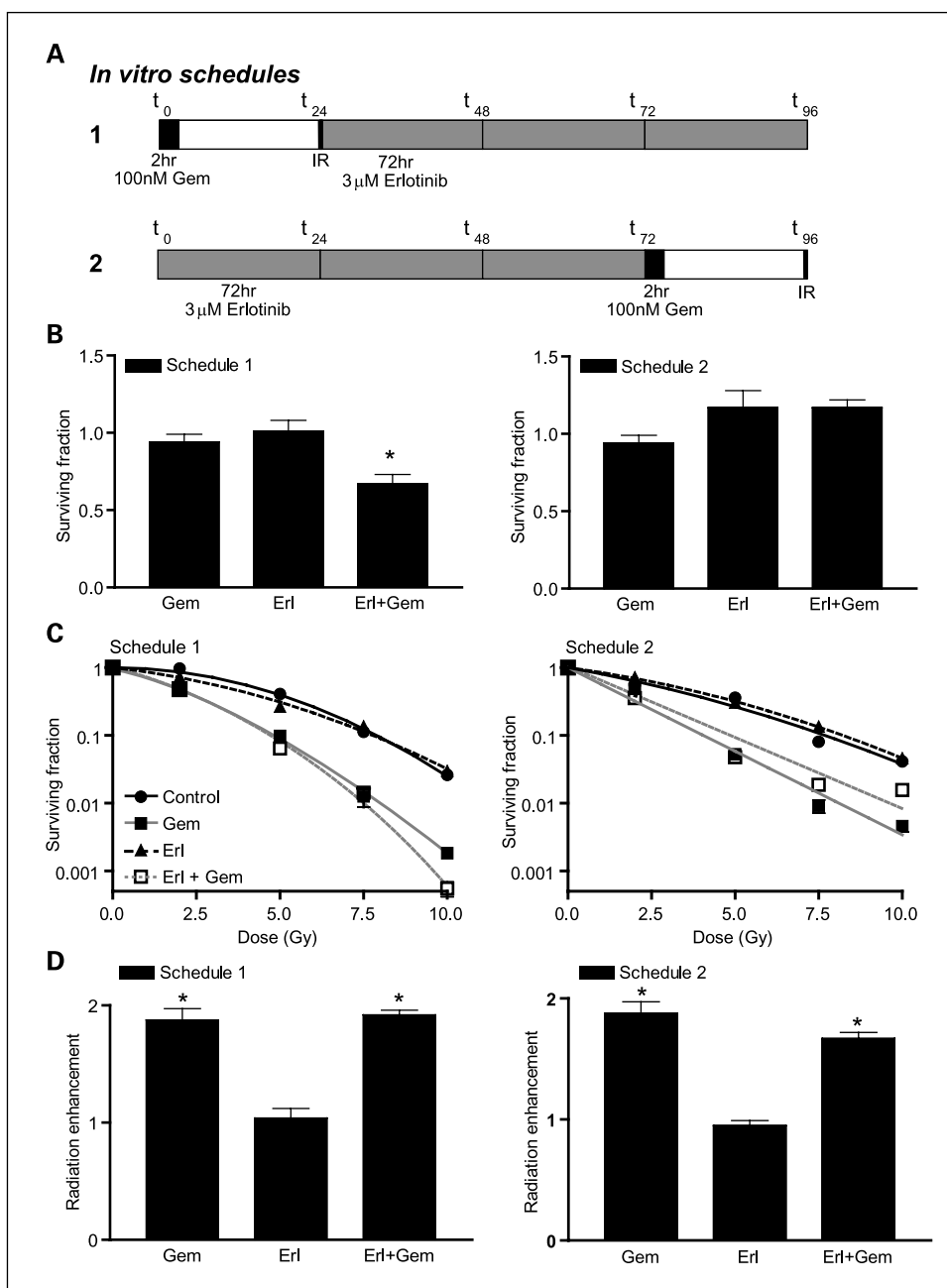


Fig. 1. The effects of gemcitabine (*gem*) and erlotinib (*erl*) on EGFR in pancreatic cancer cells. BxPC-3, Panc-1, or MPanc-96 cells were treated for 2 h with 100 nmol/L (G₁), 300 nmol/L (G₂; BxPC-3), 1 μmol/L (G₁) or 3 μmol/L (G₂; Panc-1 and MPanc-96) gemcitabine. Cells were treated for 24 h with 3 μmol/L erlotinib beginning 24 h after gemcitabine exposure. Cells were harvested for immunoblotting at 24 and 48 h post-gemcitabine (t₂₄, 48) or immediately after erlotinib (t₂₄, 48). The amounts of the indicated proteins are shown from a single experiment for each cell line that is representative of at least three independent experiments.

Fig. 2. The effects of erlotinib on cytotoxicity and radiosensitization in response to gemcitabine. BxPC-3 cells were treated for 2 h with 100 nmol/L gemcitabine and/or for 72 h with 3 μ mol/L erlotinib according to schedules 1 or 2 as illustrated (A). Cytotoxicity was calculated as the fraction of surviving colonies in treated cells relative to the number of colonies in the untreated control cells (surviving fraction, 1; B). The radiation survival curves in response to gemcitabine and/or erlotinib (C) were used to calculate the mean inactivation dose. Radiation enhancement was calculated as the ratio of the mean inactivation dose for drug treated cells to nondrug treated cells (radiation enhancement, 1). Columns and points, mean of three independent experiments (B and D) or a single experiment (C); bars, SE. Statistically significant differences between gemcitabine versus erlotinib plus gemcitabine (B) or radiation versus radiation plus gemcitabine and/or erlotinib (D) are shown (*, $P < 0.05$).



was the triple combination of either cetuximab or erlotinib plus gemcitabine-radiation (30.1 ± 3.3 days or 27.5 ± 3.3 days, respectively). The addition of cetuximab or erlotinib to gemcitabine and radiation produced minimal weight loss (Table 2), suggesting that these combinations did not produce marked normal tissue toxicity. Taken together, these results show that EGFR antagonists such as cetuximab or erlotinib can improve the antitumor efficacy of gemcitabine-radiation (without additional normal tissue toxicity) in a pancreatic cancer model.

To characterize the molecular phenotype of tumors treated with cetuximab or erlotinib and gemcitabine-radiation, we analyzed EGFR signaling in BxPC-3 tumor xenografts both at the beginning (day 2; Fig. 4A-D) and end (day 12; Fig. 4E-H) of the 2-week treatment cycle. Consistent with our *in vitro*

findings, we found that phosphorylation of EGFR at Y845 was significantly reduced by treatment with cetuximab or erlotinib by treatment day 2 (both $P < 0.01$), and we observed a trend for gemcitabine ($P = 0.11$) or radiation ($P = 0.20$) alone to increase EGFR^(Y845), although the combination of gemcitabine and radiation did not ($P = 0.18$). Interestingly, at this early time point, neither cetuximab nor erlotinib inhibited EGFR^(Y845) in the presence of gemcitabine-radiation, despite the result that these treatments produced the greatest tumor growth inhibition. We therefore investigated EGFR^(Y1173) and found that cetuximab or erlotinib alone significantly reduced EGFR^(Y1173) relative to control (both $P < 0.01$). In addition, the combination of either cetuximab or erlotinib with gemcitabine-radiation significantly reduced EGFR^(Y1173) at the 2-day time point relative to control ($P < 0.03$ and < 0.02 ,

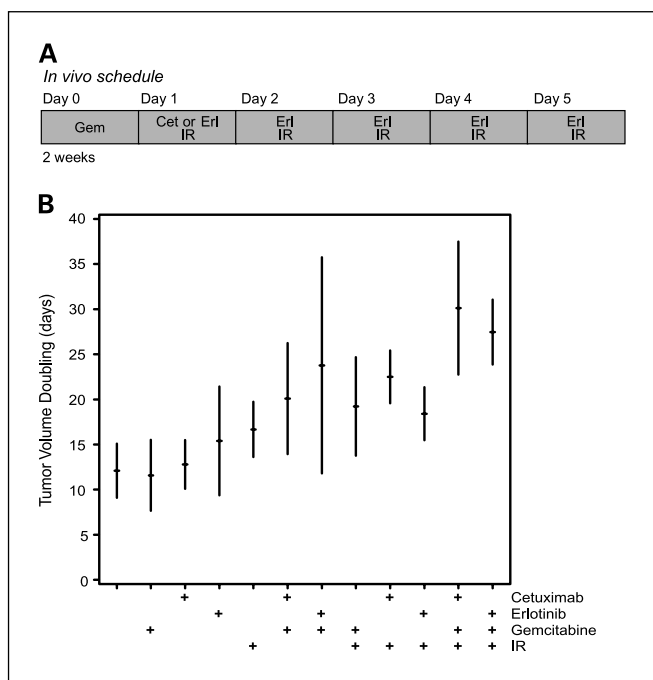


Fig. 3. The effects of cetuximab or erlotinib on gemcitabine-mediated radiosensitization *in vivo*. Athymic nude mice bearing s.c. BxPC-3 xenografts were treated with the indicated combinations of gemcitabine (120 mg/kg), cetuximab (50 mg/kg), erlotinib (100 mg/kg), or radiation (1 Gy × 10) as illustrated (A). Data are expressed as the time to tumor volume doubling in response to treatments that began on day 0 (B). Tic marks, mean time to tumor volume doubling; vertical bars, 95% confidence intervals. Data are from two tumors per mouse with six to seven mice per treatment group. Statistically significant differences are indicated in Table 1.

respectively). There were significant differences between EGFR^(Y845) and EGFR^(Y1173) phosphorylation in tumors treated with the combination of either cetuximab ($P < 0.02$) or erlotinib ($P < 0.010$) with gemcitabine-radiation; both cetuximab and erlotinib in combination with gemcitabine-radiation produced significant inhibition of EGFR^(Y1173) but not EGFR^(Y845), suggesting that these two phosphorylation sites respond differently to these combinations. When we assessed

Table 1. Tumor volume doubling

	Days
Control	12.1 ± 3.1
Gemcitabine	11.6 ± 3.1
Cetuximab	12.8 ± 3.1
Erlotinib	15.4 ± 3.3
IR	16.7 ± 3.1
Gem+IR	19.2 ± 3.3
Cet+Gem	20.1 ± 3.1
Erl+Gem	23.8 ± 3.3* †
Cet+IR	22.5 ± 3.3* ‡
Erl+IR	18.4 ± 3.1
Cet+Gem+IR	30.1 ± 3.3*§
Erl+Gem+IR	27.5 ± 3.3*

* $P < 0.05$ versus Control.

† Gemcitabine.

‡ Cetuximab.

§ Gem+IR.

|| Cet+Gem.

Table 2. Relative animal weight during and after therapy

Treatment	Day 7	Day 15	Day 21
Control	1.11 ± 0.01	1.22 ± 0.02	1.25 ± 0.01
Gemcitabine	1.03 ± 0.02	1.16 ± 0.01	1.20 ± 0.01
Cetuximab	1.06 ± 0.02	1.15 ± 0.02	1.21 ± 0.02
Erlotinib	1.08 ± 0.01	1.17 ± 0.01	1.24 ± 0.01
IR	1.02 ± 0.02	1.09 ± 0.03	1.16 ± 0.02
Gem+IR	0.99 ± 0.01	1.08 ± 0.02	1.17 ± 0.01
Cet+Gem	1.07 ± 0.02	1.17 ± 0.02	1.19 ± 0.01
Erl+Gem	1.05 ± 0.02	1.15 ± 0.02	1.20 ± 0.02
Cet+IR	1.06 ± 0.03	1.11 ± 0.02	1.15 ± 0.01
Erl+IR	1.00 ± 0.03	1.11 ± 0.03	1.23 ± 0.02
Cet+Gem+IR	1.02 ± 0.01	1.16 ± 0.02	1.24 ± 0.01
Erl+Gem+IR	0.94 ± 0.01	1.04 ± 0.02	1.09 ± 0.02

NOTE: Weights are relative to the first day of therapy (day 0).

tumors at the end of the treatment cycle (day 12), we found significant inhibition of both EGFR^(Y845) and EGFR^(Y1173) in response to the single agents, gemcitabine, cetuximab, erlotinib, or radiation (all $P < 0.01$). More importantly, the combination of cetuximab or erlotinib with gemcitabine-radiation resulted in significant inhibition of EGFR^(Y845) and EGFR^(Y1173) (relative to control; both $P < 0.01$) demonstrating efficient inhibition of EGFR phosphorylation under the conditions that produced the greatest reduction in tumor growth. Together, these results suggest that inhibition of pEGFR^(Y1173) in early treatment or EGFR^(Y845) and pEGFR^(Y1173) in late treatment are qualitatively (but not quantitatively) associated with tumor growth inhibition.

We also examined the effects of cetuximab or erlotinib with gemcitabine and radiation on a prosurvival molecule downstream of EGFR, pAKT^(S473). We found that the amount of pAKT^(S473) in tumors after treatment with gemcitabine, cetuximab, erlotinib, radiation, or gemcitabine plus radiation was significantly reduced by the second day of treatment (all $P < 0.01$). Furthermore, in response to either cetuximab or erlotinib in combination with gemcitabine-radiation, pAKT^(S473) was also significantly reduced (relative to control; both $P < 0.01$). The levels of pAKT^(S473) remained repressed through out the duration of the treatment as evidenced by repression of pAKT^(S473) compared with control pAKT^(S473) levels at day 12 ($P < 0.002$). Together, these data show that cetuximab or erlotinib, as well as gemcitabine and radiation inhibit pAKT^(S473).

Discussion

In this study, we have shown *in vitro* that the EGFR antagonist erlotinib can enhance the toxicity of gemcitabine in a schedule-dependent manner without abrogating gemcitabine-mediated radiosensitization. In mouse BxPC-3 pancreatic tumor xenografts, cetuximab or erlotinib in combination with gemcitabine-radiation produced significant tumor growth inhibition relative to untreated tumors. Most importantly, EGFR inhibition enhanced the growth delay produced by combination gemcitabine-radiation. Cetuximab or erlotinib alone, or in combination with gemcitabine-radiation, resulted in significant inhibition of the autophosphorylation site at

Y1173 early in treatment, and both the Y845 and Y1173 EGFR phosphorylation sites by the end of treatment. This work supports the clinical use of EGFR antagonists, such as cetuximab or erlotinib, in combination with gemcitabine

radiotherapy for the treatment of pancreatic cancer. Furthermore, this work suggests that pEGFR^(Y1173) and pAKT^(S473) might be useful qualitative markers for predicting response early in treatment.

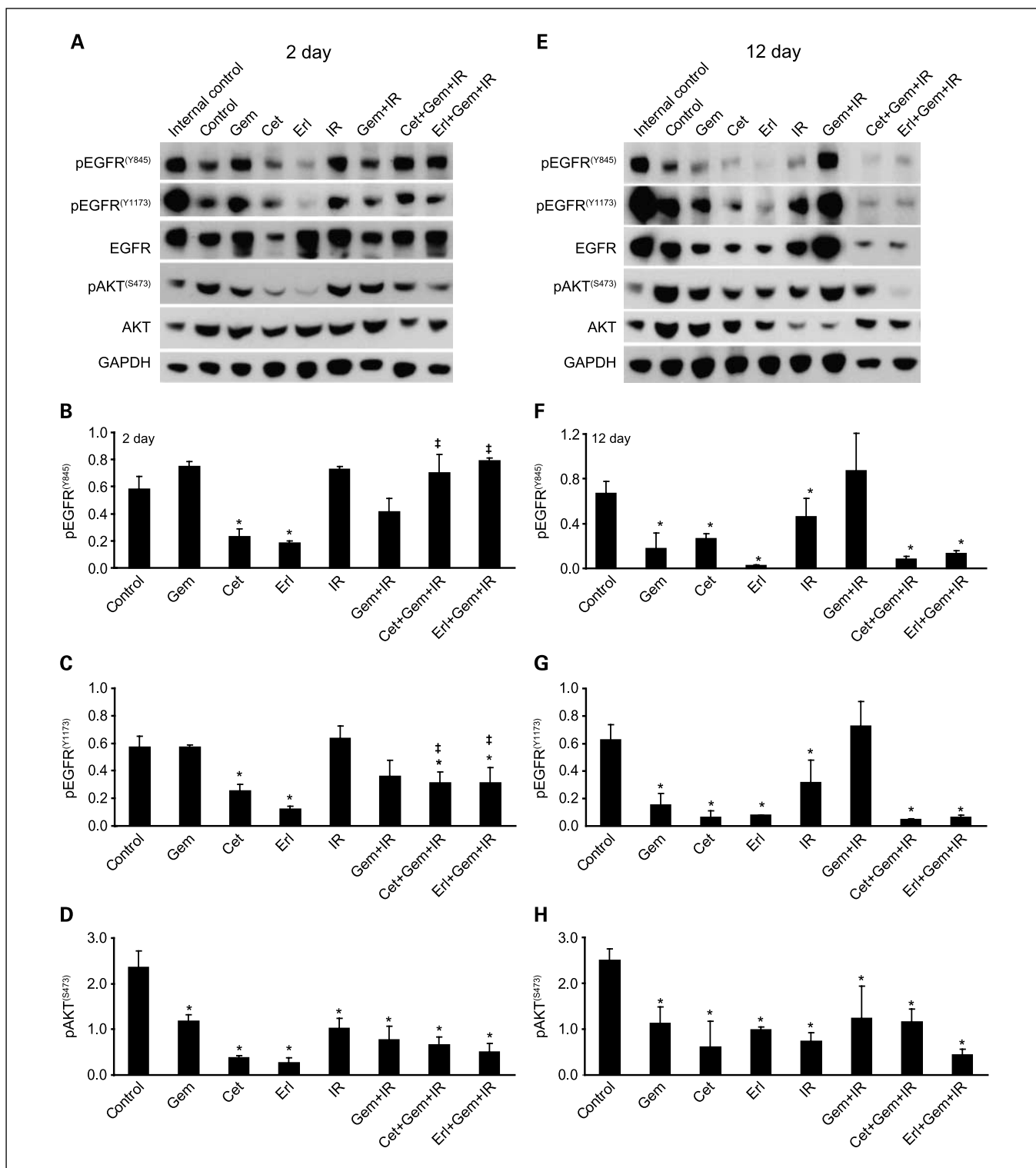


Fig. 4. The effects of cetuximab or erlotinib, gemcitabine, and radiation on EGFR signaling *in vivo*. Mice were treated as described in Fig. 3. Tumors were harvested on day 2 (A-D) or 12 (E-H) of treatment for immunoblotting. Columns, mean of single experiment (A and E) or of 2 to 5 tumors (B-D and F-H); bars, SE. Statistically significant differences of control versus treated (*) or EGFR^(Y845) versus EGFR^(Y1173) ‡ are indicated (P < 0.05).

A variety of preclinical studies have shown that EGFR inhibitors can increase radiation sensitivity in both *in vitro* and *in vivo* model systems (28–32). Although the majority of studies have reported additive effects resulting from the combination of EGFR antagonists and radiotherapy *in vitro*, the same combinations produce synergistic effects in xenograft models (25, 28, 29, 33–35). This might be because EGFR inhibitors and radiation affect several downstream signaling pathways (36). These include pathways regulating cellular proliferation and apoptosis, which would be evident in both *in vitro* and *in vivo*, and pathways regulating angiogenesis (28, 35) and tumor invasion (37), which might be detectable only in tumor xenograft models. Although we have not addressed the effects of EGFR inhibitors on angiogenesis and invasion, it is possible that the effect of erlotinib on gemcitabine radiosensitization *in vivo* may be attributable to effects on angiogenesis and/or invasion.

Our finding that the addition of EGFR inhibition produces only a modest improvement over gemcitabine (alone or with concurrent radiation) is consistent with the recent clinical results obtained by the addition of erlotinib to gemcitabine therapy in the treatment of pancreatic cancer (21). The relative insensitivity of pancreatic cancers to EGFR inhibitors as well as the lack of correlation between tumor growth and pEGFR inhibition observed in this study could be explained by a number of factors. Mutant ras is detected in >85% of pancreatic cancers (38), which makes cells resistant to EGFR inhibition (39–42). Although this is not the case in BxPC-3, which bear wild-type ras, BxPC-3 cells have been reported to express constitutively active Ras (43) that is also insensitive to cetuximab treatment. Furthermore, BxPC-3 cells have been shown to express elevated levels of ErbB3 (relative to other pancreatic cancer cells) and constitutively phosphorylated ErbB3 (44). It has been suggested that this ErbB3 expression results in heterodimerization of EGFR with ErbB3 and recycling of the heterodimer complex back to the cell surface, which may explain resistance to cetuximab. Thus, it is clear that constitutive expression of EGFR and its inhibition by an antibody or a small molecule may not always be sufficient to produce a response in the presence of other activated pathways.

Phosphorylation of EGFR at Y845 in response to gemcitabine has previously been shown in head and neck cancer cells (45). Src-dependent phosphorylation of EGFR has also been shown to occur in response to cellular stress by cisplatin (46), H₂O₂ (10), and UV (47). In addition, phosphorylation of EGFR has been observed in response to a variety of chemotherapeutic agents including gemcitabine (45), cisplatin (46), oxaliplatin, 5-fluorouracil (48), paclitaxel (49), doxorubicin (50), and irinotecan (51). EGFR^(Y845) as well as EGFR⁽⁹⁹²⁾ were shown to be similarly inhibited by cetuximab or erlotinib (52). In this study, we found that cetuximab or erlotinib combined with gemcitabine-radiation inhibited EGFR^(Y1173) but not EGFR^(Y845) at the beginning of the treatment cycle. This study is the first to our knowledge to show a fundamental difference

between two EGFR phosphorylation sites in response to EGFR inhibition. The finding that cetuximab or erlotinib combined with gemcitabine-radiation inhibited EGFR^(Y1173) but not EGFR^(Y845) suggests that differing mechanisms underlie phosphorylation of these two sites. Although this difference was not observed at the end of the treatment cycle, it suggested that EGFR^(Y845) and EGFR^(Y1173) may have different functions and, furthermore, that multiple phosphorylation sites are required to fully assess EGFR signaling.

In this study, we were interested in the effects that cetuximab or erlotinib would have on tumor growth in response to gemcitabine-radiation. Thus, a limitation of this study is that we used low doses of gemcitabine (120 mg/kg) and radiation (1 Gy × 10) that would permit us to observe a further delay of tumor growth by cetuximab or erlotinib. It is conceivable that the results of this study might have differed if we had used the maximally tolerated doses of gemcitabine and radiation, as would be the case in a clinical study. In fact, we attribute the difference between the gemcitabine-mediated induction of pEGFR observed *in vitro* (Fig. 1) and the lack of significant pEGFR induction *in vivo*, to the low relatively noncytotoxic dose of gemcitabine we chose for the *in vivo* studies. We designed the scheduling of EGFR inhibitors, gemcitabine, and radiation for the *in vivo* tumor growth experiments based on scheduling experiments done *in vitro*. We have not extensively investigated other schedules *in vivo*. We found this approach to be necessary for the efficient comparison of the number of variables involved when combining 4 agents. Furthermore, the schedule of EGFR inhibitor with gemcitabine-radiation selected in this study is amenable to integration with the current clinical regimen of once weekly gemcitabine with daily fractioned radiation therapy.

The finding that cetuximab or erlotinib in combination with gemcitabine-radiation produced a modest delay in tumor growth is consistent with the clinical data combining EGFR inhibitors with gemcitabine (21). Although this clinical trial produced only a modest improvement in survival (0.3 months) compared with gemcitabine alone, it is exceptional in that no previous trials combining agents such as oxaliplatin, cisplatin, irinotecan, 5-fluorouracil, marimastat (matrix metalloproteinase inhibitor), and tipifarnib (farnesyltransferase inhibitor) with gemcitabine produced significant survival improvements over gemcitabine alone (53). It will be important in future studies to investigate molecular agents targeted to other pathways activated in pancreatic cancer with gemcitabine and radiation as well as to explore the combination of EGFR inhibitors with other chemoradiotherapy regimens, such as 5-fluorouracil and gemcitabine-oxaliplatin-based chemoradiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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