Radiosensitization of Epidermal Growth Factor Receptor/HER2–Positive Pancreatic Cancer Is Mediated by Inhibition of Akt Independent of Ras Mutational Status

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Abstract

Purpose: Epidermal growth factor receptor (EGFR) family members (e.g., EGFR, HER2, HER3, and HER4) are commonly overexpressed in pancreatic cancer. We investigated the effects of inhibition of EGFR/HER2 signaling on pancreatic cancer to elucidate the role(s) of EGFR/HER2 in radiosensitization and to provide evidence in support of further clinical investigations.

Experimental Design: Expression of EGFR family members in pancreatic cancer lines was assessed by quantitative reverse transcription-PCR. Cell growth inhibition was determined by MTS assay. The effects of inhibition of EGFR family receptors and downstream signaling pathways on in vitro radiosensitivity were evaluated using clonogenic assays. Growth delay was used to evaluate the effects of nelfinavir on in vivo tumor radiosensitivity.

Results: Lapatinib inhibited cell growth in four pancreatic cancer cell lines, but radiosensitized only wild-type K-ras–expressing T3M4 cells. Akt activation was blocked in a wild-type K-ras cell line, whereas constitutive phosphorylation of Akt and extracellular signal-regulated kinase (ERK) was seen in lines expressing mutant K-ras. Overexpression of constitutively active K-ras (G12V) abrogated lapatinib-mediated inhibition of both Akt phosphorylation and radiosensitization. Inhibition of MAP/ERK kinase/ERK signaling with U0126 had no effect on radiosensitization, whereas inhibition of activated Akt with LY294002 (enhancement ratio, 1.2-1.8) or nelfinavir (enhancement ratio, 1.2-1.4) radiosensitized cells regardless of K-ras mutation status. Oral nelfinavir administration to mice bearing mutant K-ras–containing Capan-2 xenografts resulted in a greater than additive increase in radiation-mediated tumor growth delay (synergy assessment ratio of 1.5).

Conclusions: Inhibition of EGFR/HER2 enhances radiosensitivity in wild-type K-ras pancreatic cancer. Nelfinavir, and other phosphoinositide 3-kinase/Akt inhibitors, are effective pancreatic radiosensitizers regardless of K-ras mutation status. Clin Cancer Res; 16(3); 912–23. ©2010 AACR.
inhibitor, is an effective radiosensitizer for breast cancer, a cancer that frequently expresses high levels of HER2 and/or EGFR (14). Interestingly, the signaling pathway(s) downstream of EGFR/HER2 responsible for radiosensitization seems to vary by cancer subtype. Although numerous compounds have been used successfully in laboratory studies to directly inhibit signaling pathways located downstream of EGFR and/or HER2, translation to efficacious and tolerable clinical use has been difficult.

Nelfinavir (Viracept, Pfizer), a type 1 HIV protease inhibitor, may downregulate Akt signaling with minimal side effects. HIV protease inhibitors were first noted to inhibit the growth of Kaposi’s sarcoma independent of their antiretroviral effect soon after receiving Food and Drug Administration approval in 1997 (15, 16). Several groups then showed that these compounds radiosensitize several tumor cells through the blockade of Akt signaling (17) and/or proteasome inhibition (18). The exact mechanism of this effect remains unclear, as nelfinavir has been shown to increase caspase-dependent apoptosis, nonapoptotic (caspase-independent) death, endoplasmic reticulum stress, and autophagy (19, 20).

We initiated this study to determine whether the inhibition of EGFR/HER2 signaling could sensitize pancreatic cancer to ionizing radiation to provide data in support of a clinical trial. We expanded the study to determine the downstream signaling pathways involved in radiosensitization and to show that nelfinavir, and other agents that inhibit the phosphoinositide 3-kinase (PI3K) →Akt pathway, is an effective radiosensitizer in the majority of pancreatic cancers.

Materials and Methods

**Inhibitors and growth factors.** Lapatinib (Tykerb®) was provided by GlaxoSmithKline. LY294002 was obtained from Sigma Co. and U0126 was from Calbiochem. Inhibitors were reconstituted in DMSO, subdivided into working solutions, and stored at −20°C. Tablets of nelfinavir mesylate (650 mg) were purchased from the University of North Carolina inpatient pharmacy and were ground into fine powder before being dissolved into 100% ethanol before each use. EGF ligand was obtained from Invitrogen. Control cells were treated with equal concentrations of DMSO or ethanol.

**Quantitative reverse transcription-PCR.** Gene-specific 5′-3′ oligonucleotides and intervening fluorescent dye-labeled probes for human genes encoding EGFR, HER2, HER3, and HER4 (Supplementary Table S1) were designed, synthesized, labeled, and purified using standard techniques. Real-time fluorescence quantitative PCR was performed with an ABI PRISM 7900 instrument from Applied Biosystems. Messenger RNA sequences for each gene were transcribed in vitro using MEGAscript (Ambion), and used as positive controls and absolute quantitation standards for the assays. Amplification of 2-fold serial dilutions of RNA was used to construct standard linear curves that permitted accurate measurements of 200 to 90 million template copies. Total RNA was isolated from each cell line in triplicate by using a QIAGEN RNeasy kit and was treated with RNase-free DNase. Total RNA (10 ng) isolated from each cell line was assayed.

**Cell lines and culture conditions.** Pancreatic cancer cell lines (T3M4, Capan-2, MiaPaCa-2, and PANC-1) were obtained from the American Type Culture Collection and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL). No additional authentication was done, although the American Type Culture Collection performs DNA profiling of cell lines and all cell lines were cultured for <6 mo before being reconstituted from frozen stocks. Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere of 5% CO2.

**Cell proliferation assay and IC50 determination.** Cells (1.5-5 × 104 cells per well) were plated on 96-well plates in 100 μL media with increasing concentrations of lapatinib (0.01-50.0 μmol/L) or nelfinavir (0.001-20.0 μmol/L). After 72 h, cell viability was measured by the MTS assay according to the manufacturer’s directions (Promega). IC50 values and 95% confidence intervals were calculated according to the manufacturer’s directions (Promega). IC50 values and 95% confidence intervals were calculated according to the nonlinear curve fit and compared by the extra sum-of-squares F test using GraphPad Prism version 5.01 (GraphPad Software).

**Immunoprecipitation and Western blot analysis.** Cells were initially starved overnight followed by 1 h lapatinib (1 or 5 μmol/L) pretreatment and then EGF (10 ng/mL) stimulation for 15 min. Cellular extracts were prepared by washing cells with cold PBS and by lysing them in cold NLB buffer [20 mmol/L HEPES (pH 7.3), 50 mmol/L sodium fluoride, 10% glycerol, 1% Triton X-100, 5 mmol/L EDTA, 0.5 mol/L NaCl, 1 mmol/L sodium vanadate, aprotonin (6 μg/mL), and leupeptin (10 μg/mL)]. Receptor proteins were precipitated from cell lysates (1 mg) with a commercial antibody against HER2 (clone
9G6.10; NeoMarkers, Inc./Thermo Scientific) or with a noncommercial antibody against HER1/EGFR (V22) kindly provided by S. Earp (University of North Carolina, Chapel Hill, NC; ref. 14) at 4°C overnight. Immune
complexes were then pulled down with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) for 3 h at 4°C, washed thrice with NLB, and analyzed by Western blot analysis using an antiphosphotyrosine antibody (PY20-HRP; Santa Cruz Biotechnology, Inc.).

Western blot analyses were done to determine the effect of pharmacologic inhibitors on MAP/extracellular signal-regulated kinase kinase (MEK)/ERK1/2 and PI3K/Akt activation. Cells were plated at 40% confluence in 60-mm dishes and, on the following day, were treated with inhibitors against EGFR/HER2 (lapatinib, 1-5 μmol/L), PI3K (LY294002, 5-20 μmol/L), MEK1/2 (U0126, 1-5 μmol/L), or vehicle alone (DMSO) for 1 h, or with nelfinavir (1 μmol/L) or vehicle alone (ethanol) for 4 or 28 h before lysis. Thirty micrograms of protein lysates (harvested as described above) were separated over 15% SDS-PAGE gels, were transferred to Immobilon-P membrane (Millipore), and immunoblots were performed using phospho-ERK1/2- (#9106), ERK1/2- (#9122), phospho-Akt(Ser473)- (#9271), or Akt- (#9282), specific antibodies (all from Cell Signaling Technology, Inc.). Anti-α-tubulin (TIU-02) was obtained from Santa Cruz Biotechnology.

**Colonogenic survival assays.** Cells were trypsinized to create single-cell suspensions, seeded into T-25 flasks at defined densities, and incubated overnight to ensure log phase of growth. The next day, 2 h preirradiation, cells were fed with fresh media supplemented with either lapatinib (5 μmol/L), U0126 (5 μmol/L), or LY294002 (10 μmol/L). Cells treated with nelfinavir (1 or 5 μmol/L) received 2 or 26 h of pretreatment before irradiation. Control cells were maintained in media containing the biologically optimal dose of nelfinavir with regard to inhibition of Akt activation, mice (n = 1 per group) were injected s.c. in the flanks with Capan-2 cells (5 × 10^7) resuspended in 200 μL of a 1:1 ratio of PBS/Matrigel (BD Biosciences) and treated with nelfinavir (50, 100, or 150 mg/kg once daily) or vehicle alone by oral gavage for a total of 5 d. Mice were euthanized by CO2 inhalation and tumors were harvested using sterile technique 4 h after the last dose on day 5. Excised tumors were flash frozen and pulverized with a mortar and pestle under liquid nitrogen before transfer into 1 to 2 mL of radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.25% deoxycholate, 1% NP40, 1 mmol/L sodium orthovanadate, and complete protease inhibitor cocktail (Roche Applied Science)]. Lysates were then homogenized using a Polytron homogenizer (Brinkmann), incubated on ice for 30 min, and centrifuged at 14,000 rpm at 4°C for 15 min; supernatants were stored at −80°C before immunoblotting for P-Akt, Akt, and α-tubulin as described above.

To assess the tumor growth delay induced by nelfinavir, mice (n = 6 per group) bearing Capan-2 xenografts prepared as described above were randomly assigned to one of four treatment groups: (a) vehicle alone, (b) nelfinavir alone (150 mg/kg daily by oral gavage) × 10 d, (c) radiation alone (200 cGy/d on days 6, 7, and 8), or (d) radiation plus nelfinavir (as above). Radiation was delivered by a linear accelerator (Primus, Siemens) to anaesthetized mice using 6 MeV electrons and a custom lead cutout. Tumors were measured at 2- to 3-d intervals using Vernier calipers and the tumor volumes were calculated (V = π × length × width^2/6). Tumor volumes were fit using a squares F test. Each point represents the mean surviving fraction calculated from three independent experiments done in triplicate for each treatment condition; error bars represent the SD. The mean inactivation dose was calculated according to the method of Fertil (21), and the cell survival enhancement ratio (ER) was calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose after drug exposure as described by Morgan (22). A value significantly >1 indicates radiosensitization. For the drug dose-response comparison, two-way ANOVA followed by Bonferroni posttests was done using GraphPad Prism.

**Ectopic expression of mutant K-ras.** T3M4 cells, which are wild-type for K-ras, were transfected with pCGN-K-ras (G12V)-HA or an empty vector control as previously described (23) using FuGENE 6 Transfection Reagent (Roche Diagnostics). Clonogenic survival assays were done 24 h after transfection. Protein lysates were prepared as described above for Western blot analysis with anti-K-ras serum (Calbiochem).

**Xenografts.** Four to 5-wk-old athymic BALB/c female nude mice were purchased from Charles River Laboratories, housed in filter-topped cages in an aseptic environment, and maintained per defined protocol approved by and in accordance with the University of North Carolina Institutional Animal Care and Use Committee. To determine the biologically optimal dose of nelfinavir with regards to inhibition of Akt activation, mice (n = 1 per group) were injected s.c. in the flanks with Capan-2 cells (5 × 10^7) resuspended in 200 μL of a 1:1 ratio of PBS/Matrigel (BD Biosciences) and treated with nelfinavir (50, 100, or 150 mg/kg once daily) or vehicle alone by oral gavage for a total of 5 d. Mice were euthanized by CO2 inhalation and tumors were harvested using sterile technique 4 h after the last dose on day 5. Excised tumors were flash frozen and pulverized with a mortar and pestle under liquid nitrogen before transfer into 1 to 2 mL of radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.25% deoxycholate, 1% NP40, 1 mmol/L sodium orthovanadate, and complete protease inhibitor cocktail (Roche Applied Science)]. Lysates were then homogenized using a Polytron homogenizer (Brinkmann), incubated on ice for 30 min, and centrifuged at 14,000 rpm at 4°C for 15 min; supernatants were stored at −80°C before immunoblotting for P-Akt, Akt, and α-tubulin as described above.

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straight-line nonlinear regression (GraphPad Prism) and were compared using the extra sum-of-squares F test.

To assess antagonistic, additive, or synergistic effects, we used the fractional product method at day 25 (24, 25). The observed fractional tumor volume (FTV) is equal to the mean tumor volume of each treated group (nelfinavir, radiation, or nelfinavir + radiation) divided by the mean tumor volume of the control group. The expected FTV from the combined treatment (FTV_{nelfinavir + radiation}) is calculated by multiplying the observed FTV_{nelfinavir} by the observed FTV_{radiation}. Dividing the expected FTV_{nelfinavir + radiation} by the observed FTV_{nelfinavir + radiation} yields a synergy assessment ratio in which a value of >1 suggests that the combined treatments are effectively synergistic, a value of <1 suggests that treatments are antagonistic, and a value of 1 suggests that the treatments are additive.

Results

Lapatinib blocks EGFR and HER2 activation. We have shown previously that both lapatinib and erlotinib, an EGFR-selective tyrosine kinase inhibitor, block the soft agar growth of several pancreatic cancer cell lines (26). Because EGFR inhibition has been shown to radiosensitize other cancers, including head and neck squamous cell carcinomas (HNSCC) and breast cancer (13), we sought to determine whether these compounds could also radiosensitize pancreatic cancer cells and whether this radiosensitization correlated with EGFR and HER2 expression.

We first evaluated by quantitative reverse transcription-PCR the relative expression levels of all four members of the EGFR family of receptors among a panel of four pancreatic cancer cell lines (Table 1). Although HER2 levels were similar among all four lines, EGFR levels were 10-fold higher in the PANC-1 and T3M4 cells relative to that observed in the Capan-2 and MIA PaCa-2 cells. Expression of HER3, a family member that lacks kinase activity, was increased in the PANC-1 and T3M4 cells compared with the Capan-2 and MIA PaCa-2 cell lines, indicating the importance of the HER family in pancreatic cancer cell lines. Despite a mean elevation in HER3 mRNA levels in the PANC-1 and T3M4 cells, levels of HER4, the final family member, had very low mRNA expression levels across all four cell lines.

All cell lines showed an antiproliferative effect in response to increasing concentrations of both erlotinib and lapatinib (Table 1). The dual EGFR/HER2 inhibitor lapatinib showed improved growth-inhibitory activity compared with erlotinib in Capan-2 and MIA PaCa-2 cell lines (P = 0.0008 and 0.0001, respectively), suggesting that low levels of EGFR mRNA in these cell lines. PANC-1 and T3M4 cells had higher levels of EGFR than HER2 expression, and showed similar growth inhibition by lapatinib and erlotinib (P = not significant). To show that lapatinib blocks ligand-stimulated EGFR and HER2 activation in our pancreatic cells, the activation of receptors was analyzed by immunoprecipitation followed by Western blot analysis. Consistent with what we and others have previously reported using in vitro, in vivo, and patient samples (refs. 14, 27 and reviewed in ref. 28), lapatinib blocked the activation of both EGFR and HER2 in all four pancreatic cell lines (Fig. 1A). Pancreatic cancer cell lines harboring K-ras mutations are resistant to lapatinib-mediated radiosensitization. Due to the improved antiproliferative and ligand-stimulated receptor inhibition of lapatinib in the tested cell lines, we chose to investigate whether lapatinib could radiosensitize pancreatic cancer cells. Clonogenic survival assays were done on our panel of cells that were either treated with lapatinib (5 μmol/L) or vehicle alone for the 2 hours preceding and 2 hours after irradiation. We chose this short duration of drug treatment because the clonogenic survival and cell cycle distribution of nonirradiated cell lines that were pretreated in this fashion with either lapatinib or DMSO control were not statistically different (data not shown), suggesting that the 4-hour exposure to lapatinib did not radiosensitize cells simply by inhibiting proliferation or by redistributing cells to a more radiosensitive phase of the cell cycle. T3M4 cells were radiosensitized by lapatinib, whereas MIA PaCa-2, PANC-1, and Capan-2 cells were not radiosensitized.

### Table 1. EGFR family expression levels and IC50 values for growth inhibition with lapatinib

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Messenger RNA copy number (SD, n = 3)</th>
<th>Lapatinib IC50 (μmol/L, 95% CI)</th>
<th>Erlotinib IC50 (μmol/L, 95% CI)</th>
<th>Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGFR</td>
<td>HER2</td>
<td>HER3</td>
<td>HER4</td>
</tr>
<tr>
<td>Capan-2</td>
<td>6525 (814)</td>
<td>40171 (7341)</td>
<td>44006 (2098)</td>
<td>37 (10)</td>
</tr>
<tr>
<td>MIA PaCa-2</td>
<td>4076 (1092)</td>
<td>36356 (8665)</td>
<td>2554 (1364)</td>
<td>33 (2)</td>
</tr>
<tr>
<td>PANC-1</td>
<td>65918 (6071)</td>
<td>25144 (1720)</td>
<td>4213 (3214)</td>
<td>341 (33)</td>
</tr>
<tr>
<td>T3M4</td>
<td>72154 (1856)</td>
<td>42408 (17906)</td>
<td>25220 (2426)</td>
<td>86 (14)</td>
</tr>
</tbody>
</table>

NOTE: Absolute levels of EGFR, HER2, HER3, and HER4 mRNA for each cell line were measured by quantitative real-time RT-PCR. Equal numbers of cells from each line were plated and cultured with increasing concentrations of lapatinib. Cell viability was measured by MTS assay and IC50 values calculated by GraphPad Prism.

 Abbreviations: 95% CI, 95% confidence interval; wt, wild-type.

*Comparison of lapatinib versus erlotinib, P < 0.001.

†Comparison of lapatinib versus erlotinib, P = not significant.
Lapatinib-mediated radiosensitization occurred in a dose-dependent manner (Supplementary Fig. S1) and at doses unlikely to have significant off-target effects (29). The ER of 1.3 for T3M4 cells is consistent with that reported for known radiosensitizers such as gemcitabine or cisplatin (30, 31). Suggestive of the importance of K-ras mutations in the radiation response, T3M4 cells express wild-type K-ras whereas MIA PaCa-2, PANC-1, and Capan-2 cell lines all express mutant K-ras (32).

The presence of constitutively active, mutant forms of K-ras, a molecular abnormality seen in ~90% of pancreatic cancers (33, 34), has previously been shown to confer radioresistance (35–38). Thus, we hypothesized that the inhibition of EGFR/HER2 signaling by lapatinib with resulting radiosensitization was conferred through the inhibition of specific downstream signaling pathways that are directly activated in the presence of constitutively active Ras. We first evaluated the ability of lapatinib to inhibit the downstream signaling of the PI3K/Akt and Raf/MEK/
ERK pathways, two pathways capable of being activated by both EGFR/HER2 and Ras. Activation of Akt, but not ERK1/2, was completely inhibited by lapatinib in the T3M4 cells, whereas neither ERK1/2 nor Akt were inhibited by lapatinib in cells with mutant K-ras (Fig. 1C). Taken together, these data suggest that resistance to lapatinib radiosensitization in the MIA PaCa-2, PANC-1, and Capan-2 cells may be mediated by activation of PI3K/Akt by mutant Ras.

**K-ras expression blocks radiosensitization by lapatinib.** To determine the role of mutant Ras in conferring radioresistance in these cells, we next evaluated whether ectopic expression of mutant K-ras could abrogate lapatinib-mediated radiosensitization of T3M4 cells. Cells treated with lapatinib that were expressing K-ras(G12V), but not vector control, exhibited sustained Akt activation and no change in ERK activation (Fig. 2A). This correlated with a lack of radiosensitization by lapatinib in cells expressing K-ras(G12V), but not vector control (Fig. 2B). These results support a model in which the presence of mutant K-ras can render pancreatic cancer cells resistant to lapatinib-mediated radiosensitization.

**Pancreatic cancer cells are radiosensitized by inhibition of PI3K/Akt, but not MEK/ERK.** If activated Ras could block the radiosensitization observed with lapatinib-mediated inhibition of EGFR/HER2 in the T3M4 cells, we reasoned that radiosensitization by lapatinib was being mediated by the inhibition of a downstream signaling pathway(s) that is activated by both EGFR/HER2 and Ras. In addition, we hypothesized that in the mutant K-ras cell lines (MIA PaCa-2, PANC-1, and Capan-2), activation of these downstream pathways by Ras could be responsible for their observed resistance to lapatinib-mediated radiosensitization. Downstream signaling from EGFR/HER2 and Ras are both known to activate several key pathways in common, including the Raf/MEK/ERK and PI3K/Akt pathways (13–39). To determine whether inhibition of Raf/MEK/ERK and/or PI3K/Akt could radiosensitize pancreatic cancer cells, we evaluated the ability of U0126, a MEK inhibitor and known breast cancer radiosensitizer (44), and LY294002, a PI3K inhibitor (45), to sensitize our panel of pancreatic cancer cell lines to radiation-induced cell death. Despite effective inhibition of ERK1/2 phosphorylation in all cell lines by U0126 (Supplementary Fig. S2A), this inhibition of MEK/ERK activation did not radiosensitize any of the pancreatic cancer cell lines (Supplementary Fig. S2B). A modest increase in Akt activation was seen in some cell lines in response to U0126 treatment, a result consistent with feedback-signaling loops described by others (41, 42) and consistent with the role of Akt in the radioresponse. In contrast, treatment with LY294002 resulted in the effective inhibition of Akt with consequent radiosensitization (ERs ranged from 1.3-2.1) of all cells regardless of their K-ras mutational status (Fig. 3A and B).

**Nelfinavir blocks Akt phosphorylation and radiosensitizes both wild-type and mutant K-ras cell lines.** Several Food and Drug Administration–approved HIV protease inhibitors including nelfinavir and ritonavir have been shown to block Akt signaling and radiosensitize HNSCC, breast, lung, and brain tumor cell lines (19, 43–47). Because currently available PI3K inhibitors have shown unacceptable clinical toxicity, we sought to evaluate whether inhibition of the PI3K/Akt pathway with nelfinavir would radiosensitize pancreatic cancer cells. Cells treated with a clinically attainable dose of nelfinavir (48) or vehicle alone showed decreased Akt activation after 28 hours, but not after a 4-hour exposure (Fig. 4A). Little change in ERK1/2 activation (Fig. 4A) or cell cycle distribution (data not shown) was seen at either time point.

To determine the effect of nelfinavir on radiation response, cells were similarly pretreated with nelfinavir for either 2 or 26 hours before and 2 hours after irradiation.
and their ability to proliferate in clonogenic survival assays determined. Both mutant and wild-type K-ras cells were radiosensitized (ERs ranged from 1.3-1.5; Fig. 4B) after 26 hours of nelfinavir pretreatment. Consistent with effects on Akt activation, no radiosensitization was seen after 2 hours of pretreatment. To exclude the possibility that nelfinavir treatment induces growth arrest, MTS assay was used to monitor proliferation after exposure to nelfinavir for either 2 or 26 hours. No significant difference in proliferation was seen with either length of exposure in any of the four cell lines tested (data not shown).

Nelfinavir inhibits Akt activation and results in tumor growth delay of Capan-2–bearing xenografts. We next assessed the ability of nelfinavir to radiosensitize a mouse xenograft model using Capan-2 cells, chosen based on their robust ability to form tumors. First, to determine the optimal dose of nelfinavir required to inhibit Akt activation in vivo, Capan-2 cells (5 × 10^6) were injected into the flanks of athymic BALB/c nude mice. After palpable tumors developed, mice were treated with indicated doses of nelfinavir or vehicle control by gastric gavage for 5 consecutive days. On the 5th day, mice were sacrificed, tumor lysates were prepared, and Akt activation was assessed by Western blot analysis. At a dose of 150 mg/kg, phospho-Akt levels in vivo were significantly decreased (Fig. 5A). With this optimized dose, tumor growth in cohorts (n = 6 mice per treatment group) were compared with mice either sham-treated or treated with nelfinavir, radiation, or nelfinavir plus radiation. A clinically relevant dose of radiation (2 Gy per fraction) was chosen to provide meaningful assessment of any radiosensitization. Tumor growth following treatment was significantly slower in mice treated with nelfinavir and radiation than with either treatment alone (P < 0.0001; Fig. 5B) and was consistent with synergy between radiation and nelfinavir as shown by a synergy assessment ratio (±SEM) of 1.5 ± 0.27 as determined by the fractional product method (Fig. 5C). In addition, the slopes of the tumor volume curves after completion of all treatments (day 10) differed significantly (P ≤ 0.0001) consistent
with the synergy between radiation and nelfinavir. Consistent with the survival of some tumor cells after the initial treatment, a repopulation with similar growth rates was observed after day 20. However, tumor volumes in the nelfinavir plus radiation treatment were consistently significantly reduced compared with controls consistent with synergy between radiation and nelfinavir. Collectively, these data support a model in which blockade of an activated PI3K/Akt prosurvival pathway mediates radiation sensitization and provides evidence that drugs such as nelfinavir or other novel agents targeting this pathway may be efficacious radiosensitizers worthy of further study.

**Discussion**

EGFR and/or HER2 are overexpressed in a significant number of pancreatic cancers (4–8), and blockade of EGFR or HER2 inhibits the growth of pancreatic cancer cells *in vitro* (9–11). Erlotinib has been approved for the treatment of pancreatic cancer (3) and its role as a radiosensitizer is currently being studied in clinical trials. Due to the growing evidence supporting the ability of pharmacologic inhibitors of EGFR and HER2 to radiosensitize multiple types of cancers including breast, HNSCC, colon, and pancreas (reviewed in ref. 13), and due to the overexpression of both EGFR and HER2 in pancreatic cancer, we hypothesized that dual inhibition of EGFR and HER2 with lapatinib would sensitize pancreatic cancer to radiation.

In our preliminary studies, we sought to determine whether the inhibition of both EGFR and HER2 with lapatinib would be superior to the inhibition of EGFR alone with erlotinib. As expected, lapatinib had an improved proliferative IC₅₀ in cell lines with high HER2 mRNA levels and had a similar IC₅₀ as erlotinib in cells with high...
levels of EGFR mRNA. Thus, we chose to study the ability of lapatinib to radiosensitize pancreatic cancer. Intriguingly, we found that lapatinib was an effective radiosensitizer in only the T3M4 line that did not harbor a mutant form of K-ras despite its ability to block EGFR and HER2 activation (Fig. 1A), cellular proliferation (Table 1), and soft agar growth in multiple cell lines. This was consistent with the results reported recently by Morgan et al. (22) in which erlotinib radiosensitized a single cell line expressing wild-type K-ras. Due to the expression of mutated K-ras in >90% of pancreatic cancers (33, 34), our data suggest that targeting EGFR and HER2 in a clinical trial is unlikely to be a successful strategy for the radiosensitization of pancreatic cancer. Given the wealth of evidence supporting the resistance of K-ras–mutated cancers to EGFR-targeted therapies (reviewed in ref. 49), this finding is not surprising.

The differential effect of lapatinib on growth inhibition and radiosensitization adds to the evidence that the downstream signaling pathways responsible for these biological responses can be uncoupled. We have previously shown that ERK inhibition correlates with both growth inhibition and radiosensitization in EGFR-overexpressing breast cancer cell lines (14), whereas HER2-overexpressing breast cancer cell lines show growth delay but not radiosensitization in response to therapies that inhibit Akt. These differences may depend on the alternative activation of intracellular feedback loops through the collateral pathway activation, a mechanism of resistance to tyrosine kinase inhibitors recently described by several groups (41, 42).

We have shown that lapatinib decreased Akt-activation in T3M4 cells and that overexpression of activated K-ras in these cells abrogated the ability of lapatinib to both inhibit Akt and radiosensitize these cells. Direct inhibition of the PI3K/Akt pathway radiosensitized all cells independent of their K-ras mutational status, whereas inhibition of MER/ERK signaling had no effect on the radiation sensitivity of any cell line tested. These results add support to the growing body of evidence that the PI3K/Akt signaling pathway plays a vital role in radiosensitization (17, 35, 43, 50–53) and provides further evidence that Akt inhibitors may be promising clinical radiosensitizers.

Finally, we show that nelfinavir, an HIV protease inhibitor, blocked Akt activation and radiosensitized both wild-type and mutant K-ras–containing cells at concentrations attainable in humans (48). The radiation ER of nelfinavir ranged from 1.2 to 1.4, values that can result in a large cumulative effect when applied over many daily fractions of radiation (Fig. 5D). Using a xenograft system, we showed that oral nelfinavir decreased intratumor Akt activation in vivo and synergized with clinically relevant fractionated radiation doses. The exact mechanism of action of nelfinavir remains unclear. In addition, whether all HIV protease inhibitors share a common mechanism of radiosensitization remains untested. Saquinavir, a compound in the class of HIV protease inhibitors, has been shown to block proteasome function stabilizing IκB and decreasing NFκB in glioblastoma and prostate cancer cell lines (18). Others have pointed to a role of the ER stress response and/or the unfolded protein response, a mechanism of resistance to tyrosine kinase inhibitors recently described by several groups (41, 42).

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6 B. Calvo and K. Baerman, unpublished data.
7 Manuscript in preparation.
response resulting in phosphatase activation and Akt dephosphorylation in a HNSCC cell line (45). Both decreased Akt and NFκB activation can contribute to radiosensitization. In addition, HIV protease inhibitors may enhance tumor oxygenation through inhibition of HIF-1 and vascular endothelial growth factor as shown in glioblastoma, lung carcinoma, and HNSCC cell lines, thus rendering tumors more sensitive to radiation regardless of effects on intracellular signaling pathways (44, 46). The potential cell line–specific differences in mechanism highlight the importance of studying potential treatments in multiple systems.

These results provide valuable information in support of the use of nelfinavir as a clinically relevant radiosensitizer for pancreatic cancer. Although a small phase I trial combining radiation and nelfinavir with increasing doses of gemcitabine has recently been completed (54), this trial was not designed to determine the biologically effective dose of nelfinavir. In addition, the tolerability of adding nelfinavir, or other novel Akt inhibitors, to radiation and 5-fluorouracil or capcitabine, a common regimen used in the treatment of pancreatic cancer, deserves further study.

Although we have delineated the PI3K/Akt pathway as an important component of radiation sensitization in pancreatic cancer, other signaling pathways downstream of EGFR/HER2, Ras, or yet undefined signaling node proteins may also play an important role in this response. It is also possible that the off target effects may play a role in radiosensitization. Several groups have shown that LY294002 inhibits not only PI3K but, at concentrations higher than used in our studies, can also inhibit PI3K-like kinases such as DNA-PK, a key regulator of DNA double-strand break repair (55, 56). The concomitant use of multiple targeted therapies is being investigated in our laboratory and others and may result in improved tumor control both locally and distantly. Care must be used in these cases, as drug combinations may result in unexpected therapeutic antagonism, have increased toxicities, and lead to unexpected clinical consequences. Although the treatment of metastatic disease remains of critical importance in the treatment of pancreatic cancer, a substantial portion of patients are still dying of local disease, underlying the importance of both improved local and systemic therapies (57).

Conclusion

We have provided evidence that the high incidence of K-ras mutations in pancreatic cancer makes the use of EGFR and/or HER2 inhibitors as radiosensitizers in this disease unlikely to be efficacious. This is consistent with findings reported by several groups that mutations in K-ras render non–small cell lung cancer and colorectal cancer resistant to EGFR-targeted therapy (reviewed in ref. 58). It also complements the data provided by Morgan and colleagues (22) that erlotinib is a radiosensitizer for a wild-type K-ras–containing pancreatic cancer cell line. Furthermore, we show that persistent activation of the PI3K/Akt pathway through constitutively active K-ras correlates with a lack of radiosensitization and that direct inhibition of the PI3K/Akt pathway results in radiosensitization regardless of K-ras mutational status. Most importantly, nelfinavir, an HIV protease inhibitor, both decreases Akt phosphorylation and radiosensitizes several pancreatic cancer cell lines regardless of K-ras mutation status. Although most inhibitors of the PI3K/Akt pathway are too toxic for routine clinical use, nelfinavir is routinely used long-term for the treatment of HIV with relatively few side effects. Additional studies into the tolerability and efficacy of combined treatment with nelfinavir, traditional cytotoxic chemotherapy, and radiation for the treatment of pancreatic cancer are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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