Cotargeting MAPK and PI3K Signaling with Concurrent Radiotherapy as a Strategy for the Treatment of Pancreatic Cancer

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Abstract

There is an urgent need for the development of novel therapies to treat pancreatic cancer, which is among the most lethal of all cancers. KRAS-activating mutations, which are found in more than 90% of pancreatic adenocarcinomas, drive tumor dependency on the Ras/MAPK and Akt signaling pathways. Radiation is currently being explored as a component of the standard treatment regimen for pancreatic cancer. This study’s purpose was to test the hypothesis that MAP kinase kinase (MEK or MAP2K) inhibitors will offer clear therapeutic benefit when integrated into radiotherapy treatment regimens for treatment of this disease. We explored the activation of the mitogen-activated protein kinase (MAPK) and Akt pathways in response to radiation in multiple pancreatic tumor cell lines. Small molecule inhibitors of MEK (PD0325901) and Akt (API-2) were subsequently evaluated for their radiosensitizing potential alone and in combination. In vivo efficacy was tested in subcutaneous MIA-PaCa2 xenografts. Phosphorylated levels of extracellular signal–regulated kinase (ERK)-1/2 and Akt were found to increase in response to radiation treatment in our pancreatic tumor cell line panel. MEK inhibitor–induced radiosensitization was observed in vitro and in vivo. The further addition of an Akt inhibitor to the MEK inhibitor/radiation regimen resulted in enhanced therapeutic gain as determined by increased radiosensitization and tumor cell death. In conclusion, MEK inhibition results in growth arrest, apoptosis, and radiosensitization of multiple preclinical pancreatic tumor models, and the effects can be enhanced by combination with an Akt inhibitor. These results provide rationale for further testing of a treatment regimen in pancreatic cancer that combines MEK inhibition with radiation, optimally in conjunction with Akt inhibition. Mol Cancer Ther; 11(5); 1193–202. ©2012 AACR.

Introduction

Aberrant KRAS signaling is a hallmark of the vast majority of pancreatic cancers, which exhibit an especially high incidence (>90%) of KRAS mutations. Consequently these cancers display activation of the RAF/MEK/MAPK (ERK) signaling cascade. Phosphorylation of these kinases drives proliferation of pancreatic cancer cells and impacts their survival and metastatic spread (1, 2). Consequently, a growing number of MAP kinase kinase (MEK or MAP2K) inhibitors have now entered clinical testing against a variety of solid tumor types, including pancreatic cancer (3–7). However, the large number of genetic aberrations in pancreatic cancer makes it unlikely that single-agent therapy will produce meaningful therapeutic benefit to this patient population.

Multiple, potentially attractive strategies exist for combining MEK inhibitors with other therapies. Specifically, combined targeting of both MEK and phosphoinositide 3-kinase (PI3K) has attracted much interest for the treatment of KRAS driven tumors (8, 9). Oncogenic KRAS drives activation of both the mitogen-activated protein kinase (MAPK) as well as PI3K/Akt pathways, which are important for proliferation, survival, and tumorigenesis. Compensatory signaling arising from crosstalk between these pathways can reduce the therapeutic effectiveness of targeting either pathway alone. Specifically, PI3K/Akt pathways have been implicated in mediating resistance to MEK inhibitors (9–11). Conversely, inhibition of Akt/mTOR signaling in human cancer cells can lead to extracellular signal–regulated kinase (ERK) pathway activation through a PI3K-dependent mechanism (12).

Cotargeting both the MAPK and PI3K/Akt pathways is also potentially advantageous in the radiotherapy setting.
Numerous lines of evidence point to hyperactivation of either of these pathways leading to the development of radioresistance (13–17). These findings have led to the discovery that MEK and Akt inhibitors as single agents possess radiosensitizing properties in a broad spectrum of human tumors (18–20).

Molecularly targeted approaches that enhance the effectiveness of radiation are particularly attractive for the treatment of pancreatic cancer. There are presently few therapeutic options for patients diagnosed with this disease. Approximately 80% of patients are diagnosed with locally advanced or metastatic disease that precludes surgical intervention. Radiation therapy significantly improves local control and is considered a standard of care for patients with locally advanced pancreatic cancer. Thus, strategies aimed at improving radiation efficacy could play a major role in the design of improved therapies for this disease.

We hypothesized that activation of PI3K/Akt signaling would compromise the full potential of MEK inhibitors to sensitize pancreatic cancer cells to the lethal effects of radiation. The purpose of this study was to explore the response of a panel of pancreatic tumor models to MEK inhibition with concurrent radiation treatment. We show here that radiation and MEK inhibition independently upregulate Akt activity and that cotargeting both the MAP kinase and PI3K/Akt pathways results in improved radiosensitization and tumor control both in vitro and in vivo.

Materials and Methods

Antibodies, chemicals, and cell culture

Akt, p-Akt (Ser473), ERK-1/2, p-ERK-1/2 (Thr202/Tyr204), and cleaved PARP (Asp214) antibodies were purchased from Cell Signaling Technology. Ki-67 antibody was purchased from Dako. API-2/triciribine was purchased from Tocris. PD0325901 was purchased from LC Laboratories. The structures for PD0325901 and API-2 are shown in Fig. 1. MIA-PaCa2 [Dulbecco’s Modified Eagle’s Medium (DMEM) and 10% FBS], Panc-1 (RPMI-1640 and 10% FBS), BxPC-3 (RPMI-1640 + 10% FBS), Capan-1 (Iscove’s Modified Dulbecco’s Medium + 20% FBS), Capan-2 (McCoy’s 5a Modified Medium + 10% FBS), AsPC-1 (RPMI-1640 + 10% FBS), and HepG2 (DMEM:Ham’s F-12 + 10% FBS) cells were purchased from American Type Culture Collection, expanded upon receipt and numerous vials of low-passage cells were banked in liquid nitrogen. Cells were never passaged more than 3 months. Cells were grown in a 37°C incubator with 5% CO2.

Immunoblotting

Cell lysates were prepared immediately in radioimmunoprecipitation assay lysis buffer (1% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-HCL pH 7.4, 0.25% Na-deoxycholate, 1 mmol/L EDTA) supplemented with 1× protease inhibitor (cOmplete; Roche Applied Science) and phosphatase inhibitors (PhosSTOP, Roche Applied Science). Protein concentration was determined with a DC Protein Assay Kit (BioRad). Proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Primary antibodies were allowed to bind for 2 hours at room temperature and used at a dilution of 1:500 to 1:2,000, except for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used at 1:10,000. After washing in TBS-Tween, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies diluted 1:10,000 for 1 hour. Membranes were washed with TBS-Tween and incubated for 1 minute with enhanced chemiluminescence reagent (Amersham Pharmacia) before exposing film.

Clonogenic survival assays

Cells were trypsinized to generate single-cell suspensions, and cells were seeded into 6-well or 60-mm tissue culture plates (in triplicate). After allowing 6 hours for adherence, cells were incubated with dimethyl sulfoxide (DMSO), PD0325901 (10 or 100 nmol/L), or various concentrations of API-2 (0.1 to 1 μmol/L) for 1 hour before irradiation. Ten to 14 days after seeding, colonies were stained with 0.5% crystal violet, and the number of colonies containing at least 50 cells was determined. Plating
efficiency, survival fractions, and dose enhancement ratios (DER) were calculated according to previously described methods (21). For each condition, 6 wells were plated in replicate for experiments carried out in a 6-well plate and in triplicate for experiments carried out in 60-mm culture plates. Experiments were repeated multiple, independent times.

**Tumor xenograft studies**

All animal procedures were approved by the University of Michigan Committee for Use and Care of Animals. Four- to 6-week-old athymic CD-1 female mice were obtained from Charles River Laboratories and acclimatized for at least 1 week before use. The mice were injected subcutaneously with 5 × 10^6 MIA-PaCa-2 cells in 100 μL serum-free RPMI per flank. Tumors were allowed to grow to the size of approximately 100 mm³, as measured by MRI, before randomization to groups consisting of treatment with vehicle, PD0325901 (10 mg/kg daily via oral gavage), API-2 (1 mg/kg daily injected intraperitoneally), and/or radiation (2 Gy) for a total of 10 days (days 1–10). PD0325901 was prepared in 0.2% Tween-80 with 0.5% hydroxypropylmethlcellulose in sterile water, whereas API-2 was prepared in 15% DMSO in 0.9% sterile saline. Baseline (pretreatment) MRI scans were conducted on day 0, days 4 and 7 (during treatment), day 11 (1 day after completion of treatment), followed by weekly thereafter (day 18, 25, 32). Mice were weighed on the day of each MRI scan to monitor for toxicity.

**Experimental radiation**

Radiation was carried out at 320 kVp, 10 mA with an IC-320 orthovoltage irradiator (Kimtron Medical). For *in vitro* experiments, a 20 × 24 cm² cone was used at a source-to-surface distance (SSD) of 50 cm at a dose rate of approximately 434 cGy/min. For animal irradiation, a 6 × 8 cm² cone was used at an SSD of 40 cm, at a dose rate of approximately 138 cGy/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 (NIST) calibration. Mice were anesthetized with isoflurane and placed in cardboard restraints. Flank irradiation was carried out with a custom cut lead secondary collimator.

**Xenograft tumor volume**

During MRI examinations, animals were anesthetized with 1% to 2% isoflurane/air, with body temperature maintained at 37°C, using an Air-Therm air heater (World Precision Instruments). MRI scans were conducted immediately before first treatment (day 0), days 4, 7, and 11, and weekly thereafter using a 9.4T, 16-cm horizontal bore Agilent Direct Drive system with a quadrature rat head coil (Doty Scientific, Inc.). Axial T2-weighted images were acquired using a fast spin-echo sequence with the following parameters: repetition time (TR)/effective echo time (TE), 4,000/60 ms; echo spacing, 15 ms; echo train length, 4; field of view (FOV), 30 × 30 mm²; matrix, 256 × 128; slice thickness, 1 mm; and 25 contiguous slices. Tumor regions of interest (ROI) were contoured on T2-weighted images and used for volumetric analysis. Image postprocessing and analysis was conducted using “in-house” program developed in Matlab (MathWorks).

**Immunohistochemistry**

Tumors were harvested and fixed in 10% neutral-buffered formalin for at least 48 hours (n = 4 per group). Tumors were sectioned and paraffin-embedded and 5-micron sections were cut onto slides. Paraffin was removed in xylene and slides were rehydrated through gradually decreasing alcohol concentrations 2-minute per step before ending in tap water (100% ethanol, 95% ethanol, 70% ethanol, water). Antigen retrieval was conducted by microwaving slides for 10 minutes in pH 6.0 citrate buffer, followed by a 10-minute cooling period, and a 10-minute running water wash. Immunoperoxidase staining was carried out on a DAKO AutoStainer at room temperature by applying peroxidase block (5 minutes), buffer rinse, primary antibody (30 minutes), buffer rinse, secondary antibody (EnVision + anti-rabbit) 30 minutes, buffer rinse, 3,3′-diaminobenzidine (DAB) 5 minutes, buffer rinse, followed by hematoxylin counterstain (5 minutes), and water rinse. Slides were then dehydrated through gradually decreasing alcohol concentrations (70% ethanol, 95% ethanol, 100% ethanol, 2 minutes each), 3 xylene washes (2-minute each), and followed by placement of a coverslip. Images were captured on an Olympus BX-51 microscope (×20 magnification).

**Data analysis**

Data are represented as the mean ± SEM for clonogenic survival and xenograft tumor growth experiments. Statistical comparisons were made using the unpaired 2-tailed Student *t* test with *P* values less than 0.05 being judged significant.

**Results**

**PD0325901, a potent MEK inhibitor, radiosensitizes pancreatic cancer cells**

The impact of radiation on MAPK pathway activation was determined in a panel of 6 human pancreatic adenocarcinoma cell lines (MIA-PaCa-2, BxPC-3, PANC-1, AsPC-1, Capan-1, and Capan-2), and a hepatocellular carcinoma cell line (HepG2). A time-dependent increase in p-ERK-1/2 (p-ERK) activity in response to radiation was observed in every model. Representative data for 4 of the cell lines are shown in Fig. 2A. Some cell lines showed activation of ERK-1/2 as early as 2 hours, but all cells showed activation by 24 hours. These effects were also observed at a lower radiation dose of 3 Gy (data not shown).

Clonogenic assays were carried out to test the radiosensitivity of these cell lines under conditions where ERK activation is suppressed by MEK inhibitor treatment. Cells were pretreated with the MEK inhibitor PD0325901...
followed by irradiation in the continued presence of the MEK inhibitor. The concentration of PD0325901 used in these studies (10–100 nmol/L) was previously determined to result in near complete loss of detectable p-ERK activity by 3 hours in all cell lines tested, and as early as 1 hour in the majority of the cell lines studied (Supplemental Fig. S1).

As shown in Fig. 2B, treatment with PD0325901 resulted in significant radiosensitization in multiple pancreatic cancer cell lines, including MIA-PaCa-2 and Capan-2 cell lines, with dose enhancement factors of 1.34 and 1.25, respectively. Because these cell lines are KRAS mutant, we also tested HepG2 cells, an NRAS mutant cell line, to determine whether PD0325901-mediated radiosensitization was dependent on RAS isoform or tissue of origin (i.e., pancreas vs. liver). We again observed significant radiosensitization, at a dose sufficient for target inhibition, with a dose enhancement factor of 1.51. As expected, radiation induced G2–M arrest at 24 hours (Table 1). However, radiation did not induce a substantial increase in the sub-G1 fraction at 48 hours relative to that found in control or PD0325901-treated cells, consistent with the concept that radiation predominantly functions by inducing arrest and postmitotic death. The G1 block observed under conditions of MEK inhibition is consistent with previous reports (22, 23).

Concurrent treatment with PD0325901 and radiation improves therapeutic response in vivo

MIA-PaCa-2 cells were subcutaneously implanted in athymic nude mice and tumors allowed to reach a size of approximately 100 mm³ before mice were randomized to 1 of 4 groups: (i) control (vehicle), (ii) radiotherapy, (iii) PD0325901, and (iv) PD0325901 + radiotherapy. For radiation, 2 Gy per day was chosen as the daily dose, similar to standardly used clinical practice guidelines. Treatments occurred daily for 10 consecutive days (days 1–10). Baseline MRI scans were conducted on days 0, days 4 and 7 (during treatment), day 11 (at end of therapy), and then weekly thereafter (days 18, 25, 32). As shown in Fig. 3, control

Figure 2. Radiation upregulates ERK-1/2 activity, and a MEK-1/2 inhibitor radiosensitizes multiple cell lines. Six pancreatic cancer cell lines were irradiated, including MIA-PaCa-2, BxPC-3, PANC-1, AsPC-1, Capan-1, and Capan-2 (first 4 cell lines shown). p-ERK-1/2 expression was determined by immunoblotting at various time points after 6 Gy of irradiation (0, 2, 6, and 24 hours). Irradiation resulted in increased p-ERK-1/2 in all cell lines, maximal at 24 hours. Total ERK-1/2 levels shown as an equal loading control. A, clonogenic survival assays were conducted by pretreating cells with DMSO or MEKi (100 nmol/L for MIA-PaCa-2, 10 nmol/L for HepG2) for 1 hour before increasing doses of radiation, then changing the medium 24 hours later, and allowing the cell lines to form colonies for more than 1 to 2 weeks. MEKi treatment resulted in substantial radiosensitization in MIA-PaCa-2, Capan-2, and HepG2 cells. Asterisks indicate P value less than 0.05 compared with DMSO-treated cells. Error bars represent SEM.
and monitored closely during therapy administration, had concurrent MEK inhibition and radiation treatment results in single modality arm, these results provide evidence that volume reductions were not observed in the radiotherapy 80% reduction in tumor volume by day 11. Given that greatest therapeutic response with approximately an 24 h 48 h 200 300 400 500 600 700 800 % volume change

<table>
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<th>Table 1. Cell-cycle analysis on pancreatic cancer cell lines</th>
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NOTE: Cell-cycle analysis conducted by treating pancreatic cancer cell lines with combinations of DMSO, MEKi (100 nmol/L), API-2 (8 μmol/L), and radiotherapy (RT, 6 Gy), and determining sub-G₁, G₁, S, and G₂-M phase fractions at 24 and 48 hours.

Radiation and PD0325901 independently upregulate Akt activity

As shown in Fig. 5A, radiation induces a rapid and transient activation of Akt in 5 of 6 pancreatic cancer cell tumors continued to grow at a rapid pace after randomization. Radiotherapy-treated tumors grew initially, but then experienced essentially no change in tumor volume throughout therapy, consistent with induction of growth arrest and postmitotic death. PD0325901-treated tumors experienced rapid regressions during treatment, with the nadir corresponding to an approximately 35% reduction in volume at day 11 and resumed rapid growth immediately after treatment was discontinued. Tumors treated concurrently with PD0325901 and radiotherapy exhibited the greatest therapeutic response with approximately an 80% reduction in tumor volume by day 11. Given that volume reductions were not observed in the radiotherapy single modality arm, these results provide evidence that concurrent MEK inhibition and radiation treatment results in therapeutic sensitization. Mice, weighed twice weekly and monitored closely during therapy administration, had no significant toxicity with only a maximum 6% decline in body weight (Supplementary Fig. S2).

Immunohistochemical staining was carried out on tumors excised after 4 days of treatment. As shown in Fig. 4A, radiation produced marked upregulation of ERK-1/2 activity compared with control tumors. PD0325901 treatment resulted in a profound loss of p-ERK activity, confirming effective target inhibition of MEK. Less than 3% of cells showed any p-ERK expression in both MEK inhibitor–treated groups. Tumors from the combination arm further exhibited a significant decrease in cellularity, consistent with the improved efficacy of this treatment regimen relative to single-agent/modality treatment alone. To analyze the functional impact of reduced p-ERK expression, Ki-67 staining was also carried out. Surprisingly, despite the enhanced reduction in cellular density induced by concurrent radiation and MEK inhibitor treatment, the proliferative index seemed to be comparable for cells treated with the combination versus MEK inhibitor alone (Fig. 4B). This led us to explore whether activation of the PI3K pathway could be compromising overall effectiveness of MEK inhibitor–based radiotherapy regimens.

Figure 3. Combination MEKi and radiation induces maximal decreases in tumor volume, correlating with therapeutic efficacy, in a pancreatic cancer xenograft model. MIA-PaCa-2 cells were injected into the flanks of athymic nude mice, and tumors were allowed to reach a size of approximately 100 mm³ (by serial MRI scans), before randomization to: (i) control (vehicle), (ii) radiotherapy (RT, 2 Gy × 10 days), (iii) MEKi (10 mg/kg once daily × 10 days), and (iv) MEKi + RT (MEKi delivered 2–3 hours before RT). Treatments occurred from days 1 to 10. MRI sequences were obtained on days 0, 4, 7, 11, 18, 25, and weekly thereafter until study endpoint. Curves represent percentage of change in tumor volume during study period by treatment group: (i) control (n = 16 tumors, 8 mice), (ii) RT (n = 16 tumors, 9 mice), (iii) MEKi (n = 12 tumors, 6 mice), (iv) MEKi + RT (n = 14 tumors, 8 mice). Error bars represent SEM, and asterisk represents P < 0.05 compared with the control group.
lines tested beginning within 2 hours after radiation that is maintained for at least 6 hours. By 24 hours after radiation, p-Akt levels have returned to their preirradiation levels. It is interesting to note that Akt activation occurs earlier than ERK activation (Fig. 2A). We also examined the effect of PD0325901 treatment on PI3K/Akt activation. In Fig. 5B, 1 hour of MEK inhibitor treatment produced a significant increase in p-Akt expression. The amount of p-Akt returned to control levels by 6 hours (data not shown). Taken together, treatment of pancreatic cancer cells with either radiation or MEK inhibitor induces activation of Akt, perhaps suggesting that these cells activate prosurvival mechanism(s) in response to cellular damage or stress.

### Dual inhibition of MEK and Akt inhibition promotes apoptosis in multiple pancreatic tumor models

On the basis of the above results, we hypothesized that Akt inhibition could potentially sensitize cells to MEK-1/2 inhibition and radiation. Consequently, a panel of 4 pancreatic tumor cell lines (MIA-PaCa-2, BxPC-3, Capan-1, and AsPC-1) were treated with API-2, a selective Akt inhibitor (24). Treatment with API-2 for 1 hour resulted in greater than 95% reduction in p-Akt levels at doses of 8 μmol/L and higher, which occurred regardless of the presence or absence of PD0325901 (Supplementary Fig. S3). We next treated these pancreatic cancer cell lines with PD0325901 and API-2, either alone or in combination. Twenty-four hours after treatment, we carried out immunoblotting to detect cleaved PARP (a marker of cells undergoing apoptosis). In all but one cell line, combination treatment with PD0325901 and API-2 produced a striking degree of enhanced apoptosis compared with that elicited by either agent alone (Fig. 6A). Flow cytometric assessment of cell viability showed clear evidence that combination therapy resulted in the highest proportion of nonviable cells in the sub-G₁ fraction (Table 1). This result is consistent with the immunoblotting data showing a significant hyperactivation of apoptotic pathways. These data led us to further explore the impact on overall therapeutic effectiveness of cotargeting both of these major signaling pathways in the radiation setting.

**Akt inhibition further improves therapeutic efficacy of radiation administered concurrently with PD0325901**

The same panel of 4 models tested in Fig. 5 was also treated with radiation alone or in combination with PD0325901 and/or API-2. None of the models exhibited a significant increase in cleaved PARP levels in response to radiation treatment (Supplementary Fig. S4). This result is consistent with prior evidence showing that radiotherapy does not induce apoptosis by 24 hours and predominantly exerts antineoplastic effects by inducing growth arrest and postmitotic death.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Radiation upregulates ERK-1/2 activity, which is abrogated by MEKi treatment, and MEKi treatment results in substantially decreased Ki-67 staining within tumors. Immunohistochemical staining on tumors isolated at day 4 of treatment from the 4 groups of mice were stained with p-ERK-1/2 (A) or Ki-67 (B). Three to 4 tumors from 2 mice from each group were fixed, embedded, and stained. Scale (yellow line) is 200 μm.

![Figure 5](https://example.com/fig5.png)

**Figure 5.** Akt is activated by radiation or MEKi. A, immunoblots showing activation of Akt as measured by p-Akt in response to radiation (3 Gy) in a time-dependent fashion in multiple pancreatic cancer cell lines. B, immunoblots showing rapid activation of Akt after 1 hour of MEKi treatment in multiple pancreatic cancer cell lines. For A and B, total Akt is shown as an equal loading control.
Clonogenic assays were then carried out to explore the ability of API-2 to radiosensitize cells. A dose of 1 μmol/L was found to elicit a significant degree of radiosensitization (Fig. 6B). Furthermore, a subeffective dose of API-2 (0.2 μmol/L) when combined with PD0325901 further enhanced the degree of radiosensitization compared with the MEK inhibitor alone (Fig. 6C).

We next tested whether Akt inhibition in vivo would further enhance the tumor inhibitory effects of MEK inhibition and radiation. Mice bearing MIA-PaCa-2 xenografts reached approximately 100 mm³ in size were irradiated after dosing of either PD0325901 or API-2 alone versus coadministration of both agents. API-2 was administered daily for 10 consecutive days at a dose that previously has been shown to be effective in other tumor models (1 mg/kg intraperitoneal daily; ref. 24). However, this dose of API-2 proved to be ineffective at retarding the growth of MIA-PaCa-2 tumors as reflected by a delayed and modest reduction in tumor volume relative to the vehicle-treated controls (Supplementary Fig. S5). In

**Figure 6.** Combination of API-2 and MEKi induces activation of apoptotic pathways, radiosensitizes pancreatic cancer cells, and maximizes therapeutic efficacy in a xenograft model. A, various cell lines were treated with various combinations of API-2 (8 μmol/L) and/or MEKi (100 nmol/L) for 24 hours, lysed, and then subjected to immunoblotting for cleaved PARP (cPARP), as a measure of activation of the apoptotic pathway. GAPDH shown as an equal loading control. B, clonogenic survival assay was conducted by pretreating cells with DMSO or increasing concentrations of API-2 for 1 hour before increasing doses of radiation, then changing the medium 24 hours later, and allowing the cell lines to form colonies for more than 1 to 2 weeks. API-2 treatment resulted in substantial radiosensitization in MIA-PaCa-2 cells at 1 μmol/L, but not at lower concentrations. C, clonogenic survival assay was conducted by pretreating cells with DMSO, MEKi (100 nmol/L), or the combination of MEKi and API-2 (0.2 μmol/L). The addition of a lower, subeffective dose of API-2 resulted in further radiosensitization of MIA-PaCa-2 cells (arrow). D, MIA-PaCa-2 xenograft model experiments were carried out with various combinations of API-2, MEKi, and RT. Percentage of change in tumor volume during study period by treatment group: (i) MEKi (n = 12 tumors, 6 mice), (ii) MEKi + RT (n = 14 tumors, 7 mice), (iii) API-2 + MEKi (n = 9 tumors, 5 mice), (iv) API-2 + MEKi + RT (n = 12 tumors, 6 mice), and (v) RT (n = 16 tumors, 9 mice). The percentage of change in tumor volume during study period by treatment group is shown. Error bars in A and B represent SEM, and asterisks indicate P value less than 0.05 compared with DMSO-treated cells. Error bars in D represent SEM and asterisks represent differences with a P value less than 0.05 when comparing MEKi + RT group to API-2 + MEKi + RT group. All 4 treatment groups in D had P less than 0.05 compared with the control (no treatment) group at all time points.

Clonogenic assays were then carried out to explore the ability of API-2 to radiosensitize cells. A dose of 1 μmol/L was found to elicit a significant degree of radiosensitization (Fig. 6B). Furthermore, a subeffective dose of API-2 (0.2 μmol/L) when combined with PD0325901 further enhanced the degree of radiosensitization compared with the MEK inhibitor alone (Fig. 6C).

We next tested whether Akt inhibition in vivo would further enhance the tumor inhibitory effects of MEK inhibition and radiation. Mice bearing MIA-PaCa-2 xenografts that reached approximately 100 mm³ in size were irradiated after dosing of either PD0325901 or API-2 alone versus coadministration of both agents. API-2 was administered daily for 10 consecutive days at a dose that previously has been shown to be effective in other tumor models (1 mg/kg intraperitoneal daily; ref. 24). However, this dose of API-2 proved to be ineffective at retarding the growth of MIA-PaCa-2 tumors as reflected by a delayed and modest reduction in tumor volume relative to the vehicle-treated controls (Supplementary Fig. S5). In
contrast, API-2 when administered along with PD0325901 and concurrent radiotherapy produced a significant delay in tumor growth (Fig. 6D). The added therapeutic activity of crippling both MEK and Akt became evident after the cessation of treatment (day 18). Statistically significant differences between the PD0325901/radiation and PD0325901/API-2/radiation groups did not occur until day 39 and continued until the end of the study (day 60). As before, there were no remarkable clinical signs of toxicity in any of the groups and weight loss never exceeded 6% (Supplementary Fig. S2).

Discussion

It is well established that KRAS is mutated in more than 90% of pancreatic cancers, and the high frequency of this genetic aberration is virtually unique to pancreatic cancer (25, 26). The high frequency of KRAS mutations in pancreatic cancer makes the RAS/MAPK pathway an attractive target for intervention. The emergence of highly potent and selective small-molecule inhibitors of MEK, a critical downstream player in the RAS/ERK pathway, enables effective pathway suppression to produce meaningful therapeutic activity in a broad spectrum of human tumors (27).

Preclinical data suggest that roughly half of KRAS mutant tumors are susceptible to MEK inhibitor-based therapy and the subset of these tumors most sensitive to MEK inhibition are wild-type for PIK3CA (9). Effective use of MEK inhibitors to treat pancreatic cancer will need to address activation of the PI3K pathway, which tracks with the aggressiveness of this disease. Indeed, activated Akt and PI3K/p110γ overexpression bear importance for pancreatic cancer progression and survival (28–30). Collectively, these findings provide strong impetus to design treatment regimens that block signaling through both the MEK/ERK and PI3K/Akt pathways.

There is a growing body of evidence showing substantial crosstalk between the RAS/ERK and PI3K/Akt pathways, and that compensatory activation of either pathway mediates resistance to inhibition of the other pathway (9–12, 31). Our results show that MEK inhibition activates the PI3K/Akt pathway in multiple pancreatic models. Our findings further show that a combination approach targeting both pathways results in an enhancement of apoptosis and is highly efficacious in MIA-PaCa-2 tumors.

As radiation is an important component of local therapy for locally advanced pancreatic cancer, we have further explored the concept of combining MEK and Akt inhibitors to enhance the effects of radiotherapy. We found that radiation results in time-dependent activation of ERK in vitro and in vivo, and that upstream MEK inhibition results in significant radiosensitization in multiple pancreatic cancer cell lines. Importantly, the radiosensitizing potential of MEK inhibition was confirmed in vivo. Recently, other groups have shown that another MEK inhibitor (AZD6244) also radiosensitizes cancer cell lines with a broad range of histologies (18, 19). Ongoing studies in our laboratory are exploring the mechanistic basis of MEK inhibitor-induced radiosensitization and early results suggest that the mechanism may be related to inability to promote or repair DNA damage. It has also been proposed that a reduction in hypoxia-inducible factor (HIF)-1 signaling under hypoxic conditions occurs in response to MEK inhibition thereby circumventing hypoxia-induced radioresistance (18, 32).

Focusing on pancreatic cancer models, we show that radiation activates both RAS/MAPK and PI3K/Akt signaling, providing a strong rationale for investigating radiotherapy regimens that incorporate agents targeting both pathways. Our subsequent in vitro and in vivo combination studies provide further evidence that this is a viable approach warranting further investigation. Combination of PD0325901 with API-2 and concurrent radiotherapy produced a statistically significant enhancement in radiosensitization in clonogenic survival assays, and in tumor reduction compared with all other treatment arms, and occurred without additional toxicity. We believe that these data argue that ERK-1/2 and Akt activation after radiation serve as survival mechanisms to correct the DNA-damaging effects of radiation. In a similar fashion, radiation activates Akt, and blockade of signaling through Akt with API-2 also radiosensitizes cells. Likewise, there is evidence in the literature that hyperactivation of the RAS/MAPK or Akt pathways makes cells more resistant to the effects of radiation, therefore providing more evidence that these pathways are important for radiation survival, and not a bystander effect of radiation damage. Our unifying hypothesis is that cells have compensatory signaling pathways, which promote resistance not only to the effects of chemotherapy or targeted agents, but also to radiation. Radiation activates both PI3K/Akt and RAS/MAPK pathways, independently promoting cell survival through different pathways. However, there is evidence emerging for considerable crosstalk occurring between the PI3K/Akt and RAS/MAPK pathways, such that blockage of one pathway with a targeted agent results in compensatory activation of the other. We have also shown that this likely occurs in the context of radiation, as the combination of MEK-1/2 and Akt inhibition further radiosensitizes cells beyond MEK-1/2 inhibition alone. Furthermore, the earlier activation of Akt compared with ERK-1/2 activation after radiation may have important implications for the proper sequencing and design of treatments incorporating targeted agents in combination with radiation.

The in vivo studies reported here have relied on the use of subcutaneously implanted xenografts. There are divergent views on the relative values of subcutaneous and orthotopic models in predicting clinical outcome (33). One position is that subcutaneous models when properly used and interpreted are immensely valuable. An example is the encouraging clinical activity seen with MEK inhibitors in BRAF-mutated tumors, an outcome predicted on the basis of subcutaneous models, which further predicted diminished or no activity of these agents...
MEK and Akt Pathway Radiosensitization in Pancreatic Cancer

Our results provide rationale for exploring a regimen combining MEK inhibition and radiation, optimally in conjunction with PI3K/Akt inhibition for the treatment of pancreatic cancer.

Disclosure of Potential Conflicts of Interest
J. Sebolt-Leopold has served as a consultant/advisory board member for Wilex AG.

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References


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