Synergistic Effect between Erlotinib and MEK Inhibitors in KRAS Wild-Type Human Pancreatic Cancer Cells

Caroline H. Diep, Ruben M. Munoz, Ashish Choudhary, Daniel D. Von Hoff, and Haiyong Han

Abstract

Purpose: The combination of erlotinib and gemcitabine has shown a small but statistically significant survival advantage when compared with gemcitabine alone in patients with advanced pancreatic cancer. However, the overall survival rate with the erlotinib and gemcitabine combination is still low. In this study, we sought to identify gene targets that, when inhibited, would enhance the activity of epidermal growth factor receptor (EGFR)-targeted therapies in pancreatic cancer cells.

Experimental Design: A high-throughput RNA interference (RNAi) screen was carried out to identify candidate genes. Selected gene hits were further confirmed and mechanisms of action were further investigated using various assays.

Results: Six gene hits from siRNA screening were confirmed to significantly sensitize BxPC-3 pancreatic cancer cells to erlotinib. One of the hits, mitogen-activated protein kinase (MAPK) 1, was selected for further mechanistic studies. Combination treatments of erlotinib and two MAP kinase kinase (MEK) inhibitors, RDEA119 and AZD6244, showed significant synergistic effect for both combinations (RDEA119–erlotinib and AZD6244–erlotinib) compared with the corresponding single drug treatments in pancreatic cancer cell lines with wild-type KRAS (BxPC-3 and Hs 700T) but not in cell lines with mutant KRAS (MIA PaCa-2 and PANC-1). The enhanced antitumor activity of the combination treatment was further verified in the BxPC-3 and MIA PaCa-2 mouse xenograft model. Examination of the MAPK signaling pathway by Western blotting indicated effective inhibition of the EGFR signaling by the drug combination in KRAS wild-type cells but not in KRAS mutant cells.

Conclusions: Overall, our results suggest that combination therapy of an EGFR and MEK inhibitors may have enhanced efficacy in patients with pancreatic cancer.

Introduction

With a 5-year survival rate of less than 5%, pancreatic cancer remains the most deadly of all major cancer types (1, 2). Gemcitabine has been the standard first line therapy for patients with advanced pancreatic cancer since 1996. Since then, multiple clinical trials combining gemcitabine with other chemotherapeutics have failed to show improvement in overall survival compared with gemcitabine alone (3–5). Until recently in 2005, the FDA (Food and Drug Administration) approved the regimen of gemcitabine and erlotinib (Tarceva), an epidermal growth factor receptor (EGFR) inhibitor, based on increased survival.

The EGFR tyrosine kinase signaling pathway has been implicated in several cellular processes pertinent to cancer progression including cell survival, proliferation, invasion, and metastasis. Dysregulation of EGFR signaling occurs in as much as one-half of all pancreatic cancers (6). Erlotinib is a small molecular weight inhibitor of the EGFR tyrosine kinase that has shown clinical activity in patients with advanced non–small cell lung or pancreatic cancer. Patients with advanced pancreatic cancer treated with the combination of erlotinib and gemcitabine have shown statistical significant survival advantage over patients treated with gemcitabine alone. However, the overall response and survival rate with the erlotinib and gemcitabine combination is still low with the survival rate at one year increased from 17% to 23% with the combination (7).

The RAS–RAF–MAPK (mitogen-activated protein kinase) pathway is one of the downstream effector pathways of the EGFR tyrosine kinase signaling. It is constitutively activated in multiple human tumors due to gain-of-function mutations in RAS or RAF. Of the RAS family, KRAS is more often mutated with approximately 20% of all human tumors possessing an activating mutation in codon 12, 13, and more rarely 61 (8). KRAS mutations are the most prevalent in pancreatic ductal adenocarcinomas (70%–90%; ref. 9–11), followed by colorectal adenocarcinomas (35%) and...
were cultivated in a humidified incubator at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 30% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells were harvested with 0.05% trypsin at 70% to 80% cell density.

Cell line identities were verified by short tandem repeat (STR) profiling (16) using the AmpFISTR Identifiler PCR amplification Kit (Applied Biosystems). This method simultaneously amplifies 15 STR loci and amelogenin in a single tube, using 5 dyes, 6-FAM, JOE, NED, PET, and LIZ which are then separated on a 3100 Genetic Analyzer (Applied Biosystems). GeneMapper ID v3.2 software was used for analysis (Applied Biosystems). AmpFISTR control DNA and the AmpFISTR allelic ladder were run concurrently. Results were compared with published STR sequences from the ATCC. The STR profiling was repeated once a cell line has been passaged more than 6 months after previous STR profiling.

siRNA library screening and hit selection

An RNAi screen using a library of siRNA duplex oligonucleotides targeting 588 known human kinase genes (2 siRNAs per gene; QIAGEN) was carried out to identify sensitizing targets for erlotinib using a reverse transfection protocol as described previously (17). Two nonsilencing siRNAs were used as negative controls, whereas the AllStars Negative Control (Qiagen) was included as a positive control. The siRNAs were first arrayed into 384-well plates for a final assay concentration of 20 nmol/L in duplicates. The arrayed siRNAs were then incubated with 20 μL serum-free RPMI 1640 cell culture media (Invitrogen) containing 0.04 μL siLentfect lipid reagent (Bio-Rad) at room temperature for 30 minutes. Next, BxPC-3 cells were plated to the siRNA-transfection reagent mix at 1,200 cells per well and serum supplemented at a final concentration of 5%. The plates were incubated in a humidified incubator at 37°C for 24 hours. Afterward, a serial dilution of erlotinib (6 concentrations between 0 and 100 μmol/L) was added to the wells and incubated for 96 hours. Cell viability was determined by CellTiter-Glo Luminescent Assay (Promega) and the luminescence was recorded with the Synergy HT Microplate Reader (BioTek).

The percent cell survival of the siRNA and erlotinib combination was normalized to the percent cell survival of corresponding siRNA alone control. The IC50 values for each siRNA and erlotinib treatment were calculated by fitting the data to a sigmoid dose–response model using nonlinear regression with the Matlab Software (2007a; The MathWorks Inc). Each siRNA was evaluated and ranked by the following qualifiers as potential hits: (i) the R2 for the sigmoid fitting curve was greater than 0.9, (ii) the effect on cell viability of the siRNA itself was less than 50%, and (iii) 2-fold or higher decrease in the IC50 value compared with the Neg siRNA control. To further validate the positive gene hits, an additional 2 siRNA sequences (4 total) were obtained for each gene and subjected to a confirmation screen with 8 erlotinib concentrations using the same procedures described above. Genes that have at least 2 out of 4 siRNA sequences showing 2-fold or higher reduction in erlotinib IC50 value compared with the Neg siRNA control were selected as confirmed hits.

Translational Relevance

With a 5-year survival rate of less than 5%, pancreatic cancer is among the most lethal types of human cancers. Current therapies are mostly ineffective and new therapies are desperately needed. This article describes the synergistic effect between erlotinib and MAP kinase kinase (MEK) inhibitors in pancreatic cancer cells and animal models. We show that the combination of erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, and several MEK inhibitors have enhanced efficacy in pancreatic cancer cells with wild-type KRAS. This work is highly translational, as further evaluation of the combination treatment may result in new and improved treatment for patients with pancreatic cancer.

Lung carcinomas (17%; ref. 12). Of the RAF family, mutations only in BRAF have been reported but are mutually exclusive from a KRAS mutation. It is a rare exception that a tumor possesses both KRAS and BRAF mutations (13).

Downstream of RAS and RAF is MEK1/2, which is critical to transmitting signals to extracellular signal–regulated kinase (ERK) and has become an attractive pharmaceutical target in tumors harboring aberrant signaling in the MAPK pathway. AZD6244 (ARRY-142886) and RDEA119 (BAY 869766) are 2 potent MEK1/2 inhibitors that are currently being evaluated in at least 3 studies: a phase I dose-escalation study, a phase I monotherapy in Japanese xenograft models (14). RDEA119 is an allosteric, selective ATP-competitive inhibitor of MEK1/2, which has been reported to inhibit cellular growth and induce apoptosis in vitro and reduce tumor growth in the BxPC-3 and HT-29 xenograft models (15). RDEA119 is an allosteric, selective inhibitor of MEK1/2, which has been reported to inhibit cell proliferation in vitro and reduce tumor growth in various in vivo models (15). Clinically, RDEA119 is currently being evaluated in at least 3 studies: a phase I dose-escalation study, a phase I monotherapy in Japanese patients, and a phase I/II study in combination with sorafenib in advanced cancer patients (http://www.clinicaltrials.gov).

In this study, we employed high-throughput RNA interference (RNAi) screening approach to identify targets that would enhance the activity of erlotinib in pancreatic cancer cells. We determined that the combination of a MAP kinase kinase (MEK) inhibitor and erlotinib has significant anti-tumor activity in a subset of pancreatic cancer cells that harbor wild-type KRAS in in vitro and in vivo models.

Materials and Methods

Cell line culture

The pancreatic cancer cell lines BxPC-3, Hs 700T, MIA PaCa-2, and PANC-1 were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells were cultivated in a humidified incubator at 37°C and 5% CO2.
Verification of siRNA-mediated mRNA and protein expression knockdown using real-time PCR and Western blotting

To evaluate the gene-specific knockdown of mRNA expression by siRNA, cancer cells (250,000 cells per well) were reverse transfected with 20 nmol/L of the MAPK1 siRNAs (Qiagen) for 72 hours in 6-well plates. The cells were then washed with Dulbecco’s PBS (DPBS) twice and harvested with trypsinization. For RNA extraction, the RNaseasy Mini Kit (Qiagen) was utilized following the manufacturer’s recommended protocol. One microgram of total RNA was used in a 20 μL cDNA synthesis reaction (Quanta Biosciences). One microliter of the cDNA reaction mix, 10 μL of FastStart SYBR Green Master mix (Roche), and 4 μL of MAPK1 primer mix (5'-CGACC-3' and 5'-TCAATGCTGTGGACCTTC-3', final concentration 0.4 μmol/L each) were combined. A separate reaction for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was included as a control and the primers used were 5'-ATTGCCCTCAACGACCACTT-3' and 5'-GTCCACACCCTGTGCG-3'. The real-time quantitative PCR was carried out using the MyIQ Real-Time PCR Detection System (Bio-Rad). The cycling program used was 95°C for 10 minutes, 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for cycles. Each sample was run in duplicates, normalized to GAPDH, and analyzed by the comparative C_{T} method (18).

For protein expression, the cells were washed with DPBS twice and subsequently lysed with RIPA (radioimmunoprecipitation assay) buffer (Cell Signaling Technology) supplemented with Complete Mini protease cocktail and PhosSTOP (Roche). The lysates were incubated on ice for 30 minutes prior to centrifugation at 14,000 x g for 10 minutes at 4°C. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce Biotechnology). A total of 30 μg of protein per lane was separated by 10% SDS-PAGE electrophoresis (Invitrogen) and transferred to nitrocellulose membrane blotts. The membranes were blocked for 1 hour with 5% fat-free milk at room temperature and then incubated with a rabbit polyclonal p42 MAP kinase (Erk2) antibody (1:1,000 dilution; Cell Signaling Technology) overnight at 4°C. The blots were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer for 3 times and then exposed to anti-rabbit IgG HRP (horseradish peroxidase)-linked antibody (1:4,000 dilution; Cell Signaling Technology) at room temperature for 1 hour. The protein bands were visualized using the chemiluminescence detection method (Millipore).

Drug combination treatment and immunoblotting analysis

Erlotinib, AZD6244, and RDEA119 were purchased from ChemieF'ek and dissolved in DMSO (dimethyl sulfoxide) to make 50 mmol/L (erlotinib) or 20 mmol/L (AZD6244 and RDEA119) stock solutions. Cells were seeded into 384-well plates at 1,200 cells per well, allowed to grow for 24 hours, and then treated with drugs for another 96 hours. Ten concentrations of AZD6244 and RDEA119 were made in 1:3 serial dilutions in serum-free medium. Three drug concentrations of erlotinib were added in combination to the MEK inhibitors at 1:1 ratio concurrently. At the end of treatment, cell viability was determined by the CellTiter-Glo Assay. The percent cell survival of erlotinib alone at the indicated drug concentration was normalized to the percent cell survival of the respective drug combination. The growth curves were plotted in the GraphPad Prism v5 software and the IC_{50} was determined by the software using the nonlinear regression (curve fit) analysis.

For Western blot analysis, 1 x 10^6 cells were seeded in T25 flasks for overnight growth. The cells were treated with drugs in serum conditions similar to the 384-well assays. The drugs at concentrations indicated in the figure legends were added directly to the medium for incubation at hours indicated. Cell lysates were prepared as described previously. The cell lysates (30 μg per lane, except 15 μg for AKT) were separated on NuPAGE protein precast gels (Invitrogen), transferred to nitrocellulose (Bio-Rad), and then probed with specific antibodies using manufacturer’s recommended dilutions. The antibodies, phospho-MAPK (Thr202/Tyr204; pMAPK), MAPK, phospho-EGFR (Tyr1068; pEGFR), EGFR, phospho-AKT (Ser473; pAKT), AKT, BIM, PARP, cyclin D1, p27, and β-actin were purchased from Cell Signaling Technology.

Apoptosis and cell-cycle analysis

Apoptosis analysis was conducted using the Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences) following the manufacturer’s protocol. Briefly, cells were seeded and treated with the drugs as described previously for the immunoblotting studies. Afterward, the cells were washed twice with DPBS and 1 x 10^6 cells were resuspended in 1 mL of 1 X Annexin V-binding buffer. Cells undergoing apoptotic cell death was analyzed by counting the cells that stained positive for Annexin V/FITC (fluorescein isothiocyanate) and negative for propidium iodide (PI), and late stage of apoptosis, necrosis, or already dead as Annexin V/FITC and PI positive using FACScalibur (BD Biosciences). Differences of the drug combination group compared with the single drug group was confirmed using an independent 2-tailed t test and considered statistically significant when P < 0.05.

For cell-cycle analysis, cells were treated with erlotinib (3 and 12.5 μmol/L) and RDEA119 (100 nmol/L) alone or in combination as described above and harvested by trypsinization. The cells were resuspended and stained with PI (Sigma-Aldrich) in a modified Krishan buffer (19) for 1 hour at 4°C. The PI-stained samples were then analyzed with a FACScan flow cytometer (BD Biosciences Immuno-cytometry systems). Histograms were analyzed for cell-cycle compartments and the percentage of cells at each phase of the cell cycle was calculated using FlowJo (Tree Star, Inc.) analysis software.

In vivo studies

Animal studies were conducted at the Translational Genomics Research Institute Drug Development Services.
Inhibition of MAPK1 expression by siRNA sensitizes pancreatic cancer cells to erlotinib

To identify gene targets that, when inhibited, sensitize pancreatic cancer cells to the treatment of erlotinib, we carried out siRNA screening in the presence of a serial dilution of erlotinib in BxPC-3 cells using a kinase-focused siRNA library (see Materials and Methods). A total of 6 genes were confirmed (Table 1). Of the 6 genes, the siRNA silencing of GCK and MAPK1 (ERK2) had the highest sensitizing effects on erlotinib (6- to 16-fold and 6- to 8-fold reduction in IC_{50} respectively; Supplementary Fig. S1A and B), whereas BMPR2 (bone morphogenetic protein receptor), BRAF, FLT3, and PRKAB2 had moderate effects (ranging from 2- to 7-fold reduction in IC_{50}; Supplementary Fig. S1C–F).

Due to the high sensitizing effect of its siRNAs and its well known involvement in cancer, MAPK1 was selected for further validation. The specific knockdown of MAPK1 expression by the siRNAs was evaluated by quantitative PCR and Western blotting analysis. Two MAPK1 siRNA sequences (MAPK1_1 and MAPK1_2) were able to achieve more than 80% efficiency in reducing mRNA transcript expression (data not shown) and protein expression levels at 72 hours in all 4 cell lines (Fig. 1A and Supplementary Fig. S3). The MAPK1 siRNA and erlotinib treatment in BxPC-3 cells were further repeated with 10 serial dilutions of erlotinib, nontargeting siRNA control (Neg siRNA), and a drug alone control. The MAPK1 siRNA and erlotinib combinations yielded an IC_{50} of a 6-fold difference compared with both controls (Fig. 1B). The siRNA and erlotinib treatment was repeated in additional well-characterized pancreatic cancer cell lines, Hs 700T, Mia PaCa-2, and Panc-1, to determine if the sensitizing effects of MAPK1 siRNA were cell line specific. Of the 3 cell lines, Hs 700T had an IC_{50} fold difference of 2.3 and 1.4 compared with the Neg siRNA control (Supplementary Fig. S2A), whereas Mia PaCa-2 and Panc-1 exhibited no sensitizing effects in either MAPK1 siRNA (Supplementary Fig. S2B and C).

It is interesting to note that BxPC-3 and Hs 700T have previously been reported to have wild-type KRAS, whereas Mia PaCa-2 and Panc-1 harbor a mutation at codon 12 (21). Sequencing of the KRAS open reading frame (ORF) of the 4 cell lines used in this study confirms the mutational status (data not shown). We therefore hypothesized that the sensitizing effect of MAPK1 siRNA might be specific to cells harboring wild-type KRAS.

MEK inhibitors synergize with erlotinib to inhibit the proliferation of KRAS wild-type pancreatic cells

Because MEK proteins phosphorylate and activate MAPK1, we postulated that small molecular weight inhibitors of MEK1/2 might be able to sensitize pancreatic

<table>
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</tr>
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</tr>
<tr>
<td>PRKAB2_6</td>
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<td>5.4</td>
</tr>
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</table>

*Number after underscore indicates the siRNA sequence number designated by QIAGEN.
cancer cells to the treatment of erlotinib. We obtained 2 MEK inhibitors, RDEA119 and AZD6244, and evaluated their synergism with erlotinib in multiple pancreatic cancer cell lines. The synergistic effect of the combination of RDEA119 and erlotinib was evaluated in the same 4 pancreatic cancer cell lines used in siRNA studies. It is noted that BxPC-3 and Hs 700T are moderately sensitive to erlotinib, whereas MIA PaCa-2 and PANC-1 are relatively less sensitive, which is consistent with other studies (Supplementary Fig. S4; ref. 22). As to RDEA119, PANC-1 is the least sensitive with an IC50 of 1.5 μmol/L, BxPC-3 and Hs 700T are moderately sensitive at with IC50 ranging between 300 and 700 nmol/L, and MIA PaCa-2 is exquisitely sensitive with an IC50 of 50 nmol/L (15). Similar sensitivity profiles were observed for AZD6244 in these pancreatic cancer cell lines (data not shown).

When fixed concentrations of erlotinib (12.5, 3.0, or 0.8 μmol/L) were added to a dose–response curve of RDEA119, a significant synergistic effect was observed in BxPC-3 and Hs 700T cells (Fig. 2A and B). As the concentration of erlotinib decreased from 12.5 to 0.8 μmol/L, the level of synergy (left shift of the dose–response curves) decreased as well, indicating that the level of synergy was dose dependent between erlotinib and RDEA119. The synergistic effect is much more dramatic in BxPC-3 cell line (672-fold at 12.5 μmol/L of erlotinib) than in Hs 700T (14-fold at 12.5 μmol/L of erlotinib; Table 2). No significant synergy was observed in MIA PaCa-2 and PANC-1 cells at the same drug concentrations (Fig. 2C and D). Higher concentrations of erlotinib (up to 50 μmol/L) did not show any significant synergy with RDEA119 (data not shown) in these 2 cell lines. Table 2 lists the IC50 values of RDEA119 alone and RDEA119 and erlotinib as well as the fold reduction when compared with RDEA119 alone in the 4 pancreatic cancer cell lines. Other pancreatic cancer cell lines, such as AsPC-1, CFPAC-1, and L3.6pl, are similar in sensitivity to EGFR inhibitors as BxPC-3 and Hs 700T, yet contain mutant KRAS (23, 24). No significant synergy was observed in these KRAS mutant cell lines at the same drug combination concentrations (Supplementary Fig. S5A–C).

Similar to RDEA119, AZD6244 showed a significant synergistic effect with erlotinib in BxPC-3 and Hs 700T (Supplementary Fig. S6A and B). The synergism between AZD6244 and erlotinib is also dose dependent, although it is more apparent in Hs 700T than in BxPC-3. Interestingly, the synergistic effect between AZD6244 and erlotinib is more dramatic in Hs 700T cells than in BxCP-3 cells (64- vs. 15-fold at 12.5 μmol/L of erlotinib; Supplementary Table S1). There was no significant synergy between the 2 drugs in MIA PaCa-2 and PANC-1 cells (Supplementary Fig. S6C and D).

On the basis of our current observations, we hypothesized that the efficacy of the EGFR and MEK inhibitor combination was limited to cells with wild-type KRAS. To determine whether mutant KRAS was the determining factor for the lack of synergy, siRNA targeting the specific KRAS mutation in MIA PaCa-2 and PANC-1 (25) was transfected in combination with the treatment of erlotinib and RDEA119. In MIA PaCa2, the inhibition of mutant KRAS yielded a synergistic effect between RDEA119 and 50 μmol/L of erlotinib (2.4-fold compared with erlotinib and RDEA119; Supplementary Fig. S7A). As the dose of erlotinib was reduced to 25 μmol/L (Supplementary Fig. S7B), the synergistic effect was lost. In PANC-1, sensitivity with the KRAS mutant–specific siRNA and drug combination was restored to by 1.5-fold compared RDEA119 in combination with erlotinib at 50 μmol/L (Supplementary Fig. S7C), but no difference at 25 μmol/L (Supplementary Fig. S7D).

Together, these results indicate that the synergism between erlotinib and the MEK inhibitors is restricted to pancreatic cancer cell lines with wild-type KRAS, further supporting the hypothesis that inhibition of MAPK1 function specifically sensitizes KRAS wild-type pancreatic cancer cells to the treatment of erlotinib.
The erlotinib and MEK inhibitor combination disrupts a negative feedback loop of the EGFR–MAPK signaling pathways

On the basis of the observed synergistic effect of the erlotinib and RDEA119 combination in inhibiting cell proliferation, we assessed the phosphorylation activities within the EGFR–MAPK signaling pathway. The MAPK signaling pathway regulates cell proliferation and survival, and we hypothesized that the synergy observed in the KRAS wild-type cells were, at least in part, due to the increased inhibition of the MAPK signaling. To examine the effect of drug treatment on MAPK signaling, both BxPC-3 and MIA PaCa-2 cells were treated with 12.5 μmol/L of erlotinib, 100 nmol/L of RDEA119, or the combination for a time course of 8, 24, and 48 hours. The erlotinib concentration used in our experiments (12.5 μmol/L) is comparable to the pharmacokinetic variables documented in patients, which showed the trough, peak plasma, and average steady-state plasma concentrations of 9.6, 15.1, and 11.0 μmol/L, respectively (26–28). The 100 nmol/L concentration of the MEK inhibitor is below pharmacokinetic plasma concentrations obtained in recent clinical studies (29, 30).

In BxPC-3, erlotinib significantly decreased the pMAPK level over the time course, whereas in MIA PaCa-2, there was little effect (Fig. 3), which is expected on the basis of their KRAS mutational status. With the treatment of RDEA119 alone, the pMAPK level was effectively suppressed at 8 hours in both BxPC-3 and MIA PaCa-2. As time progressed, the phosphorylation of MAPK was recovered in both cell lines but at a higher level in BxPC-3 to near basal levels by 48 hours. In the combination treatment, BxPC-3 showed sustained ablation of pMAPK activity throughout the time course, whereas MIA PaCa-2 recovered activity similar to RDEA119 alone by 48 hours.

Figure 2. Combination treatment of RDEA119 and erlotinib (E) in pancreatic cancer cells. Three erlotinib concentrations (12.5, 3, and 0.8 μmol/L), each of which was concurrently added on to a serial dilution of RDEA119 in 4 pancreatic cancer cell lines, BxPC-3 (A), Hs 700T (B), MIA PaCa-2 (C), and PANC-1 (D). The drug dose–response curves for the combinations have been normalized to the percent cell viability of corresponding erlotinib alone treatment (denoted as V = % in the legends). Data are means ± SD from 3 independent experiments and each of which had 4 replicates per drug concentration.

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In addition, MEK inhibition led to a marked increase in pEGFR in BxPC-3 cells indicating a feedback between MEK inhibition and EGFR activation (Fig. 3A). This was most apparent at 48 hours of RDEA119 treatment in BxPC-3, when the level of pEGFR dramatically increased compared with basal levels, whereas the total EGFR levels remain the same. This observation was previously also reported in breast and lung cancer cell lines when treated with another MEK inhibitor, AZD6244 (31). The level of pEGFR was not changed in MIA PaCa-2 in any of the treatments. With the increased activation of EGFR by MEK inhibitors, the ratio-

tionale of the combination of erlotinib and RDEA119 is evident in BxPC-3 as it inhibits both the activity from the receptor tyrosine kinase and its signaling cascade to MAPK.

It has been noted in the literature that disruption of the EGFR–MAPK signaling pathway can shift signaling to AKT as a mode to maintain cell survival (32–34). In either of the drug treatments in MIA PaCa-2, pAKT levels did increase when compared with basal levels of the cells only in control, yet the levels were not differentially affected in each respective group as time progressed (Fig. 3B). For BxPC-3, the combination increased pAKT levels at 8 hours of treatment by 20% above the basal control, but as time progressed to 48 hours, the combination reduced pAKT levels by 20% compared with basal control levels. RDEA119 alone increased pAKT in BxPC-3, whereas the erlotinib treatment slightly reduced it (Fig. 3A).

The exquisite sensitivity of MIA PaCa-2 to the MEK inhibitors in our study is comparable to previous reports (35, 36). Results from a recent study reported that sensitivity to MEK inhibition was inversely correlated with the basal level of pAKT (37). In MIA PaCa-2, the basal level of pAKT was comparably lower than BxPC-3 (Fig. 3). Despite its sensitivity, the combination of MEK inhibition and erlotinib was not synergistic in MIA PaCa-2.

Overall, these results showed that the synergy of the drug combination observed in BxPC-3 is likely due to the complete repression of the EGFR–MAPK signaling pathway and reduction of pAKT activity, whereas the lack of synergy in MIA PaCa-2 may be attributed to the residual activity of pMAPK and the increased levels of pAKT.

**Dual EGFR and MEK inhibition cooperatively induces apoptotic cell death**

To further understand the mechanism of the synergism between EGFR and MEK inhibitions, we evaluated the degree of apoptosis with Annexin V/FITC flow cytometric analysis and the expression of BIM and PARP in cells treated with erlotinib, RDEA119, or the combination. As shown in Figure 4A, the percentage of cells that were undergoing apoptosis (Annexin V positive/PI negative) in BxPC-3 were significantly higher in the combination (28%) than the respective single drug treatments (erlotinib, 14% and RDEA119, 12%). The combination of erlotinib and RDEA119 cooperatively induces and enhances apoptotic cell death more than the single agents. The percentage of BxPC-3 cells in late apoptosis or already dead (Annexin V positive/PI positive) was consistent with 26% with erlotinib, 21% with RDEA119, and 41% with combination treatment. Comparing RDEA119 and the combination, the level of apoptosis observed in the combination treatment in MIA PaCa-2 is

<table>
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<th>Cell line</th>
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<td>–</td>
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<td>0.4</td>
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<td>RDEA + erlotinib</td>
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<td>(3.0)</td>
<td></td>
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<tr>
<td></td>
<td>RDEA + erlotinib</td>
<td>24.5</td>
<td>11.0</td>
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<tr>
<td></td>
<td>(0.8)</td>
<td></td>
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<tr>
<td>Hs 700T</td>
<td>RDEA119</td>
<td>684.1</td>
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</tr>
<tr>
<td></td>
<td>RDEA + erlotinib</td>
<td>48.3</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>(12.5)</td>
<td></td>
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<tr>
<td></td>
<td>RDEA + erlotinib</td>
<td>166.8</td>
<td>4.1</td>
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<tr>
<td></td>
<td>(3.0)</td>
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<td>RDEA + erlotinib</td>
<td>459.1</td>
<td>1.5</td>
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<tr>
<td></td>
<td>(0.8)</td>
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<tr>
<td>MIA PaCa-2</td>
<td>RDEA119</td>
<td>53.3</td>
<td>–</td>
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<td>RDEA + erlotinib</td>
<td>107</td>
<td>0.5</td>
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<tr>
<td></td>
<td>(12.5)</td>
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<tr>
<td></td>
<td>RDEA + erlotinib</td>
<td>60.3</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>(3.0)</td>
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<tr>
<td></td>
<td>RDEA + erlotinib</td>
<td>58.5</td>
<td>0.9</td>
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<tr>
<td></td>
<td>(0.8)</td>
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<tr>
<td>PANC-1</td>
<td>RDEA119</td>
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<tr>
<td></td>
<td>RDEA + erlotinib</td>
<td>1,620.6</td>
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<tr>
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<td>(12.5)</td>
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<td>RDEA + erlotinib</td>
<td>1,608.1</td>
<td>0.9</td>
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<tr>
<td></td>
<td>RDEA + erlotinib</td>
<td>1,597.2</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>(0.8)</td>
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*aIC50 was calculated after the RDEA119 dose–response curve was normalized to the effect of the erlotinib only control.*
due to mostly the effect of RDEA119 alone. Erlotinib does not synergistically nor additively enhance apoptosis in the combination in MIA PaCa-2.

It has been reported that MAPK can inhibit the activity of several proapoptotic proteins by promoting its ubiquitination for proteasome-dependent degradation, more notably the BIM protein (38–40). Several studies have reported that BIM is a major target of MAPK-dependent survival signaling, and the upregulation of BIM was required for committed apoptosis (41, 42). As single agents, erlotinib and RDEA119 had different effects on the proapoptotic BIM protein levels (Fig. 4C). In the cell lines treated with the inhibitors, BIMEL was the predominant isofrom expressed. BIME1 expression moderately increased in BxPC-3 and MIA PaCa-2 cells treated with either erlotinib or RDEA119, yet the drug combination induced the dephosphorylation, stabilized, active form at 48 hours more so in BxPC-3 than in MIA PaCa-2. Erlotinib or RDEA119 induced comparable levels of BIM expression and moderate apoptosis, but the combination is necessary to potently inhibit the EGFR–MAPK signaling pathway in BxPC-3 to proapoptotic signaling as seen by the upregulation of BIM expression.

Next, the analysis of cells undergoing apoptosis was evaluated by PARP cleavage. In MIA PaCa-2, there exists a basal level of PARP cleavage in the cells only control, but there was not a significant differential change in PARP cleavage with either drug treatments (Fig. 4C). The basal level of PARP cleavage in MIA PaCa-2 was similar in a previous study examining the in vitro activity of AZD6244 and rapamycin (43). It is of note that the study used a high dose of AZD6244 at 1 μmol/L to induce PARP cleavage in both BxPC-3 and MIA PaCa-2. In this study, we used 100 nmol/L of RDEA119, which increased PARP cleavage slightly in MIA PaCa-2 compared with those in the cells only control, erlotinib, and the combination. In contrast, PARP cleavage was observed only in the drug combination in BxPC-3, which is consistent with the results from the Annexin V/FITC analysis.

Finally, the effects of erlotinib and RDEA119 on the cell-cycle distribution were examined. Consistent with the apoptotic immunoblot profiles, a substantial increase in the sub-G1 population was only observed in BxPC-3 in the combination treatment (Supplementary Table S2). As the concentration of erlotinib was reduced from 12.5 to 3 μmol/L, the differential effect on cell cycle was not significant. In MIA PaCa-2, the sub-G1 population was minimal in all of the drug treatments but did exhibit some cell-cycle arrest with an increase of G0/G1 population (Supplementary Table S3). Accordingly, cyclin D1 levels were downregulated with an increase of p27 (Supplementary Fig. S8).

The combination of erlotinib and RDEA119 shows antitumor efficacy in vivo

The antitumor efficacy of the erlotinib and RDEA119 combination was evaluated in BxPC-3 and MIA PaCa-2 mouse xenografts. Consistent with in vitro observations, a significant effect of tumor growth suppression was seen in BxPC-3 in the combination treatment of erlotinib at 50 mg/kg and RDEA119 at 6 mg/kg with a TGI of 67% when compared with vehicle (P < 0.001), and single drug dose controls of erlotinib had a TGI of 42% (P < 0.05) and for RDEA119 of 52% (P < 0.01) by day 24 (Fig. 5A). Similar antitumor effects were observed for a second dosing regimen (erlotinib at 50 mg/kg and RDEA119 at 12 mg/kg; data not shown). In MIA PaCa-2, no significant effect of tumor growth suppression was observed in the drug combination compared with vehicle (P = 0.18) and RDEA119 (P = 0.2; Fig. 5B).

Because the combination of erlotinib and gemcitabine has shown small yet statistically significant survival advantage over patients treated with gemcitabine alone, we evaluated if the addition of gemcitabine to the erlotinib and RDEA119 combination treatment would enhance tumor growth suppression. The addition of gemcitabine significantly reduced tumor growth in BxPC-3 when compared with the combination treatment (P < 0.01;
Supplementary Fig. S9A). In MIA PaCa-2, gemcitabine did not enhance tumor suppression in addition to the erlotinib and RDEA119 combination treatment ($P = 0.13$; Supplementary Fig. S9B).

The effects of drug treatment did not notably affect the body weight of the mice during the treatment course. All treatment regimens were well tolerated with minimal weight losses (<10%) that are similar to that of vehicle control (Fig. 5C and D and Supplementary Fig. S9C and D).

**Discussion**

There have been multiple investigations of chemotherapeutics that target EGFR and thereby inhibiting the EGFR-MAPK signaling pathway in solid tumors. However, response rates for single agent remain relatively modest unless the EGFR-targeted therapy is combined with other chemotherapeutics or radiation (44). The use of high-throughput siRNA (HT-siRNA) screening is a powerful method in the development of therapeutic compounds,
from target discovery to validation and elucidating mechanisms of action (45). In this study, we presented evidence of how efficacious the HT-siRNA screen was in discovering a rational drug combination to erlotinib in pancreatic cancer cells. Results from the screen identified several gene targets, which when inhibited, would enhance the cytotoxic activity of erlotinib. The targets identified in the screen may also present potential use molecular targets and provide interesting insight into molecular interactions of EGFR signaling inhibition in pancreatic cancer. BMP-2 and its receptors BMPR1 and BMPR2 are overexpressed in pancreatic cancer cells. The aberrant activation of BMP-2 and its receptors have been linked to significantly activate MAPK1 in pancreatic cancer cell lines, and treatment of a MAPK inhibitor blocks the BMP-2 mitogenic effects (46). Recently, in BRAF wild-type cells, RAF inhibitors can increase CRAF activity resulting in an increase of MEK–ERK phosphorylation and enhanced growth (47). The identification of the inhibition of BMPR2 and BRAF as enhancers to erlotinib further highlights how the activation of alternative protein kinases in the EGFR–MAPK pathway enables cancer cells to maintain the oncogenic potential when EGFR is inhibited.

Sunitinib, a multitargeted receptor tyrosine kinase inhibitor, FLT3 among them (48), has been reported to sensitize pancreatic cancer cells to ionizing radiation by attenuating pAKT and pERK levels (49). GCK phosphorylates glucose to provide glucose-6-phosphate for the synthesis of glycogen and preferentially expresses in hepatocytes and pancreatic beta cells. PRKAB2 is a regulatory subunit of AMP-activated protein kinase, which regulates the intercellular metabolism of fatty acids and glycogen. Although both GCK and PRKAB2 have not been studied in the context of pancreatic cancer, both genes have been associated to diabetes (50–52).

The use of MEK inhibitors to perturb the activity of MAPK has been reported to have efficacy in pancreatic models (36, 53). Other groups have reported on the synergistic effect of

Figure 5. Inhibition of tumor growth by erlotinib and RDEA119 as single agents or in combination in the BxPC-3 and MIA PaCa-2 xenograft model. Tumor growth curves for the different treatment groups in BxPC-3 (A) and MIA PaCa-2 (B). Mouse body weight curves for different treatment groups in BxPC-3 (C) and MIA PaCa-2 (D). Arrows indicate the treatment start and end dates.
EGFR and MEK inhibition. Jimeno and colleagues reported on the efficacy of the combined EGFR and MAPK inhibitors in biliary and pancreatic cancer. Their discovery was based on global analysis of gene expression profiles on biliary cancer cell lines that were resistant to EGFR-targeted therapeutics, gefitinib and erlotinib, and then confirmed the activity in vivo in human pancreatic cancer xenografts with minimal mechanistic studies to explain the synergy in their pancreatic cancer model (54). In EGFR-dependent lung cancer cell lines, Balko and colleagues observed synergistic cytotoxicity with the combination of EGFR and MEK inhibitors only in cells with wild-type KRAS (55). Yoon and colleagues recently reported the synergistic effect between gefitinib and AZD6244 in gastric and lung cancer cells (56, 57). The authors found that the combination treatment repressed AKT and ERK activation and consequently enhanced apoptosis.

The occurrence of somatic KRAS mutations is a highly predictive marker of resistance to anti-EGFR chemotherapies. In colorectal cancer, KRAS mutations were associated with resistance to anti-EGFR therapies (cetuximab and panitumumab) in patients with metastatic disease, whereas KRAS wild-type predicted efficacy in terms of tumor response and patient survival (58–60). In non–small cell lung cancer, KRAS mutations are attributed to the poor response to erlotinib and gefitinib (61). In pancreatic cancer, the combination of gemcitabine and erlotinib exhibited modest (6.24 vs. 5.91 months) but statistically significant improvement in overall survival (7). As KRAS mutations are reported so prevalently in pancreatic cancer, our study does raise the issue of the status of KRAS for patients that may benefit from the treatment of erlotinib and a MEK inhibitor.

In summary, we have shown a synergistic effect between erlotinib and 2 clinically relevant MEK inhibitors, RDEA119 and AZD6244, in a subset of KRAS wild-type pancreatic cancer cells in vitro and in vivo. Our results provide a clear biological rationale for the investigation of erlotinib in combination with a MEK inhibitor in KRAS wild-type pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Jill Muehling and Kristen Bisanz for assistance with the STR profiling of the cell lines, Carole Viso and Tammy Brehm-Gibson for cell-cycle analysis, and Paul Gonzales and Mario Sepulveda for animal studies.

Grant Support

This work was supported by funding from the National Foundation for Cancer Research (NFCR) and a National Cancer Institute grant CA109552.

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Received August 17, 2010; revised January 25, 2011; accepted February 27, 2011; published OnlineFirst March 8, 2011.

References


Synergistic Effect between Erlotinib and MEK Inhibitors in KRAS Wild-Type Human Pancreatic Cancer Cells

Caroline H. Diep, Ruben M. Munoz, Ashish Choudhary, et al.

Clin Cancer Res 2011;17:2744-2756. Published OnlineFirst March 8, 2011.