Pharmacologic Inhibition of RAF→MEK→ERK Signaling Elicits Pancreatic Cancer Cell Cycle Arrest Through Induced Expression of p27Kip1

Stephan Gysin,1 Sang-Hyun Lee,1 Nicholas M. Dean,2 and Martin McMahon1

1Cancer Research Institute and Department of Cellular and Molecular Pharmacology, University of California, San Francisco Comprehensive Cancer Center, San Francisco, California and 2Isis Pharmaceuticals, Carlsbad, California

Abstract

Expression of mutationally activated RAS is a feature common to the vast majority of human pancreatic adenocarcinomas. RAS elicits its effects through numerous signaling pathways including the RAF→mitogen-activated protein (MAP)/extracellular signal–regulated kinase (ERK) kinase [MEK]→ERK MAP kinase pathway. To assess the role of this pathway in regulating cell proliferation, we tested the effects of pharmacologic inhibition of MEK on human pancreatic cancer cell lines. In eight cell lines tested, MEK inhibition led to a cessation of cell proliferation accompanied by G0-G1 cell cycle arrest. Concomitant with cell cycle arrest, we observed induced expression of p27Kip1, inhibition of cyclin/cyclin-dependent kinase 2 (cdk2) activity, accumulation of hypophosphorylated pRb, and inhibition of E2F activity. Using both antisense and RNA interference techniques, we assessed the role of p27Kip1 in the observed effects of MEK inhibition on pancreatic cancer cell proliferation. Inhibition of p27Kip1 expression in Mia PaCa-2 cells restored the activity of cyclin/cdk2, phosphorylation of pRb, and E2F activity and partially relieved the effects of U0126 on pancreatic cancer cell cycle arrest. Consistent with the effects of p27Kip1 on cyclin/cdk2 activity, inhibition of CDK2 expression by RNA interference also led to G0-G1 cell cycle arrest. These data suggest that the expression of p27Kip1 is downstream of the RAF→MEK→ERK pathway and that the regulated expression of this protein plays an important role in promoting the proliferation of pancreatic cancer cells. Moreover, these data suggest that pharmacologic inhibition of the RAF→MEK→ERK signaling pathway alone might tend to have a cytostatic, as opposed to a cytotoxic, effect on pancreatic cancer cells. (Cancer Res 2005; 65(11): 4870-80)

Introduction

Adenocarcinoma of the pancreas is the fifth leading cause of cancer death in the United States, claiming the life of ~30,000 patients every year (1, 2). Effective treatment of this disease is hampered by the inability to diagnose it early in its progression and by the capacity of the disease for early invasion and metastasis. In addition, many pancreatic cancers are refractory to standard regimens of radiation and/or chemotherapy (3, 4). Consequently, the median survival time for patients diagnosed with adenocarcinoma of the pancreas is less than 6 months and the 5-year survival rate is less than 4% (5, 6).

Of all human malignancies, pancreatic cancer displays the highest frequency (~70-90%) of somatic activating mutations in RAS genes (mainly KRAS; refs. 7–9). In addition, pancreatic cancers frequently display mutations in the INK4A/ARF (~90%), TP53 (~70%), and DPC4 (~50%) genes. However, it remains largely unclear how specific signaling pathways contribute to the aberrant biochemical and biological properties of the pancreatic cancer cell. Moreover, it remains unclear how best to employ pharmacologic inhibitors of intracellular signaling pathways to improve the response of pancreatic cancers to conventional chemotherapy.

RAS-regulated signaling pathways play an important role in the initiation and progression of this disease. RAS regulates multiple intracellular signaling pathways, of which the best understood is the RAF→mitogen-activated protein (MAP)/extracellular signal–regulated kinase (ERK) kinase [MEK]→ERK MAP kinase pathway. Moreover, recent evidence suggests that this pathway may be activated in other types of cancer (melanoma, ovarian, thyroid, colorectal, and lung) as a direct consequence of somatic point mutations in the Braf gene (9–13). Because of the perceived importance of the RAF→MEK→ERK pathway in the aberrant behavior of cancer cells, it has been the subject of intense scrutiny to understand its fundamental role in cancer cell biology and as a target for therapeutic intervention (14–16). One of the key roles of the RAF→MEK→ERK pathway is the regulation of the cell division cycle (17). In response to growth factor stimulation or oncogene activation, the RAF→MEK→ERK pathway can elicit effects on gene transcription, mRNA translation, or posttranslational effects on the expression/activity of D- and E-type cyclins, cyclin-dependent kinases (cdk), and cdk inhibitors to regulate G0→G1→S phase cell cycle progression (18–20).

A number of pharmacologic agents have been described that inhibit RAF→MEK→ERK signaling in mammalian cells including PD098059, U0126, and CI-1040. These agents inhibit the activity of the protein kinase MEK in a manner that is not substrate competitive (21–25). Hence, the mechanism of action of these agents is distinct from the vast majority of protein kinase inhibitors that are ATP analogues (26). Here we have employed U0126 to explore the role of the RAS-activated RAF→MEK→ERK pathway in the proliferation of human pancreatic cancer–derived cell lines in vitro. Treatment of such cells with U0126 led to inhibition of ERK activity accompanied by a G1 cell cycle arrest, but not apoptosis. U0126-induced cell cycle arrest was invariably accompanied by the induced expression of the cdk inhibitor p27Kip1. Furthermore U0126-induced p27Kip1 expression was often, but not invariably, accompanied by elevated KIP1 mRNA, consistent with the ability of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Martin McMahon, Cancer Research Institute and Department of Cellular and Molecular Pharmacology, University of California, San Francisco Comprehensive Cancer Center, CCRB, 2340 Sutter Street, Box 0128, San Francisco, CA 94115. Phone: 415-502-5829; Fax: 415-502-3179; E-mail: mcmahon@cc.ucsf.edu.

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RAF to suppress KIP1 mRNA expression (20). Using both antisense and RNA interference techniques, we show that p27Kip1 expression is required for the inhibition of cyclin/cdk2 activity, hypophosphorylation of pRb, down-regulation of Erk activity, and for G1 cell cycle arrest that occurs in response to MEK inhibition. Consistent with an important role for cdk2 in promoting G1→S phase progression, RNA interference–mediated inhibition of cdk2 expression also led to a G1 cell cycle arrest. Interestingly, and perhaps surprisingly, we did not detect an effect of MEK inhibition on cdk4 activity. In addition, pharmacologic inhibition of cdk4 elicited apoptosis with only a modest effect on the percentage of cells in S phase. Consequently, these data are consistent with the hypothesis that the RAS-activated RAF→MEK→ERK pathway plays an important role in the regulation of cyclin/cdk2 activity, at least in part, through the regulation of p27Kip1 expression.

Materials and Methods

Cell lines, cell culture conditions, and growth curves. The pancreatic cancer cell lines Mia PaCa-2, Panc-1, CFPAC-1, HPAF II, Capan-2, MPanc-96, Hs766T, and BxPC-3 were generously provided by Drs. Paul Kirschmeier and Chandra Kumar (Scheiring Plough Research Institute, Kenilworth, NJ). Mia PaCa-2, Panc-1, CFPAC-1, HPAF II, Capan-2, and Hs766T were grown in DMEM supplemented with 10% (v/v) fetal serum, penicillin, streptomycin, and glucamin. Mia PaCa-2 was additionally supplemented with 2.5% (v/v) horse serum. MPanc-96 and BxPC-3 were grown in RPMI 1640 including 10% (v/v) fetal bovine serum, penicillin, streptomycin, and glucamin. Growth curves were done by seeding 10^5 cells at day 0 in the presence of 10 μmol/L U0126 (Panc-1 and Capan-2), 15 μmol/L U0126 (Mia PaCa-2, CFPAC-1, and HPAF II), 25 μmol/L U0126 (Hs766T), and 50 μmol/L U0126 (BxPC-3) or DMSO as a vehicle control. The cells were counted in triplicate at days 2, 5, and 6 using a hemocytometer (Neubauer chamber) and viability was estimated with trypan blue staining. A 1 mmol/L stock solution of AG12275 (kind gift of Dr. Osamu Tetsu, Cancer Research Institute, UCSF Comprehensive Cancer Center, San Francisco, CA) was prepared in DMSO and added to cells at a final concentration of 700 μmol/L in accord with the observations of others (27–29).

Protein extraction and Western blot analysis. Cells were lysed in NP40 lysis buffer containing 50 mmol/L HEPES (pH 7.5), 250 mmol/L NaCl, 1% (v/v) NP40, plus protease inhibitors (1 μmol/L phenylmethylsulfonyl fluoride and 10 μmol/L pepticatin) and phosphatase inhibitors (1 mmol/L EGTA, 10 mmol/L NaF, 1 mmol/L tetrasodium pyrophosphate, 100 μmol/L β-glycerophosphate, and 1 mmol/L sodium orthovanadate). The protein concentrations were measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Fifty-microgram aliquots of total protein were resolved by SDS-PAGE and blotted onto nitrocellulose (Bio-Rad, Hercules, CA). The Western blots were incubated with the following primary antibodies: α-phospho-ERK1/2, α-pan-ERK1, α-phospho-Rb, and α-pan-Rb (New England Biolabs, Beverly, MA); α-p27Kip1 (BD Transduction Laboratories, Lexington, KY); α-CDK2 (Upstate, Charlottesville, VA); α-CDK4 (BD Biosciences Clontech, Palo Alto, CA); α-cyclin E (Calbiochem, San Diego, CA); and α-cyclin D1 (BD PharmMingen, San Diego, CA). Antibody-antigen complexes were detected using the appropriate secondary antibody linked to horseradish peroxidase and visualized using the Supersignal chemiluminescent reagent from Pierce.

Propidium iodide staining and BrdUrd analysis. Cells were treated for the indicated times with fresh growth medium in the presence of U0126 or DMSO as a vehicle control. In the case of BrdUrd labeling, cells were treated with BrdUrd at a final concentration of 50 μmol/L for 18 to 30 hours. Cells were fixed and then stained with propidium iodide and/or with a FITC–conjugated anti-BrdUrd antibody (BD PharmMingen). Cell cycle distribution and DNA synthesis were analyzed using a Becton Dickinson FACScan (Becton Dickinson, Franklin Lakes, NJ).

Quantitative reverse transcription-PCR. Extraction of total RNA was done using RNeasy from Qiagen (Valencia, CA). Preparation of cDNA and TaqMan analysis were done as described (30). Sequences of the PCR primers and TaqMan probe were human KIP1 forward, 5′-GGTTAGCCGGG-CAATGCCG-3′; human KIP1 reverse, 5′-TCCACAGAAACGGGTATTGTTG-3′; and TaqMan probe: 5′-FAM-ACCTGAAACCGACTTTCTACT-CAAAAC-TAMRA-3′. FAM is 6-carboxy-fluorescein and TAMRA is 6-carboxy-tetramethyl-rhodamine (Integrated DNA Technologies, Skokie, IL). hGUS levels were used as an internal control. Relative expression levels were calculated as detailed previously (30).

Inhibition of p27Kip1 or cdk2 expression by antisense or RNA interference. Sequence of antisense oligos and their transfection was done as described elsewhere (31). The antisense oligonucleotide solution was prepared by mixing 20 μL Lipofectin (final concentration, 5 μg/mL; Invitrogen Life Technologies, Carlsbad, CA) with 8 μL antisense or mismatch oligonucleotides (final concentration, 200 μmol/L) and 4 mL of serum-free DMEM. This mixture was added to the cells. Twenty-four hours after transfection, the medium was changed and U0126 or DMSO was added for a further 24 hours.

For the anti-KIP1 RNA interference experiments, we designed three siRNAs and their complementary sequences: wild-type, 5′-GAUCGAGUGG-CAGAGGGUGGdTdT-3′; 4 bp mismatch, 5′-CUACGALUUGLCAGAGG-GUGGdTdT-3′ (mismatches underlined); and 6 bp mismatch, 5′-UUAAGUGAGCUAGGCGGGAGdTdT-3′ (mismatches underlined).

For the anti-CDK2 RNA interference experiments, we employed commercially available specific [wild-type (wt)] and control (mismatch) siRNAs (Qiagen) that have been previously described (27).

Cells were transfected with siRNAs in much the same way as for the antisense experiments. We used 30 μL Oligofectamine per 0.4 nmol siRNA (final concentration, 100 nmol/L). Twenty-four hours after the transfection, we replaced the medium with normal growth medium and added U0126 for a further 39 to 48 hours. The cells were labeled with BrdUrd (final concentration, 50 μmol/L) for the last 18 to 30 hours of the experiment. Subsequently the cells were prepared for protein extraction and fluorescence-activated cell sorting (FACS) analysis.

Cyclin-dependent kinase assays. Immune complex cyclin/cdk2 kinase assays were done as previously described (32). Cells were lysed in NP40 lysis buffer and lysates were precleared with protein A-Sepharose (Sigma-Aldrich, St. Louis, MO). Cdk2 was immunoprecipitated with a rabbit polyclonal antibody (Upstate). Immunoprecipitates were washed twice with NP40 lysis buffer and twice with the CDK2 kinase assay buffer [50 mmol/L Tris (pH 7.4), 10 mmol/L MgCl2, and 1 mmol/L DTT]. The reaction was carried out at 30°C for 30 minutes in 50 μL of kinase assay buffer containing 3 μg of histone H1, 10 μg [γ-32P]ATP, and 10 μmol/L ATP. The reactions were stopped by adding 5× SDS sample buffer and by boiling for 7 minutes. The samples were then loaded on a 4% to 20% gradient polyacrylamide gel and analyzed as above. Cdk4- or cyclin D1–associated kinase assays were done as previously described using a gluthathione S-transferase fusion protein (GST-Rb) that contains a carboxyl-terminal fragment of pRB (33–35). The specificity of these assays for GST-Rb phosphorylation was determined by the use of nonimmune serum. Antisera against cdk4 or cyclin D1 and GST-Rb substrate protein were a kind gift from Drs. David Parry and Emma Lees (DNAX Research Institute, Palo Alto, CA).

E2F luciferase reporter assay. In a manner similar to that described for transfection of antisense oligonucleotides, cells were transfected with 3 μg of an E2F reporter plasmid (pE2F-TA-Luc, Clontech) along with KIP1 antisense or mismatch oligos (final concentration, 200 μmol/L) in the absence or presence of U0126. Cells were harvested for protein extraction and luciferase assays (Promega, Madison, WI). Luciferase activity was quantitated using a luminometer. Equal amounts of proteins were determined with the BCA protein assay kit (Pierce).

Results

Pancreatic cancer cell cycle arrest following pharmacologic inhibition of MEK. In this study we investigated the effects of inhibition of the RAS-activated RAF→MEK→ERK MAP kinase pathway on the proliferation of human pancreatic cancer cell lines. To do so, we used the pharmacologic inhibitor U0126 that inhibits the ability of MEK1/2 to activate ERK1/2 (23, 36).
U0126 has been widely used as a tool to explore the role of the RAF→MEK→ERK signaling pathway in a variety of cellular processes (23).

Cell lines used in this study included Mia PaCa-2, Panc-1, CFPAC-1, HPAF II, Capan-2, MPanc-96, Hs766T, and BxPC-3 (37). All of these cells express a mutationally activated form of KRAS except for BxPC-3. As a first step, we treated all eight cell lines with varying concentrations of U0126 for 24 or 48 hours to determine the minimum concentration of inhibitor required for inhibition of ERK1/2 phosphorylation (data not shown). The range of concentrations effective in the inhibition of RAF→MEK→ERK signaling was 10 μmol/L (Panc-1 and Capan-2 cells) to 25 μmol/L (HPAF II, Hs766T, and BxPC-3 cells; Fig. 2 and Materials and Methods). We then measured the effects of U0126 on the proliferation of each of the pancreatic cancer cell lines over a course of 6 days (Fig. 1). U0126 treatment of all eight pancreatic cancer cell lines led to a striking inhibition of cell proliferation without a significant reduction in cell viability. To determine if U0126 was influencing progression through the cell division cycle or promoting apoptosis, we analyzed cell cycle distribution in all eight cell lines in the absence or presence of U0126 (48 hours) by propidium iodide staining followed by FACScan analysis. These experiments indicated that U0126 treatment led to an increase in the G0-G1 population and a decrease of cells in S phase, but there was no significant increase in cells with a sub-G1 DNA content (not shown). These data suggest that pharmacologic inhibition of the RAF→MEK→ERK pathway in pancreatic cancer cell lines elicits cell cycle arrest without a significant short-term increase in apoptosis (Fig. 1).

**Figure 1.** Inhibition of pancreatic cancer cell proliferation by U0126. Pancreatic cancer cells (~10^5 cells/well) were seeded in six-well dishes and then either treated with DMSO as a solvent control (○) or with U0126 (●). Cells were counted in the presence of trypsin blue in triplicate on days 2, 5, and 6 using a hemocytometer. Culture media containing DMSO or U0126 was changed every second day. Cell cycle distribution was determined by propidium iodide staining and subsequent FACS analysis. For the propidium iodide analysis the cells were treated for 48 hours with DMSO or U0126. The concentrations of U0126 used for each individual cell line were determined by Western blotting for the effects on phospho-ERK1/2 (Materials and Methods and Fig. 2).
In addition to inhibiting MEK1/2, U0126 is also reported to inhibit the MEK5—ERK5 pathway (38–40). However, we did not observe any effects of U0126 on ERK5 phosphorylation in the pancreatic cancer cells used in this study. Furthermore, obtained using U0126 were confirmed using Cl-1040 (PD184352), a structurally unrelated, nonsubstrate competitive MEK inhibitor that does not inhibit MEK5—ERK5 signal transmission (refs. 25, 38–40; data not shown). These data suggest that the effects of U0126 on pancreatic cancer cell proliferation are most likely mediated through inhibition of RAF—MEK—ERK signaling.

**Induced expression of p27<sup>KIP1</sup> following MEK inhibition.**

Next we assessed the effects of U0126 on the expression and/or activity of a panel of key components of the cell cycle machinery. Treatment of all eight cell lines with U0126 led to decreased ERK1/2 activity with no overall alteration in ERK1/2 expression (Fig. 2). In screening for effects on the expression of cdk inhibitors, we could rule out certain candidates because pancreatic cancer cells fail to express p16<sup>INK4A</sup> due either to gene mutations or epigenetic silencing by DNA methylation (41–43). Although we saw modest effects of U0126 on cyclin D1 expression in two of eight cell lines, the most consistent change that we observed in eight of eight pancreatic cancer cell lines tested was elevated p27<sup>KIP1</sup> expression (Fig. 2). Other cdk inhibitors were either not expressed in the pancreatic cancer cell lines or their expression was not influenced by U0126 (data not shown). However, we detected a modest increase in p18<sup>INK4C</sup> expression in MIA PaCa-2 cells, but only at very late times after U0126 treatment. p21<sup>CIP1</sup>, a known RAF—MEK—ERK target gene, was only weakly expressed in HPAF II, Hs766T, and BxPC-3, and its expression was not altered by U0126 (27, 33). Expression of p15<sup>INK4B</sup>, p19<sup>INK4D</sup>, or p57<sup>Kip2</sup> was not detectable by Western blotting in any cell line (data not shown).

Because p27<sup>KIP1</sup> plays an important role in G<sub>0</sub>—G<sub>1</sub>—S phase progression, we focused on the mechanism of its regulation and its role downstream of the RAF—MEK—ERK pathway in pancreatic cancer cell proliferation (20). To determine the effects of MEK inhibition on p27<sup>KIP1</sup> expression, we isolated both total RNA and protein in parallel from MIA PaCa-2 cells that were either solvent treated (DMSO) or treated for different periods of time with U0126 (Fig. 3). The expression of <i>KIP1</i> mRNA was assessed by TaqMan real-time PCR analysis and by RNase protection (data not shown). Expression of p27<sup>KIP1</sup> was assessed by Western blotting. MEK inhibition led to an increase of <i>KIP1</i> mRNA at all times analyzed. By contrast, the effects of U0126 on p27<sup>KIP1</sup> expression required 4 to 8 hours to be manifest. These data suggest that, at least in MIA PaCa-2 cells, inhibition of MEK leads to elevated expression of <i>KIP1</i> mRNA, which in turn promotes p27<sup>KIP1</sup> expression. However, it is also likely that the full effects of MEK inhibition on p27<sup>KIP1</sup> abundance may also involve alterations in protein half-life. TaqMan and Western blotsing analysis of the other seven pancreatic cancer cell lines revealed that in some cells (Panc-1, HPAF II, Capan-2, and BxPC-3) U0126 treatment led to increased <i>KIP1</i> mRNA and p27<sup>KIP1</sup> expression, whereas in other cases (CFPAC-1, MPanc-96, and Hs766T) p27<sup>KIP1</sup> was induced in the absence of detectable alterations in <i>KIP1</i> mRNA expression (Fig. 3B). These data suggest that p27<sup>KIP1</sup> expression may be regulated by the RAF—MEK—ERK pathway in at least two distinct ways: regulation of mRNA abundance and regulation of protein stability.

MEK inhibition leads to pRB hyperphosphorylation, loss of E2F activity, and decreased cyclin/cdk2 but not cyclin/cdk4 activity. U0126-induced expression of p27<sup>KIP1</sup> would be predicted to lead to inhibition of cyclin/cdk2 complexes leading to G<sub>0</sub>-G<sub>1</sub> cell cycle arrest. To test this hypothesis, asynchronously growing MIA PaCa-2 cells were treated for 4, 8, 14, 16, or 24 hours either with U0126 or DMSO (Fig. 4A). A fraction of the cells was analyzed for their stage in the cell cycle by propidium iodide staining (Fig. 4A) and a second fraction was processed for analysis by Western blotting (Fig. 4B). FACS analysis of propidium iodide–stained cells revealed that the onset of U0126-induced cell cycle arrest occurred between 8 and 14 hours after drug addition and cells continued to accumulate in G<sub>1</sub> across the time course of the experiment. As previously observed, U0126 treatment of MIA PaCa-2 cells led to a striking reduction of ERK1/2 phosphorylation (Fig. 4B). Induced expression of p27<sup>KIP1</sup> was detected 4 to 8 hours after U0126 addition and was sustained up to 24 hours. U0126 had little, if any, effect on the overall expression of cyclin E or cdk2; however, we detected a modest reduction of cyclin D1 and cdk4 expression and a shift of cdk2 to a more hypophosphorylated state at late times (24 hours) after U0126 addition. Interestingly, analysis of pRB revealed the presence of hypophosphorylated forms of the protein as early as 4 hours after U0126 addition and apparently before induction of p27<sup>KIP1</sup>. Therefore, there may be an as yet unidentified early event that precedes the induction of p27<sup>KIP1</sup> that may participate in the initial onset of the cell cycle arrest.

We also measured the protein kinase activity of cyclin/cdk2 using an in vitro kinase assay (Fig. 4C). Cdk2 was immunoprecipitated from the same set of extracts as described above and its catalytic activity was assessed using histone H1 as a substrate. We detected a modest decrease in cyclin/cdk2 kinase activity as early as 4 hours after U0126 addition at a time before p27<sup>KIP1</sup> induction and incorporation into cyclin/cdk2 complexes. However, 24 hours after U0126 addition, cyclin/cdk2 activity was significantly inhibited at a time when the association with p27<sup>KIP1</sup> was maximal. These data are consistent with an early effect of U0126 on cdk2 activity that is sustained by the subsequent induction of p27<sup>KIP1</sup> expression.

![Figure 2](https://example.com/figure2.png)
DMSO-treated cells. Total p27 Kip1 expression and ERK1/2 expression were assessed by Western blotting with appropriate antisera.

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Figure 3. Induction of KIP1 mRNA and p27Kip1 in U0126-treated pancreatic cancer cells. A, MIA PaCa-2 cells were treated for the indicated times with medium containing either DMSO (−) or 15 μmol/L U0126 (+). Total cellular RNA and protein were extracted in parallel from the same dish of cells. KIP1 mRNA (A) was assayed by TaqMan real-time PCR (Materials and Methods). The histogram indicates the abundance of KIP1 mRNA in U0126-treated cells relative to the level of KIP1 mRNA expressed in asynchronously growing DMSO-untreated MIA PaCa-2 cells that was arbitrarily set at 1. Activated P-ERK1/2, total ERK1/2, and p27Kip1 expressions were detected by Western blotting with appropriate antisera. B, total RNA was isolated from the various pancreatic cancer cell lines that were treated for 24 hours either with DMSO as a control or U0126. Total RNA and protein were isolated from the same dish of cells. The histogram shows the fold induction of KIP1 mRNA measured by TaqMan real-time PCR analysis. The fold induction was calculated by taking the ratio of KIP1 mRNA abundance in the U0126-treated cells relative to the DMSO-treated cells. Total p27Kip1 expression and ERK1/2 expression were assessed by Western blotting with appropriate antisera.

In separate experiments, we assessed the effects of U0126 on cyclin D/cdk4 activity (Supplementary Fig. S1). Cell extracts from MIA PaCa-2 cells treated with U0126 for 4, 14, or 24 hours were subjected to immunoprecipitation with antisera specific for either cdk4 or cyclin D1. Cdk4- or cyclin D1–associated kinase activity was assessed using GST-Rb as a substrate as described previously (33–35). Perhaps surprisingly, U0126 treatment had no effect on cdk4 activity and had, at best, only a modest inhibitory effect on cyclin D1–associated kinase activity. Moreover, despite reports that p27Kip1 can associate with cyclin D/cdk complexes (44, 45), we did not detect p27Kip1 in immunoprecipitates of cdk4 or cyclin D1 (data not shown). These data suggest that pharmacologic inhibition of RAF→MEK→ERK signaling, at least in Mia PaCa-2 cells, has little or no direct effect on cyclin D/cdk4 activity.

Antisense and RNA interference techniques reveal a role of p27Kip1 in the cell cycle inhibitory effects following MEK inhibition. Experiments described above suggest that p27Kip1 may be required for the maintenance of U0126-induced pancreatic cancer cell cycle arrest. To address this, we employed a combination of antisense and RNA interference techniques to address the role of p27Kip1 expression in U0126-induced cell cycle arrest. Mia PaCa-2 cells were transfected with either a KIP1-specific 20-mer antisense oligonucleotide or an 8 bp mismatch control oligonucleotide 24 hours before the addition of U0126 as previously described (46, 47). Transfection of oligonucleotides had no effect on the inhibition of ERK1/2 phosphorylation observed in response to U0126 (Fig. 5A). The mismatch oligonucleotide had little or no effect on p27Kip1 expression whereas the specific oligonucleotide (antisense) led to a striking reduction in p27Kip1 expression in cells treated with U0126. Using the same cell lysates, we assessed the activity of cyclin/cdk2 complexes. The specific oligonucleotide prevented the U0126-mediated inhibition of cdk2 activity whereas the mismatch was largely without effect. The activity of cdk2 in these assays inversely correlated with the presence of p27Kip1 in the cyclin/cdk2 complexes. Western blot analysis of cdk2 immunorecipients revealed the presence of cyclin E regardless of whether the cdk2 was active or inactive. These data suggest that U0126-induced p27Kip1 expression occurs at the right time to play a role in the inhibition of cdk2 that is observed following inhibition of RAF→MEK→ERK signaling. To address the role of p27Kip1 in the inhibition of cyclin/cdk2 activity in intact cells, we assessed the effects of abrogating p27Kip1 expression on the phosphorylation of pRb using phospho-specific anti-pRb antibodies. It is well established that the tumor suppressor pRb is a target of cyclin/cdk activity in cells leading to the phosphorylation of specific sites in pRb. Asynchronously growing Mia PaCa-2 cells display readily detectable phosphorylation of pRb on S780, S795, and S807/811. Addition of U0126 led to a striking reduction of pRb phosphorylation at each of these sites. In addition, there was a generalized reduction of pRb expression that often occurs in growth-arrested cells. Treatment of the cells with the mismatch oligonucleotide had no effect on either U0126-induced pRb hypophosphorylation or overall expression of the protein. By contrast, inhibition of p27Kip1 expression using the specific oligonucleotide prevented pRb hypophosphorylation and overall reduction of pRb expression. Hence, inhibition of p27Kip1 expression led to reactivation of cyclin/cdk complexes in intact Mia PaCa-2 cells and prevented the dephosphorylation of pRb.

As an additional test of the requirement of p27Kip1 in the regulation of pRb, we assessed the effects of U0126 on E2F activity using a luciferase reporter assay (Fig. 5B). Mia PaCa-2 cells were cotransfected with either the mismatch or antisense oligonucleotide in combination with a plasmid encoding firefly luciferase under the control of a promoter containing four E2F consensus binding sites. Cells were then treated with either U0126 or DMSO.
Control cells transfected with the mismatch oligo displayed readily detectable E2F activity that was inhibited by U0126. By contrast, inhibition of p27Kip1 expression with the specific antisense oligonucleotide restored E2F activity to the same level observed in control cells. Western blot analysis of this experiment showed that p27 Kip1 expression was induced in U0126-treated cells, unaffected by the mismatch, and strongly inhibited by the specific oligonucleotide. These data are consistent with the hypothesis that inhibition of RAF → MEK → ERK signaling leads to induced p27Kip1 expression that in turn inhibits cyclin/cdk2 activity, leading to hypophosphorylation of pRb and suppression of E2F activity, which results in G1 cell cycle arrest.

We next attempted to employ the specific antisense and mismatch oligonucleotides to test the requirement for p27Kip1 in U0126-induced cell cycle arrest. However, due to our inability to obtain consistent biological data with antisense oligonucleotides, we decided to use an RNA interference approach as an alternative to the antisense experiments (48). We designed three different siRNAs for this experiment: (a) a perfect match between the siRNA and the sequence of KIP1; (b) a similar siRNA as a but containing a 4 bp mismatch; and (c) a similar siRNA as a but containing a 6 bp mismatch. These siRNAs were transfected into MIA PaCa-2 (Fig. 5C) as double-stranded RNAs (Materials and Methods). The cells were then treated with U0126, and then harvested 48 hours later for analysis of protein expression/activity.

To assess the biochemical effects of the various siRNAs on components of the cell cycle machinery, we assessed the expression, activity, and phosphorylation status of a number of proteins in MIA PaCa-2 cells (Fig. 5C). As expected, anti-KIP1 siRNA, at least partly, restored the activity of cyclin/cdk2 complexes and led to phosphorylation of pRb. The partial restoration of cyclin/cdk2 activity by the perfect match siRNA may reflect modest levels of residual p27Kip1 present in cyclin/cdk2 complexes but may also be indicative of additional inhibitory effects of U0126 on cyclin/cdk2 activity, as suggested in Fig. 4. Consistent with its effects on p27Kip1 expression, the 4 bp mismatch siRNA elicited a modest increase in cyclin/cdk2 activity and a similarly modest increase in pRb phosphorylation. The 6 bp mismatch had largely no effect on cyclin/cdk2 activity or pRb phosphorylation.

To test the role of p27Kip1 on U0126-induced cell cycle arrest, either solvent- or U0126-treated MIA PaCa-2 cells were transfected with the various siRNAs described above. After 39 hours of U0126 treatment, cell proliferation was assessed by BrdUrd labeling combined with propidium iodide staining (Fig. 6A). In parallel with
Figure 5. Restoration of cyclin/cdk2 activity and pRb phosphorylation by antisense and siRNAs oligonucleotides against KIP1. A, MIA PaCa-2 cells were transfected with either a specific anti-KIP1 antisense oligonucleotide or a mismatch control before the addition of either DMSO (− con) or U0126 (+ con) for a further 24 hours. Cell extracts were prepared and the phosphorylation of ERK1/2 and the total expression of p27Kip1 were assessed by Western blotting. Cyclin/cdk2 kinase activity was assessed using histone H1 as a substrate. The abundance of coprecipitating cyclin E, p27Kip1, and cdk2 in each immunoprecipitate was assessed by Western blotting with the appropriate antisera as indicated. Western blotting of total cell extracts with phospho-specific α-pRb antisera was used to assess the phosphorylation of pRB on S780, S795, and S807/811 as indicated. The expression of total pRb and ERK1/2 was also assessed by Western blotting. B, MIA PaCa-2 cells were cotransfected with either a specific α-KIP1 antisense oligonucleotide or a mismatch control along with a plasmid (pE2F-TA-Luc, Clontech) carrying the luciferase gene under the control of 4 E2F binding sites. DMSO or U0126 was added for the last 39 hours before preparation of cell extracts. Luciferase reporter activity in equal aliquots of protein was measured using a luminometer. The expression of p27Kip1 and ERK1/2 in the same cell extracts was assessed by Western blotting. C, 50% confluent MIA PaCa-2 cells were transfected with α-KIP1 siRNA duplex oligonucleotides that were either a perfect match (wt), a 4 bp mismatch (4bp), or a 6 bp mismatch (6bp). Twenty-four hours after transfection, the medium was replaced with medium containing DMSO or 15 μmol/L U0126 and cells were incubated for a further 39 hours. Cell extracts were prepared and the total expression of p27Kip1 was assessed by Western blotting. Cyclin/cdk2 kinase activity was assessed using histone H1 as a substrate. The abundance of coprecipitating p27Kip1 and CDK2 in each immunoprecipitate was assessed by Western blotting with the appropriate antisera as indicated. Western blotting with phospho-specific α-pRb antisera was used to assess the phosphorylation of pRB on S780, S795, and S807/811 as indicated. The expression of total pRb was also assessed by Western blotting.
the cell biological analysis, cell extracts were also prepared for analysis by Western blotting. In MIA PaCa-2 cells the addition of the various siRNAs had no effect on the inhibition of ERK1/2 phosphorylation following U0126 addition. Addition of the perfect match siRNA led to a striking inhibition of p27Kip1 expression. Addition of the 4 bp mismatch siRNA also led to a 25% to 50% reduction of U0126-induced p27Kip1 expression in MIA PaCa-2 cells whereas addition of the 6 bp mismatch had no effect. Analysis of cell cycle progression either by BrdUrd labeling or by propidium iodide staining suggested that the perfect match siRNA reduced the percentage of U0126-treated cells that were arrested in G1 phase but an increase in the percentage of cells that were transiting through S phase. The 4 bp mismatch anti-p27Kip1 siRNA, which had an intermediate effect on p27Kip1 expression, also had an intermediate effect on cell cycle progression in MIA PaCa-2 cells. The use of anti-p27Kip1 siRNA revealed that p27Kip1 plays an important role in the maintenance of cell cycle arrest after inhibition of RAF→MEK→ERK signaling by U0126.

In addition to the experiments described above, we also tested the ability of anti-p27Kip1 siRNA to reverse the effects of U0126 in other pancreatic cancer cell lines (Supplementary Figs. S2-S4). Some of the cell lines tested were poorly transfectable such that siRNA effects could not be assessed. However, in Panc-1 cells we observed a similar restoration of proliferation as in MIA PaCa-2 cells (Supplementary Fig. S2). Hs766T showed an intermediate effect: a reduction in G1 but an accumulation in G2-M in cells treated with siRNA (Supplementary Fig. S3). In CFPAC-1 cells we did not observe any effect of anti-p27Kip1 siRNA on the observed growth arrest induced by U0126 (Supplementary Fig. S4). These data are summarized in Table 1. Interestingly, analysis of the effects of anti-p27Kip1 siRNA on U0126-treated Hs766T or CFPAC cells revealed, at best, only modest effects on phospho-RB status, consistent with the relative inefficiency of anti-p27Kip1 siRNA to reverse the growth inhibitory effects of U0126 on these cells (Supplementary Fig. S5).

Essential requirement for cdk2 in the proliferation of MIA PaCa-2 pancreatic cancer cells. Recent work from a number of laboratories has challenged the conventional view of the importance of cyclin E/cdk2 activity as an essential rate-limiting step in the progression of both normal and cancer cells into S phase (27, 49, 50). Hence, to address a potential role for cdk2 in pancreatic cancer cell line proliferation, we compared the effects of U0126 with the effects of an anti-cdk2 siRNA on MIA PaCa-2 cells. First, we confirmed that the anti-cdk2 siRNA (wt) silenced the expression of cdk2 whereas a mismatch control was without effect.
(Fig. 6B). Treatment of MIA PaCa-2 cells with either U0126 or anti-CDK2 siRNA led to a striking reduction in S-phase progression when measured either by BrdUrd incorporation or propidium iodide staining whereas the mismatch siRNA had no such effect. These data are consistent with the hypothesis that inhibition of cdk2 either by U0126-induced p27Kip1 expression or by direct blockade of cdk2 expression can elicit G0-G1 cell cycle arrest in MIA PaCa-2 cells.

To address a possible role for cdk4 in MIA PaCa-2 cell proliferation, cells were treated for 4 to 24 hours with AG12275 (700 nmol/L), a pharmacologic inhibitor that is reported to be specific and selective for MEK1/2 over MEK5 (25, 38). Although there was a modest reduction of cells in S phase 14 hours after AG12275 addition, the effects were less dramatic than those observed with MEK inhibition (Supplementary Fig. S6). Strikingly, 24 hours after AG12275 addition, there was a very significant accumulation of cells with a sub-G1 DNA content. These observations stand in contrast to that obtained in response to inhibition of MEK activity or Cdk2 expression and suggest that inhibition of cdk4 activity may predispose Mia PaCa-2 cells to apoptosis.

**Discussion**

The RAS-activated RAF→MEK→ERK pathway has been implicated in a wide variety of processes in the cancer cell including the regulation of cell mortality, apoptosis, angiogenesis, invasion and metastasis, and cell division cycle. The availability of pharmacologic inhibitors of this pathway permits an analysis of the requirement of RAF→MEK→ERK signaling in the aberrant behavior of the cancer cell. There are a number of pharmacologic agents that inhibit RAF→MEK→ERK signaling (23, 51–54). Three MEK inhibitors, U0126, PD098059, and CI-1040 (PD184352), are reported to be nonsubstrate competitive inhibitors of MEK1/2 that elicit their effects by binding to a pocket near, but not overlapping, the ATP binding site of the protein thereby stabilizing the enzyme in an inactive conformation. CI-1040 is a third-generation MEK inhibitor with a lower IC50 than U0126 or PD098059 and is more selective for MEK1/2 over MEK5 (25, 38).

The proliferative arrest in our panel of eight pancreatic cancer cell lines was primarily mediated by arrest of the cell division cycle and was not accompanied by a marked increase in apoptosis (55). These data contrast with the effects of the phosphatidylinositol 3'-kinase inhibitor LY294002, which elicited a striking apoptotic response in five of these cell lines (data not shown). Pancreatic cancer cells may use the RAF→MEK→ERK pathway for the regulation of the cell division cycle whereas the phosphatidylinositol 3'-kinase→PDK1→Akt pathway may be more closely linked to the regulation of apoptosis.

Data presented here suggest that one mechanism for U0126-induced cell cycle arrest is through induced expression of p27Kip1 leading to inhibition of cyclin/cdk2 activity. Analysis of p16 phosphorylation at early times suggested that there might be an even earlier event that initiates U0126-induced cell cycle arrest because, in MIA PaCa-2 cells, hypophosphorylated p16 was detected before the induced expression of p27Kip1. The nature of this early event remains unknown, but it is unlikely to be mediated by induced expression of known cdk inhibitors because we assessed the expression of all INK4 and CIP/KIP proteins in these experiments. However, regardless of such an early event, it is clear from antisense and RNA interference experiments that p27Kip1 plays a key role in the maintenance of cyclin/cdk2 inhibition, hypophosphorylation of p16, and G0-G1 cell cycle arrest in some pancreatic cancer cell lines. However, it is possible that in some pancreatic cancer cells additional biochemical mechanisms may account for the effects of MEK inhibition on cell proliferation and the failure of anti-KIP1 siRNA to bypass U0126-induced cell cycle arrest.

Although all of the pancreatic cancer cells tested displayed elevated p27Kip1 in response to U0126, the mechanisms of p27Kip1 induction seemed to vary between the different cell lines. Regulation of p27Kip1 expression is reported to involve alterations in gene transcription, control of mRNA translation, and posttranslational regulation of protein stability (56, 57). The precise mechanisms underlying these observations are not known. In some pancreatic cancer cell lines, U0126 elicited an increase in both KIP1 mRNA and p27Kip1, whereas in others U0126 elicited increased p27Kip1 expression in the absence of alterations in KIP1 mRNA. Hence, it seems that the RAF→MEK→ERK pathway may be able to influence both KIP1 mRNA abundance and p27Kip1 stability. Moreover, in separate studies in NIH 3T3 cells, there is clear evidence of cooperation between the RAF→MEK→ERK and phosphatidylinositol 3'-kinase→PDK1→Akt pathways in the regulation of p27Kip1 expression, which seems to be largely mediated by alterations in KIP1 mRNA expression (20).

Studies described here contrast with our colleagues' previous analysis of human colon cancer cells treated with MEK inhibitors (27). In those studies the predominant effect of U0126 on colon cancer cell proliferation was ascribed to its effects on cyclin D1/cdk4 activity. Indeed, they reported that certain colon cancers were resistant to the effects of cdk2 inhibition either through antisense- or siRNA-mediated inhibition of cdk2, overexpression of dominant-negative cdk2, or overexpression of p27Kip1 itself. In addition, antisense-mediated inhibition of p27Kip1 expression in colon cancer cells did not bypass the effects of U0126 on cell cycle progression. Our results with anti-KIP1 and anti-CDK2 siRNAs have implicated the induced expression of p27Kip1 and inhibition of cyclin/cdk2 activity, respectively, as potentially important mediators of MIA PaCa-2 cell proliferation. Moreover, perhaps surprisingly, we did not detect significant inhibition of cdk4- or cyclin D1–associated kinase activity in response to MEK inhibition in Mia PaCa-2 cells. Indeed, pharmacologic inhibition of cdk4 resulted in an apoptotic response that was distinct from that observed with
inhibition of MEK activity of cdk2 activity. Consequently, these data suggest that inhibition of RAF→MEK→ERK signaling may elicit cell cycle arrest by distinct means in colon and pancreatic cancer cell lines, with alterations in p27


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Pharmacologic Inhibition of RAF → MEK → ERK Signaling Elicits Pancreatic Cancer Cell Cycle Arrest Through Induced Expression of p27\textsuperscript{Kip1}

Stephan Gysin, Sang-Hyun Lee, Nicholas M. Dean, et al.


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