Simultaneous targeting of the epidermal growth factor receptor and cyclooxygenase-2 pathways for pancreatic cancer therapy

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Abstract

The aims of this study were to determine the effects of (*a*) combining the epidermal growth factor receptor (EGFR) blocker (erlotinib) and the cyclooxygenase-2 inhibitor (celecoxib) on cell growth and apoptosis in human pancreatic cancer cell lines, (*b*) baseline EGFR expression on the potentiation of erlotinib-induced apoptosis by celecoxib, and (*c*) the effects of the combination on the expression of the COX-2, EGFR, HER-2/*neu*, and nuclear factor-κB (NF-κB). Baseline expression of EGFR was determined by Western blot analysis in five human pancreatic cancer cell lines. BxPC-3, PANC-1, and HPAC had high EGFR and MIAPaCa had low EGFR. Cells were grown in culture and treated with erlotinib (1 and 10 μmol/L), celecoxib (1 and 10 μmol/L), and the combination. Growth inhibition was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and apoptosis was assayed by ELISA. Reverse transcriptase-PCR was used to evaluate COX-2 and EGFR mRNA. EGFR, COX-2, and HER-2/*neu* expression was determined by Western immunoblotting. Electrophoretic mobility shift assay was used to evaluate NF-κB activation. Growth inhibition and apoptosis were significantly ($P < 0.05$) higher in BxPC-3, HPAC, and PANC-1 cells treated with celecoxib and erlotinib than cells treated with either celecoxib or erlotinib. However, no potentiation in growth inhibition or apoptosis was observed in the MIAPaCa cell line with low expression of the EGFR. Significant down-regulation of COX-2 and EGFR expression was observed in the BxPC-3 and HPAC cells treated with the combination of erlotinib (1 μmol/L) and celecoxib (10 μmol/L) compared with celecoxib- or erlotinib-treated cells. Celecoxib significantly down-regulated HER-2/*neu* expression in BxPC-3 and HPAC cell lines. Significant inhibition of NF-κB activation was observed in BxPC-3 and HPAC cell lines treated with erlotinib and celecoxib. (*a*) Celecoxib can potentiate erlotinib-induced growth inhibition and apoptosis in pancreatic cell lines, (*b*) high baseline EGFR expression is a predictor of this potentiation, and (*c*) the down-regulation of EGFR, COX-2, and HER-2/*neu* expression and NF-κB inactivation contributes to the potentiation of erlotinib by celecoxib. [Mol Cancer Ther 2005;4(12):1943–51]

Introduction

The epidermal growth factor receptor (EGFR) pathway plays a central role in carcinogenesis and cell proliferation (1, 2). Pancreatic cancer cells frequently overexpress the EGFR and its known ligands (3–6). This autocrine and/or paracrine activation of the EGFR results in downstream signaling through the Ras/Raf/mitogen-activated protein kinase, phosphoinostol-3 kinase (PI3K)/Akt, and nuclear factor-κB (NF-κB) pathways (7, 8). The overexpression of the EGFR and its ligands correlates with rapidly progressive disease (6) and resistance to chemotherapy (9). Preliminary results of a phase III trial of gemcitabine with or without erlotinib in pancreas cancer revealed a modest improvement in survival with the addition of erlotinib (10). The limited clinical benefit observed with erlotinib is due to the dysregulation of the signal transduction pathways in pancreas cancer cells at multiple levels (2). Consequently, combining erlotinib with other targeted agents to augment proapoptotic and antigrowth signaling is necessary to produce a significant clinical effect.

Celecoxib is a selective cyclooxygenase-2 (COX-2) inhibitor (11). COX-2 is an inducible enzyme that is overexpressed in pancreatic cancer (12, 13). Through the conversion of arachidonic acid to prostaglandin, the COX-2 enzyme modulates angiogenesis (14) and metastasis (15). In preclinical models, selective and nonselective COX-2 inhibitors can promote apoptosis and sensitize pancreatic cancer cell lines to the effects of chemotherapeutic agents (16–18). The EGFR and COX-2 pathways interact at several levels. The EGFR pathway has a central role in the regulation of COX-2 expression. Activation of the Ras/Raf/mitogen-activated protein kinase and NF-κB transcription factor up-regulates *COX-2* gene transcription and the activation of the PI3K/Akt pathway stabilizes the COX-2 mRNA (19, 20). Similarly, overexpression of the COX-2 enzyme can potentially affect the EGFR signaling pathway. Prostaglandins transactivate the EGFR by induction of phosphorylation of the EGFR and extracellular signal-regulated kinase (21). Furthermore, COX-2 overexpression

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induces EGFR expression (22). In addition to COX-2 inhibition, celecoxib is known to directly inhibit the EGFR pathway through inhibition of the PI3K/Akt and NF-κB pathways (17, 23, 24). The interaction between the COX-2 and EGFR pathways and the COX-2-independent effects of celecoxib suggest that the addition of celecoxib to erlotinib is a rational approach for pancreatic cancer therapy.

The first aim of this study was to investigate the growth-inhibitory effects of erlotinib with celecoxib in pancreatic cancer cell lines. The second aim of the study was to evaluate the contribution of the EGFR expression on the growth inhibition of the celecoxib and erlotinib combination. To evaluate this effect, we compared the effects of the two drugs in four pancreatic cancer cell lines with different baseline EGFR expression levels. The third aim of the study was to determine the mechanisms involved in the potentiation of the effects of erlotinib by celecoxib. Three possible mechanisms of interaction were evaluated. The first mechanism was the down-regulation of Erb receptor expression by erlotinib and celecoxib. Second mechanism was the down-regulation of the COX-2 expression by erlotinib. Third, because both erlotinib and celecoxib are known to inhibit the Akt/NF-κB pathway, we hypothesized that erlotinib- and celecoxib-induced inactivation of NF-κB leads to down-regulation of EGFR and COX-2 mRNA and protein expression and in turn causes enhanced killing of pancreatic cancer cells.

Materials and Methods

Cell Culture, Drugs, and Reagents

Human pancreatic cancer cell lines BxPC-3, MIAPaCa, PANC-1, MOH-1, and HPAC cells were used in this study. BxPC-3 cells were grown in RPMI 1640 with 10% fetal bovine serum. MIAPaCa and PANC-1 cells were grown as a monolayer cell culture in DMEM containing 4.5 mg/mL D-glucose and L-glutamine supplemented with 10% fetal bovine serum, and the other two cell lines MOH-1 and HPAC cells were grown in DMEM/F12 (1:1) with 10% calf serum. Celecoxib and erlotinib were generous gifts from Pfizer (New York, NY) and OSI Pharmaceuticals (Melville, NY), respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopropanol, and DMSO were acquired from Sigma Chemical (St. Louis, MO). Apoptosis detection kit was purchased from Roche Applied Science (Indianapolis, IN). Cell culture mediums were purchased from Life Technologies Bethesda Research Laboratories (Grand Island, NY).

Immunoprecipitation for EGFR Phosphorylation

BxPC-3 cell line was used to determine the effect of erlotinib on the phosphorylation of the EGFR. BxPC-3 cells were treated with erlotinib (10 μmol/L) for 48 hours, EGF (100 ng/mL) for 15 minutes, or the combination for 48 hours. Untreated cells were used as controls. BxPC-3 cells were lysed in NP40, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 5 μg/mL pepstatin, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 100 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, and 10 mmol/L sodium pyrophosphate. Protein concentration was determined using Bicinchoninic Acid Protein assay kit from Pierce (Rockford, IL). In addition, equal amounts of proteins from erlotinib-treated and untreated cell lysates were incubated with EGFR antibody for overnight at 4°C followed by the addition of Protein-A agarose and incubated at 4°C for 1 hour. Agarose beads were washed several times with NP40 lysis buffer, resuspended in 2× SDS sample buffer, and subjected to Western blot analysis with antiphosphotyrosine antibody.

Cell Viability Assay

The viability of cells treated with erlotinib, celecoxib, or the combination was determined by the standard MTT reduction assay. BxPC-3, HPAC, PANC-1, and MIAPaCa cells were plated (3-5,000 per well) in 96-well plate and incubated overnight at 37°C. Celecoxib was dissolved in DMSO and added to cell culture medium at a concentration not exceeding 0.1% (v/v). The effects of celecoxib (1 or 10 μmol/L), erlotinib (1 or 10 μmol/L), and the combination on the four cell lines were studied. The MTT assay was done in triplicates for each drug concentration used. After the required drug treatment time, aliquots of 100 μL of MTT (1 mg/mL) were added to each well and incubated for 2 hours at 37°C. The supernatant was removed, and 100 μL of isopropanol were then added. The color intensity was measured by TECAN's microplate fluorometer (TECAN, Research Triangle Park, NC) at 595 nm. DMSO-treated cells were considered untreated control and assigned a value of 100%. Linearity of the color intensity relative to cell number within the range expected in the study was determined at the outset.

Apoptosis Assay

The Cell Death Detection ELISA kit (Roche Applied Science) was used to detect apoptosis in treated BxPC-3, HPAC, PANC-1, and MIAPaCa cells. The assay is based on a photometric enzyme immunoassay for the qualitative and quantitative determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). The assay uses anti-histone/biotin antibodies that bind to H2A, H2B, H3, and H4 histones and anti-DNA-peroxidase antibodies that react with single-stranded and double-stranded DNA. Cells seeded in six-well plates were treated with celecoxib (10 μmol/L), erlotinib (10 μmol/L), or the combination. The cells were trypsinized, and ~10,000 cells were added to 500 μL of lysis buffer and incubated at room temperature for 0.5 hour. The cells were centrifuged at 20,000 × *g* for 10 minutes, and 100 μL of the supernatant were transferred into anti-histone-coated microtiter plate and incubated at room temperature for 90 minutes. The plate was washed twice with 200 μL of washing solution provided with the kit. A solution containing 100 μL of anti-DNA-peroxidase dissolved in incubation buffer was added to the same plate and incubated for 90 minutes. After removal of the unbound antibodies, the nucleosomes were quantified by the peroxidase reaction using 2,2′-azino-di[3-ethylbenzthiazolin-sulfonat] as substrate. TECAN's microplate fluorometer (TECAN) was used to measure color intensity at 492 nm.

Immunoblotting for the Expression of EGFR, COX-2, and HER-2/*neu* Proteins

BxPC-3, MIAPaCa, MOH-1, HPAC, and PANC-1 cells were used to determine the baseline expression of the EGFR. BxPC-3 and HPAC cells treated for 48 hours with erlotinib (1 μmol/L), celecoxib (10 μmol/L), or the combination were used to evaluate the effects of treatment on COX-2 and EGFR expression. BxPC-3, MIAPaCa, HPAC, and PANC-1 cell lines were also used to determine baseline expression of HER-2/*neu*. Cells treated with celecoxib (10 μmol/L) for 48 hours were used to evaluate the effects on HER-2/*neu* expression. Cells were harvested by scraping from culture plates and collected by centrifugation. Cells were resuspended in 125 mmol/L Tris buffer (pH 6.8), sonicated twice for 10 seconds, and lysed using an equal volume of 8% SDS. Cell extracts were boiled for 10 minutes and chilled on ice. Protein concentration was then measured using Bicinchoninic Acid Protein Assay kit (Pierce). The samples were loaded on 10% SDS-PAGE for separation and electrophoretically transferred to a nitrocellulose membrane. Each membrane was incubated with monoclonal antibody against COX-2 (1:1,000; Cayman Chemical Co., Ann Arbor, MI), EGFR (1:1,000; Labvisions, Fremont, CA), HER-2/*neu* (2:1,000; Oncogene, Cambridge, MA), and polyclonal anti-β-actin (1:2,000; Sigma, St. Louis, MO). Blots were washed with phosphate buffer containing 0.05% Tween (PBST) and incubated with secondary antibodies conjugated with peroxidase. The signal intensity was then measured using chemiluminescent detection system (Pierce). Autoradiograms of the Western blots were scanned with Gel Doc 1000 image scanner (Bio-Rad, Hercules, CA) that was linked to a Macintosh computer.

Reverse Transcriptase-PCR for COX-2 and EGFR mRNA Expression

The influence of treatment on COX-2 and EGFR mRNA was determined by reverse transcriptase-PCR (RT-PCR). BxPC-3 and HPAC cells were treated with erlotinib (1 μmol/L), celecoxib (10 μmol/L), or the combination for 48 hours. Untreated cells were used as controls. Culture medium was removed, and 2 mL Trizol was added. The cells were scraped, and the lysate was passed through a pipette several times. Two hundred microliters of chloroform were added and incubated for 2 to 3 minutes before centrifugation at 12,000 × *g* for 15 minutes. Five hundred microliters of isopropanol were added to the aqueous phase, mixed, and incubated at room temperature for few minutes, then centrifuged at 12,000 × *g* for 10 minutes. The pellet was washed with 80% ethanol and dissolved in RNase-free water. Two micrograms of total RNA were reverse transcribed using 0.1 mmol/L DTT, 1 mmol/L deoxynucleotide triphosphates, 5 pmol/μL random primers, and 10 units/μL superscript II (Invitrogen, Grand Island, NY). RT-PCR for COX-2, EGFR, and β-actin amplification was done using Taq polymerase (Invitrogen), with COX-2 primer (Oxford Biomedical Research, Inc., Rochester Hills, MI), EGFR primer (Integrated DNA Technologies, Coralville, IA), and actin primer (Sigma). The PCR conditions were 94°C for 1 minute, 53°C for 1 minute, and 72°C for 2 minutes for 35 cycles. PCR products were subjected to electrophoresis on 2% agarose gel, stained with ethidium bromide, and photographed.

Real-time RT-PCR for COX-2 mRNA Expression

We also conducted real-time RT-PCR to verify the induction of *COX-2* gene expression at the mRNA level by EGFR. Two micrograms of total RNA were subjected to reverse transcription as described in the previous page. Real-time PCR reactions were then carried out in a total of 25-μL reaction mixture containing 2 μL of cDNA, 12.5 μL of 2× SYBR Green PCR Master Mix, 1.5 μL of each 5 μmol/L forward and reverse primers, and 7.5 μL of H_2O in Smart Cycler II (Cepheid, Sunnyvale, CA). The PCR conditions were 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Data were analyzed using comparative C_t method and were normalized by actin in each sample.

Electrophoretic Mobility Shift Assay for NF-κB Activation

BxPC-3 and HPAC cells were either untreated or treated with erlotinib (1 μmol/L), celecoxib (10 μmol/L), or the combination for 48 hours. The cells were suspended in 500 μL of Triton X-100 lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 100 mmol/L $MgCl_2$, 50 mmol/L levamisole, 200 mmol/L sodium butyrate, 100 mmol/L phenylmethylsulfonyl fluoride, and protein inhibitor (Roche Applied Science), which contain a broad spectrum of serine, cysteine, and metalloproteases. The cells were lysed with 20 strokes in a Dounce Homogenizer (Kontes Glass Co., Vineland, NJ) and centrifuged at 3,000 × *g* for 15 minutes at 4°C. The nuclear pellet was resuspended in an equal volume of 10 mmol/L Tris-HCl (pH 7.4), 5 mmol/L $MgCl_2$ followed by equal volume of 1 mol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), and 5 mmol/L $MgCl_2$. The nuclear suspension was then incubated on ice for 30 minutes and then centrifuged at 10,000 × *g* for 20 minutes at 4°C. The supernatant was quantified using Bicinchoninic Acid Protein Assay kit (Pierce).

Electrophoretic mobility shift assay was done using the Odyssey Infrared Imaging System with NF-κB IRDye-labeled oligonucleotide from LI-COR, Inc. (Lincoln, NE). The DNA binding reaction was set up using 5 μg of the nuclear extract mixed with oligonucleotide and gel shift binding buffer consisting of 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), and 0.25 mg/mL poly(deoxyinosinic-deoxycytidylic acid). The reaction was incubated at room temperature in the dark for 30 minutes; 2 μL of 10× orange loading dye was added to each sample and loaded on the prerun 8% polyacrylamide gel and ran at 30 mA for 1 hour. NF-κB p65 antibody and unlabeled NF-κB oligo was used to confirm the super shift and the specificity of NF-κB DNA binding activity.

The gel was scanned, and the signals were quantified using Odyssey Infrared Imaging System and Odyssey software (LI-COR). Comparison between untreated and treated was done via *t* test. Statistical significance was assumed for a $P < 0.05$.

Results

Baseline Expression of EGFR in Human Pancreatic Cancer Cells

To test our hypothesis, we measured the basal level of the EGFR in multiple pancreatic cancer cells to select the cell lines based on relative level of EGFR expression. Figure 1A shows the immunoblot for EGFR expression in BxPC-3, MIAPaCa, MOH-1, HPAC, and PANC-1 cell lines. EGFR expression was high in the BxPC-3, HPAC, and PANC-1 cell lines and low in MIAPaCa and MOH-1 cell lines.

Effects of Erlotinib on the Phosphorylation of the EGFR in Pancreatic Cancer Cells

To test the effect of erlotinib on EGFR expression, we first tested whether EGF could increase phosphorylation of the EGFR compared with the control. The addition of EGF at a concentration of 100 ng/mL significantly increased EGFR phosphorylation. Significant inhibition of phosphorylation of the EGFR was observed with erlotinib (10 μmol/L) in the EGF-treated and untreated cells (Fig. 1C). This observation confirms that the concentration of erlotinib up to 10 μmol/L used in subsequent experiments are sufficient to inhibit signaling through the EGFR in BxPC-3 cell lines.

Effects of Celecoxib and Erlotinib on the Viability of Pancreatic Cancer Cells

Overall cell growth of BxPC-3, HPAC, PANC-1, and MIAPaCa pancreatic cancer cells treated with celecoxib (1 or 10 μmol/L), erlotinib (1 or 10 μmol/L), and in combination was determined by the MTT assay. In the BxPC-3 and HPAC cell lines, a significant potentiation of the growth inhibition of erlotinib by celecoxib was observed with the 1 and 10 μmol/L concentrations of erlotinib and celecoxib (Fig. 2A and B). In the PANC-1 cell lines, significant potentiation of the growth inhibition of erlotinib by celecoxib was observed only with the 10 μmol/L concentrations of erlotinib and celecoxib (Fig. 2A and B). No growth inhibition was observed with erlotinib, celecoxib, or the combination in MIAPaCa cell line that has a low EGFR expression (Fig. 2A and B). These results suggest that each growth inhibition could be due to cell cycle arrest and/or induction of apoptosis. Hence, we have tested the effects of erlotinib and celecoxib as single agents or their combinations in the induction of apoptotic cell death.

Induction of Apoptosis by Erlotinib and Celecoxib and the Combination

The effect of celecoxib (10 μmol/L) and erlotinib (10 μmol/L) individually and in combination was tested using Cell Death Detection ELISA kit. Exposure of BxPC-3, PANC-1, and HPAC cells to either celecoxib or erlotinib for 48 hours significantly enhanced apoptosis (Fig. 3). The combination of celecoxib and erlotinib resulted in a significant increase in apoptosis compared with either agent alone. In the MIAPaCa cell line, no increase in apoptosis was noticed with celecoxib, erlotinib, or the combination (Fig. 3). Therefore, we selected the BxPC-3 and HPAC cell line for further studies evaluating the effects of celecoxib and erlotinib on the EGFR, COX-2, and HER-2/*neu* expression and NF-κB activation in an attempt to further characterize the mechanisms underlying the potentiation of apoptosis by celecoxib. Our next question was to test whether the combination of erlotinib and celecoxib could affect the expressions of COX-2 in addition to the effects on COX-2 activity.

Modulation of COX-2 Protein and mRNA Expression in BxPC-3 and HPAC Cells Treated with Celecoxib and Erlotinib

The addition of EGF at a concentration of 100 ng/mL to BxPC-3 cells significantly increased COX-2 gene transcription (Fig. 4) by both RT-PCR and real-time RT-PCR methodology. This confirmed the role of the EGFR in modulation of the expression of the COX-2 enzyme in the BxPC-3 cell line. The effect of inhibiting the EGFR pathway on COX-2 expression was then evaluated. The expression of COX-2 protein and mRNA was determined in BxPC-3 and HPAC cells treated with erlotinib (1 μmol/L), celecoxib (10 μmol/L), or both drugs (Fig. 5A and B). The data with either celecoxib or erlotinib treatment on both the cell lines on COX-2 protein and mRNA expression showed very similar results. No significant decrease was observed by either agents when used alone in both protein and mRNA expression. The experiment was repeated at least thrice with similar results. However, a significant down-regulation in the expression of both COX-2 protein and mRNA was observed in BxPC-3 and HPAC cells treated with

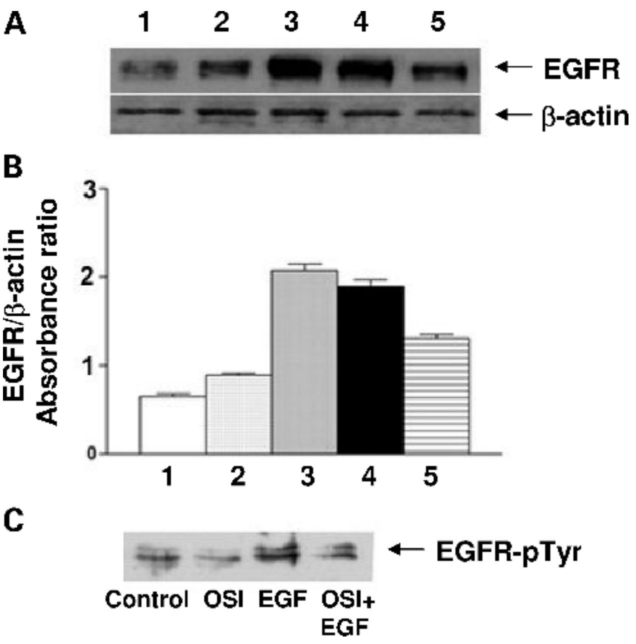

Figure 1. Expression of EGFR by Western blot analysis (**A**) and densitometric quantification (**B**) in human pancreatic cancer cell lines. **C,** effect of treatment with erlotinib (*OSI*), EGF, or the combination on EGFR phosphorylation in the BxPC-3 Cells. *1,* MIAPaCa; *2,* MOH-1; *3,* HPAC; *4,* PANC-1; *5,* BxPC-3. The level of EGFR expression was compared between a panel of pancreatic cancer cell lines relative to β-actin expression. The highest level of EGFR expression was in the HPAC cell line and lowest was in the MIAPaCa cell line. Cells were treated with DMSO, erlotinib (*OSI*; 10 μmol/L) for 48 h, EGF (100 ng/mL) for 15 min, and the combination of EGF (100 ng/mL) and erlotinib (10 μmol/L) for 48 h, respectively. Erlotinib inhibited EGFR phosphorylation in the presence or absence of EGF. Representative of three independent experiments. *Columns,* means; *bars,* SD.

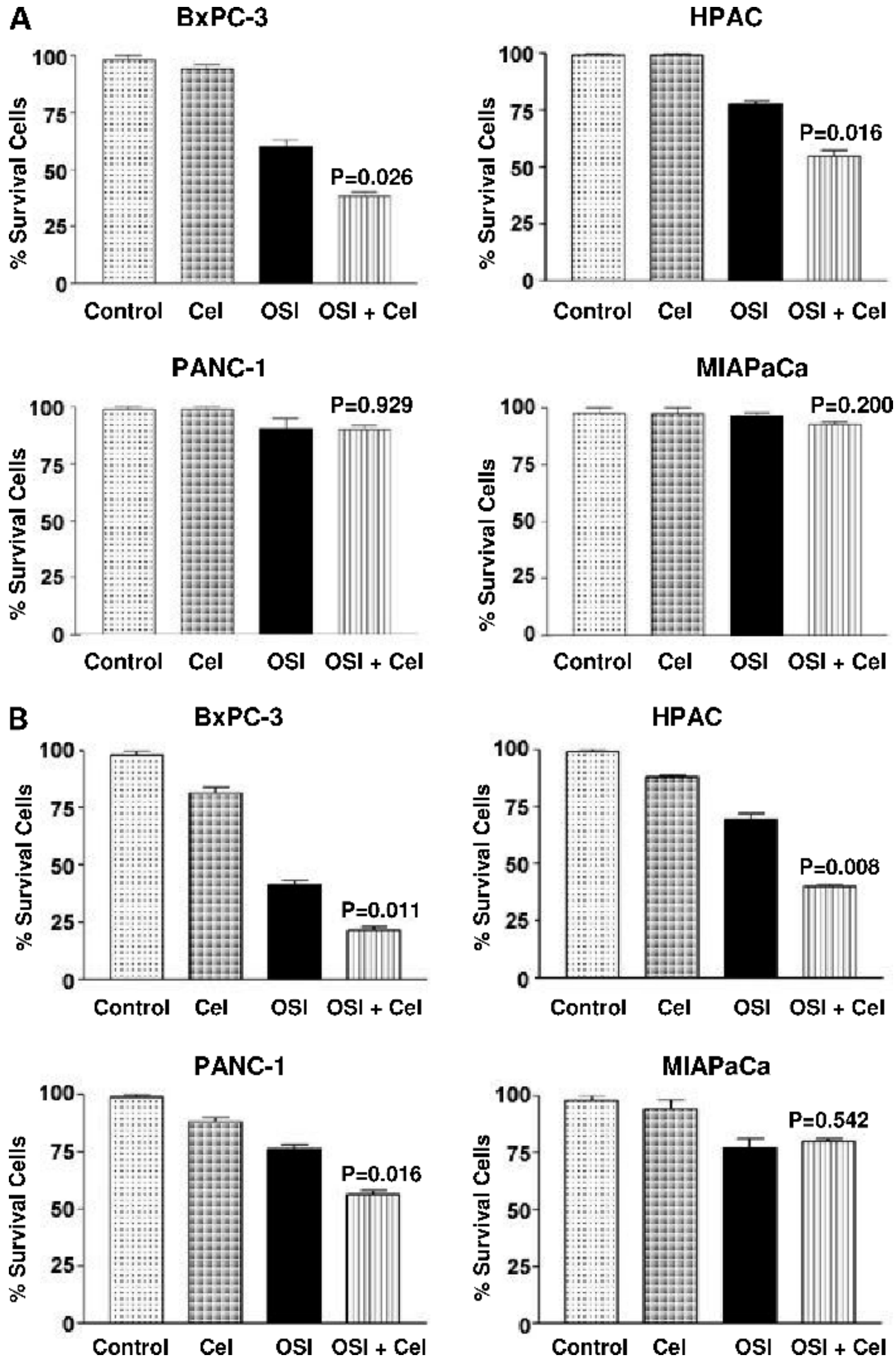

Figure 2. Effect of celecoxib, erlotinib, and the combination on the viability of human pancreatic cancer cells by the MTT assay. **A,** celecoxib (*CEL*; 1 μmol/L), erlotinib (*OSI*; 1 μmol/L), and the combination. **B,** celecoxib (10 μmol/L), erlotinib (10 μmol/L), and the combination. BxPC-3, HPAC, PANC-1, and MIAPaCa human pancreatic cancer cells were treated as described in Materials and Methods. There was a significant reduction in cell growth in the BxPC-3, HPAC, and PANC-1 cells treated with erlotinib and celecoxib compared with cells treated with either agent alone. No significant increase in growth inhibition was observed in MIAPaCa cells treated with the combination over cells treated with either agent alone. *P*s represent comparisons between erlotinib-treated cells versus cells treated with combination of erlotinib and celecoxib using *t* test.

combination of erlotinib and celecoxib. There is a minor variation observed in the results shown by Western blot for protein expression and RT-PCR for mRNA expression, which could be due to the methodologic differences in experimental procedures. Nevertheless, the data on the levels of COX-2 protein and mRNA is consistent suggesting transcriptional down-regulation in combination treatment. Subsequently, we tested the effect of erlotinib and celecoxib on EGFR expression.

Modulation of EGFR Protein and mRNA Expression in BxPC-3 and HPAC Cells Treated with Celecoxib and Erlotinib

The expression of EGFR protein and mRNA was determined in BxPC-3 and HPAC cells treated with erlotinib (1 μmol/L), celecoxib (10 μmol/L), or both drugs (Fig. 5C and D). Erlotinib resulted in a significant decrease in EGFR protein expression as well as mRNA expression in HPAC cell line. Whereas a significant down-regulation of both

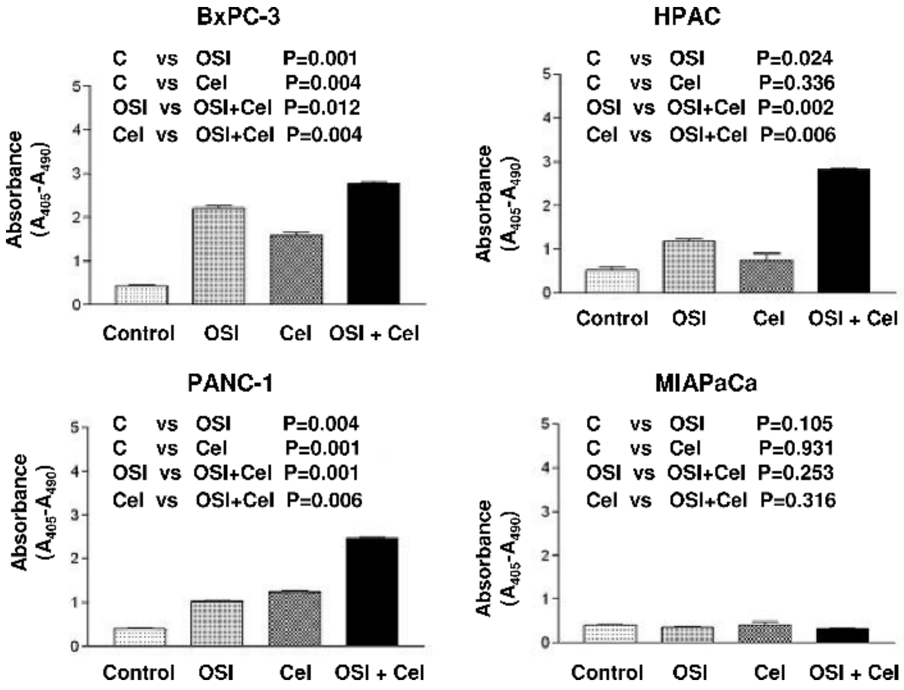

Figure 3. Induction of apoptosis in BxPC-3, HPAC, PANC-1, and MIAPaCa human pancreatic cancer cells. Cells were treated with erlotinib (*OSI*; 10 μmol/L), celecoxib (*Cel*; 10 μmol/L), or the combination as described in Materials and Methods. There was a significant potentiation of apoptosis observed in BxPC-3, HPAC, and PANC-1 cells treated with erlotinib and celecoxib compared with cells treated with either agent alone. No potentiation of apoptosis was observed in the MIAPaCa cell line.

EGFR protein and mRNA expression was observed in both BxPC-3 and HPAC cells treated with combination of erlotinib and celecoxib as compared with untreated, celecoxib-treated, or erlotinib-treated cells. Because EGFR can heterodimerize with other Erb receptors, we evaluated the effects of celecoxib on the expression of other Erb receptors.

Modulation of HER-2/*neu* Protein Expression in BxPC-3, HPAC, PANC-1, and MIAPaCa Cells Treated with Celecoxib

To evaluate the effect of celecoxib on the expression of other members of the Erb-B family, the protein expression of HER-2/*neu* was determined in BxPC-3, PANC-1, HPAC, and MIAPaCa cells at baseline and after treatment with celecoxib (10 μmol/L). Celecoxib significantly down-regulated the expression of HER-2/*neu* in the BxPC-3 and HPAC cell lines (Fig. 6).

Activation of NF-κB in BxPC-3 and HPAC Cells Treated with Celecoxib and Erlotinib

Because NF-κB plays a critical role in cell survival and transcriptional activation of COX-2 expression, our next question was to test whether erlotinib and celecoxib could mediate their effects through modulation of NF-κB activity. The DNA binding activity of NF-κB was determined in BxPC-3 and HPAC cells treated with erlotinib (1 μmol/L), celecoxib (10 μmol/L), and the combination (Fig. 7). In BxPC-3 and HPAC cell lines, the combination of erlotinib and celecoxib resulted in a significant down-regulation of the activation of NF-κB compared with untreated, erlotinib-treated, or celecoxib-treated cells.

Discussion

The activation of the EGFR pathway promotes transcription of the *COX-2* gene (19, 20). Similarly, the COX-2 signaling pathway activates EGFR phosphorylation (21) and *EGFR* transcription (22). The EGFR and COX-2 pathways are involved in carcinogenesis, angiogenesis, and chemoresistance. Therefore, targeting both EGFR and COX-2 may be an effective approach to modulate both pathways and their downstream signaling, which may result in increased therapeutic response. In the present study, we evaluated this approach by combining erlotinib, an EGFR tyrosine kinase inhibitor with celecoxib, a selective COX-2 inhibitor. The mechanisms involved in the potentiation of the effect of erlotinib by celecoxib were also explored. Three mechanisms were evaluated. The first is the effect of erlotinib on COX-2 expression; second is the effect of

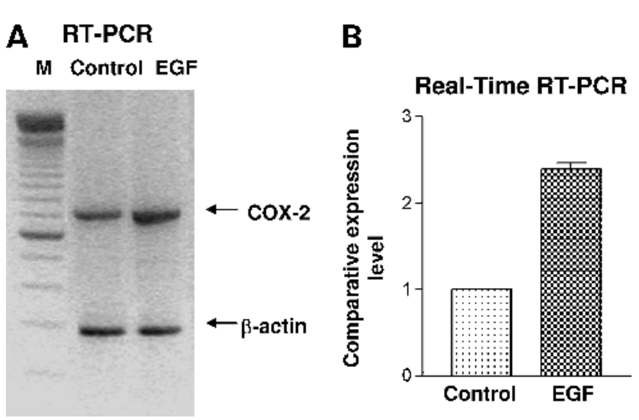

Figure 4. Induction of COX-2 gene transcription by EGF (100 ng/mL) for 15 min in BxPC-3 human pancreatic cancer cells. COX-2 mRNA expression was determined by RT-PCR (**A**) and real-time RT-PCR (**B**). Significant induction of COX-2 gene transcription with EGF was observed by both analyses.

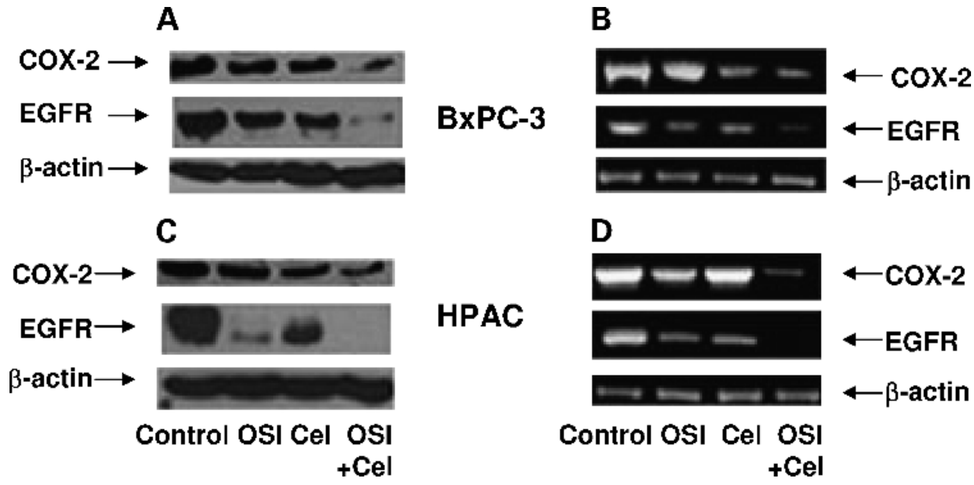

Figure 5. The expression of COX-2 and EGFR in BxPC-3 and HPAC human pancreatic cell lines treated with celecoxib (*Cel*; 10 μmol/L), erlotinib (*OSI*; 1 μmol/L), or the combination. **A,** Western blot analysis for COX-2 and EGFR in BxPC-3 cells. **B,** RT-PCR analysis for COX-2 and EGFR in BxPC-3 cells. **C,** Western blot analysis for COX-2 and EGFR in HPAC cells. **D,** RT-PCR analysis for COX-2 and EGFR in HPAC cells. Significant down-regulation of COX-2 and EGFR protein and mRNA expression was observed in cells treated with the combination of erlotinib and celecoxib compared with cells treated with either drug alone.

celecoxib on the expression of the Erb receptors, including EGFR and HER-2/*neu*; and third is the evaluation of the effects of erlotinib and celecoxib on the downstream signaling pathway involving NF-κB. The combination was evaluated in pancreatic cancer cell lines with different baseline expression levels of the EGFR protein to evaluate the role of EGFR expression on sensitivity to erlotinib.

EGFR inhibitors have shown activity in clinical trials in pancreatic (10), colorectal (25), and non–small cell lung cancers (26). The observed activity of these agents seems limited to a small proportion of patients. Therefore, clinical and preclinical trials evaluating the predictors of response to EGFR inhibitors are being conducted. The factors that can potentially influence sensitivity to EGFR inhibitors include EGFR expression, receptor mutations, heterodimerization with other growth factor receptors, and independent activation of downstream signaling pathways. Receptor mutation status has been shown to affect the response to gefitinib in non–small cell lung cancer (27). Unfortunately, the activating mutations identified in non–small cell lung cancer seem disease specific. Immunohistochemical evaluation of EGFR expression in colorectal cancer (28) has failed to reveal an interaction between receptor expression and response. Evaluation of EGFR expression using immunohistochemistry might be inaccurate due to interobserver variability, improper tissue handling, or processing. In this study, we evaluated the role of EGFR expression as evaluated by Western blot on response to erlotinib. Pancreatic cancer cell lines with higher EGFR expression responded to erlotinib, whereas cell lines with low expression, such as MIAPaCa, did not respond. This observation suggests that EGFR expression could be a predictor of response in pancreatic cancer. Future clinical trials should evaluate the reliability of immunohistochemistry as a method to evaluate EGFR expression as well as the role of EGFR expression on response to EGFR inhibitors.

Erlotinib inhibits the function of the EGFR through inhibition of the autophosphorylation of the tyrosine kinase domain of the EGFR. Down-regulation of the expression of the EGFR by celecoxib and erlotinib can further potentiate the EGFR inhibition. Heterodimerization of the EGFR with HER-2/*neu* can contribute to the resistance to erlotinib (2). Because the tyrosine kinase domain of HER-2/*neu* is not inhibited by erlotinib, EGFR signaling through HER-2/*neu* can persist in the presence of erlotinib. Celecoxib through down-regulation of HER-2/*neu* expression can further potentiate the inhibition of the EGFR pathway by erlotinib. Similarly, the down-regulation of the COX-2 expression both at the protein and mRNA by the combination of erlotinib and celecoxib could potentiate the inhibition of this pathway by celecoxib. Because both EGFR and COX-2 pathways are involved in cell growth and modulation of apoptosis,

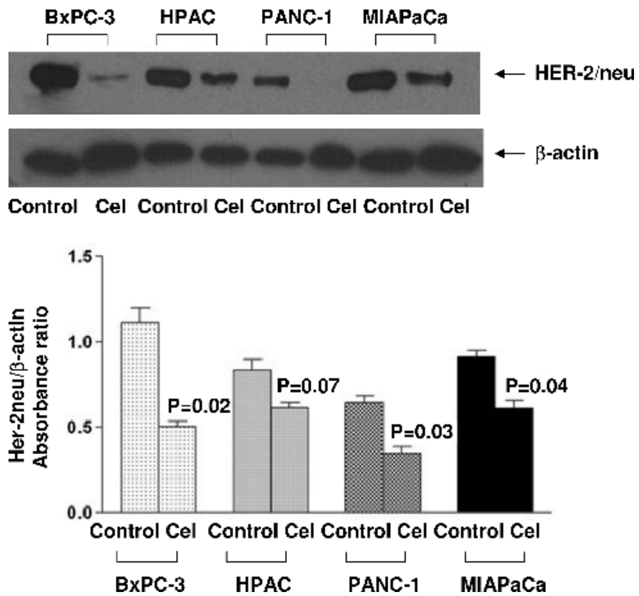

Figure 6. Effect of celecoxib (*Cel*) on HER-2/*neu* expression in BxPC3, HPAC, PANC-1, and MIAPaCa human pancreatic cell lines. Western blot analysis was done with HER-2/*neu* antibody. Down-regulation of HER-2/*neu* expression was observed with celecoxib (10 μmol/L) in treated cells.

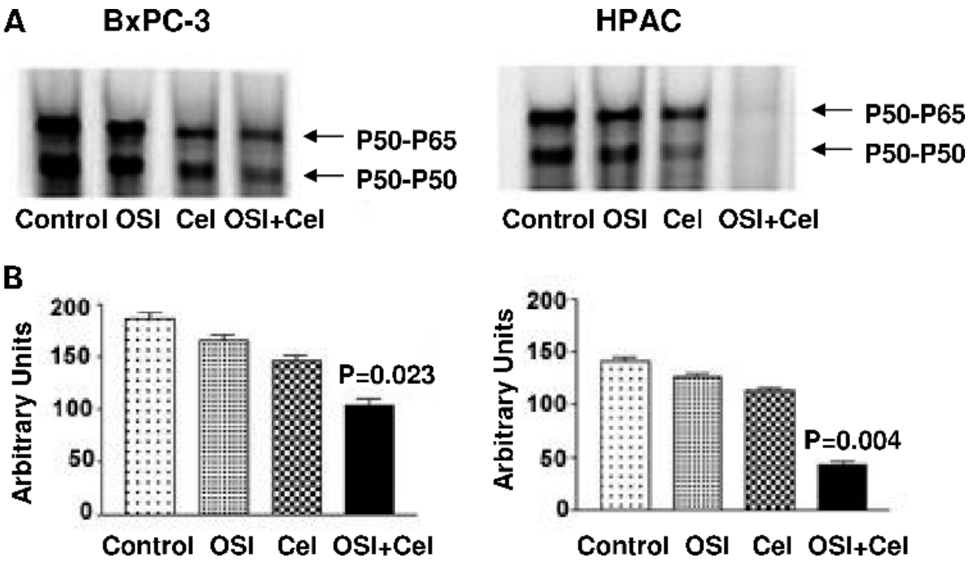

Figure 7. Determination of NF-κB activation by electrophoretic mobility shift assay in BxPC-3 and HPAC cells treated with celecoxib (*Cel*; 10 μmol/L), erlotinib (*OSI*; 1 μmol/L), or the combination. Significant down-regulation of NF-κB activation was observed in cells treated with erlotinib and celecoxib. **A,** NF-κB DNA binding activity. **B,** quantitation of the signals.

improved inhibition of these pathways by the combination could partly account for the observed potentiation of erlotinib by celecoxib. These effects could be due to inactivation of a transcription factor NF-κB, which is known to transcriptionally activate COX-2 expression.

NF-κB is a transcription factor that is involved in a wide spectrum of cellular functions, including apoptosis and cell cycle control (29, 30). Inactivation of the NF-κB can sensitize cancer cells to the effects of chemotherapy (31). In pancreatic cancer, EGFR inhibition has been shown to down-regulate NF-κB activity. The COX-2 enzyme has also been shown to activate the NF-κB pathway by the generation of prostaglandin (32, 33). Celecoxib is known to inhibit the PI3K/Akt and NF-κB pathway (23). Activated NF-κB translocates to the nucleus, resulting in the transcription of several genes, among which is *COX-2* (34), and possibly EGFR (35). The combination of erlotinib and celecoxib significantly inhibited NF-κB activation, resulting in the down-regulation of the transcription of EGFR and COX-2 enzyme and in turn increased apoptotic cell death.

The combination of an EGFR and COX-2 inhibitor has been previously evaluated in preclinical models. In squamous cell cancer cell lines, celecoxib potentiated the apoptotic effect of gefitinib (36). Tortora et al. showed significant growth inhibition in breast and colon cancer cell lines treated with a combination of gefitinib, SC-236 (a COX-2 inhibitor), and a protein kinase A antisense molecule (37). Similar effects were observed in animal models of breast and colon cancer cell lines. A potentiation of the growth inhibition of trastuzumab and cetuximab by NS-398 (COX-2 blocker) was observed in colon cancer cell lines (38). In all three studies, a down-regulation of COX-2 expression was observed with the combination of EGFR and COX-2 inhibitor. Therefore, the effects observed in this study are reproducible with different COX-2 inhibitors and in different disease models.

In conclusion, celecoxib can potentiate the growth inhibitory effects of erlotinib in pancreatic cancer cell lines. The effects of the erlotinib and celecoxib combination can be attributed to the inhibition of activation and down-regulation of expression of the COX-2, EGFR, and HER-2/*neu* as shown by the schematic diagram in Fig. 8. In addition, the combination significantly down-regulated NF-κB activation, which in turn contributes to the down-regulation of COX-2 and EGFR expression and induction of apoptotic processes. The growth inhibitory effects observed in this study have been previously reported in different disease models and with various EGFR and COX-2 inhibitors, and our study reports similar results, for the

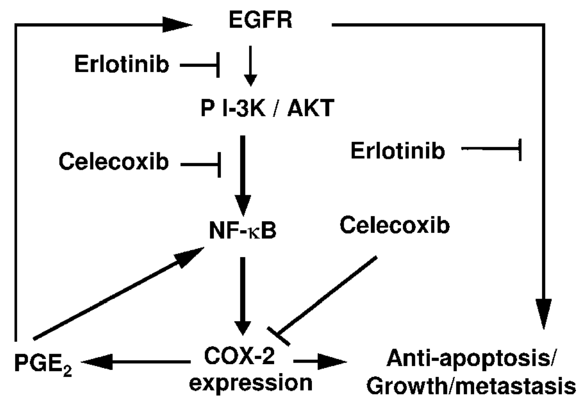

Figure 8. A hypothetical model showing the interaction among the EGFR, COX-2, and NF-κB signalling pathways. Activation of the EGFR results in downstream signaling through Akt/NF-κB, promotion of growth, and inhibition of apoptosis. NF-κB activation leads to increased transcription of the *COX-2* gene and inhibition of apoptosis. The erlotinib and celecoxib combination down-regulates EGFR and COX-2 expression and activation. NF-κB inhibition is the central role in the observed effects of the erlotinib and celecoxib combination. Inhibition of NF-κB activation is the central effect of combining erlotinib and celecoxib with respect to inhibition of proliferation and induction of apoptosis.

first time, in pancreatic cancer cells. Therefore, targeting the EGFR and COX-2 pathways seems to be a promising approach in the prevention and or treatment of pancreatic cancer.

References

1. El-Rayes BF, LoRusso PM. Targeting the epidermal growth factor receptor. Br J Cancer 2004;91:418 – 24.

2. Grandis JR, Sok JC. Signaling through the epidermal growth factor receptor during the development of malignancy. Pharmacol Ther 2004; 102:37 – 46.

3. Smith JJ, Derynck R, Korc M. Production of transforming growth factor α in human pancreatic cancer cells: evidence for a superagonist autocrine cycle. Proc Natl Acad Sci U S A 1987;84:7567 – 70.

4. Kobrin MS, Funatomi H, Friess H, Buchler MW, Stathis P, Korc M. Induction and expression of heparin-binding EGF-like growth factor in human pancreatic cancer. Biochem Biophys Res Commun 1994;202: 1705 – 9.

5. Ebert M, Yokoyama M, Kobrin MS, et al. Induction and expression of amphiregulin in human pancreatic cancer. Cancer Res 1994;54: 3959 – 62.

6. Yamanaka Y, Friess H, Kobrin MS, Buchler M, Beger HG, Korc M. Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. Anticancer Res 1993;13:565 – 9.

7. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000; 103:211 – 25.

8. Yarden Y. The EGFR family and its ligands in human cancer. Signalling mechanisms and therapeutic opportunities. Eur J Cancer 2001;37 Suppl 4:S3 – 8.

9. Wagner M, Cao T, Lopez ME, et al. Expression of a truncated EGF receptor is associated with inhibition of pancreatic cancer cell growth and enhanced sensitivity to cisplatinum. Int J Cancer 1996;68:782 – 7.

10. Moore M, Goldstein D, Hamm J, et al. Erlotinib improves survival when added to gemcitabine in patients with advanced pancreatic cancer. A phase III trial of the National Cancer Institute of Canada Clinical Trials Group. In: Miami: ASCO GI Symposium; 2005.

11. Davies NM, McLachlan AJ, Day RO, Williams KM. Clinical pharmacokinetics and pharmacodynamics of celecoxib: a selective cyclo-oxygenase-2 inhibitor. Clin Pharmacokinet 2000;38:225 – 42.

12. Yip-Schneider MT, Barnard DS, Billings SD, et al. Cyclooxygenase-2 expression in human pancreatic adenocarcinomas. Carcinogenesis 2000; 21:139 – 46.

13. Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML, Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. Cancer Res 1999;59:4356 – 62.

14. Gately S. The contributions of cyclooxygenase-2 to tumor angiogenesis. Cancer Metastasis Rev 2000;19:19 – 27.

15. Sheng H, Shao J, Washington MK, DuBois RN. Prostaglandin E_2 increases growth and motility of colorectal carcinoma cells. J Biol Chem 2001;276:18075 – 81.

16. Ding XZ, Tong WG, Adrian TE. Blockade of cyclooxygenase-2 inhibits proliferation and induces apoptosis in human pancreatic cancer cells. Anticancer Res 2000;20:2625 – 31.

17. El-Rayes BF, Ali S, Sarkar FH, Philip PA. Cyclooxygenase-2-dependent and -independent effects of celecoxib in pancreatic cancer cell lines. Mol Cancer Ther 2004;3:1421 – 6.

18. Yip-Schneider MT, Sweeney CJ, Jung SH, Crowell PL, Marshall MS. Cell cycle effects of nonsteroidal anti-inflammatory drugs and enhanced growth inhibition in combination with gemcitabine in pancreatic carcinoma cells. J Pharmacol Exp Ther 2001;298:976 – 85.

19. Elder DJ, Halton DE, Playle LC, Paraskeva C. The MEK/ERK pathway mediates COX-2-selective NSAID-induced apoptosis and induced COX-2 protein expression in colorectal carcinoma cells. Int J Cancer 2002;99: 323 – 7.

20. Sheng H, Shao J, DuBois RN. Akt/PKB activity is required for Ha-Ras-mediated transformation of intestinal epithelial cells. J Biol Chem 2001; 276:14498 – 504.

21. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E_2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med 2002;8:289 – 93.

22. Kinoshita T, Takahashi Y, Sakashita T, Inoue H, Tanabe T, Yoshimoto T. Growth stimulation and induction of epidermal growth factor receptor by overexpression of cyclooxygenases 1 and 2 in human colon carcinoma cells. Biochim Biophys Acta 1999;1438:120 – 30.

23. Arico S, Pattingre S, Bauvy C, et al. Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. J Biol Chem 2002;277:27613 – 21.

24. Zhu J, Huang JW, Tseng PH, et al. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. Cancer Res 2004;64:4309 – 18.

25. Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004;351:337 – 45.

26. Kris MG, Natale RB, Herbst R, et al. Phase II trial of ZD 1839 in advanced non-small cell lung cancer patients who have failed platinum-and docetaxel regimens. In: Orlando (FL): ASCO proceedings; 2002.

27. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129 – 39.

28. Chung KY, Shia J, Kemeny NE, et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 2005;23: 1803 – 10.

29. Aggarwal BB, Takada Y, Shishodia S, et al. Nuclear transcription factor NF-κ B: role in biology and medicine. Indian J Exp Biol 2004;42:341 – 53.

30. Li Y, Ahmad F, Ali S, Philip P, Kucuk O, Sarkar F. Inactivation of NF-kB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. Cancer Res 2005;65: 6934 – 42.

31. Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse ML, Folsch UR, Schafer H. Role of NF-κB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene 2003;22:3243 – 51.

32. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(κ)B kinase-β. Nature 1998;396:77 – 80.

33. Shishodia S, Koul D, Aggarwal BB. Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates TNF-induced NF-κ B activation through inhibition of activation of I κ B α kinase and Akt in human non-small cell lung carcinoma: correlation with suppression of COX-2 synthesis. J Immunol 2004;173:2011 – 22.

34. Schmedtje JF, Jr., Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. J Biol Chem 1997;272:601 – 8.

35. Nishi H, Neta G, Nishi KH, et al. Analysis of the epidermal growth factor receptor promoter: the effect of nuclear factor-κB. Int J Mol Med 2003;11:49 – 55.

36. Chen Z, Zhang X, Li M, et al. Simultaneously targeting epidermal growth factor receptor tyrosine kinase and cyclooxygenase-2, an efficient approach to inhibition of squamous cell carcinoma of the head and neck. Clin Cancer Res 2004;10:5930 – 9.

37. Tortora G, Caputo R, Damiano V, et al. Combination of a selective cyclooxygenase-2 inhibitor with epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 and protein kinase A antisense causes cooperative antitumor and antiangiogenic effect. Clin Cancer Res 2003;9:1566 – 72.

38. Half E, Freeburg E, Sun Y, Sinicrope F. EGFR and HER-2 receptor blopckade atenuate cyclooxygenase-2 expression and augment NSAID-mediated apoptosis in human colon cancer cells. In: Miami: ASCO GI Cancer Symposium; 2005.

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