Apricoxib, a Novel Inhibitor of COX-2, Markedly Improves Standard Therapy Response in Molecularly Defined Models of Pancreatic Cancer

Amanda Kirane¹, Jason E. Toombs¹, Katherine Ostapoff¹, Juliet G. Carbon¹, Sara Zaknoen², Jordan Braunfeld¹, Roderich E. Schwarz¹, Francis J. Burrows², and Rolf A. Brekken¹

Abstract

**Purpose:** COX-2 is expressed highly in pancreatic cancer and implicated in tumor progression. COX-2 inhibition can reduce tumor growth and augment therapy. The precise function of COX-2 in tumors remains poorly understood, but it is implicated in tumor angiogenesis, evasion of apoptosis, and induction of epithelial-to-mesenchymal transition (EMT). Current therapeutic regimens for pancreatic cancer are minimally effective, highlighting the need for novel treatment strategies. Here, we report that apricoxib, a novel COX-2 inhibitor in phase II clinical trials, significantly enhances the efficacy of gemcitabine/erlotinib in preclinical models of pancreatic cancer.

**Experimental Design:** Human pancreatic cell lines were evaluated in vitro and in vivo for response to apricoxib ± standard-of-care therapy (gemcitabine + erlotinib). Tumor tissue underwent posttreatment analysis for cell proliferation, viability, and EMT phenotype. Vascular parameters were also determined.

**Results:** COX-2 inhibition reduced the IC₅₀ of gemcitabine + erlotinib in six pancreatic cancer cell lines tested in vitro. Furthermore, apricoxib increased the antitumor efficacy of standard combination therapy in several orthotopic xenograft models. In vivo apricoxib combination therapy was only effective at reducing tumor growth and metastasis in tumors with elevated COX-2 activity. In each model examined, treatment with apricoxib resulted in vascular normalization without a decrease in microvessel density and promotion of an epithelial phenotype by tumor cells regardless of basal COX-2 expression.

**Conclusions:** Apricoxib robustly reverses EMT and augments standard therapy without reducing microvessel density and warrants further clinical evaluation in patients with pancreatic cancer. Clin Cancer Res; 18(18); 5031–42. ©2012 AACR.

Introduction

The progression of several human cancers, including pancreatic, has been linked to inflammation, which can trigger secretion of growth factors, infiltration of immune cells, and DNA damage by reactive oxygen species leading to tumor cell proliferation and escape from cell death (1). Epidemiologically, chronic use of anti-inflammatory drugs correlates with a reduction in the incidence of certain cancers, including pancreatic cancer (2, 3). Upregulation of cyclooxygenase-2 (COX-2), the rate limiting step in the synthesis of prostaglandins from arachidonic acid, is an early inflammatory response and is regulated by several growth factors and cytokines (4). Elevated COX-2 expression is frequent in many cancers, including pancreatic cancer where it is expressed highly in up to 75% of cases (5). Increased COX-2 in pancreatic tumors correlates with increased invasiveness and shorter overall survival (6–10). The most abundant product of COX-2 in tumors, prostaglandin E₂ (PGE₂), can affect multiple carcinogenic pathways and participate in proliferation, invasion, angiogenesis, chemoresistance, and metastasis (5, 11–14). In the pancreas under normal conditions, only islet cells express COX-2 (10). As pancreatic intraepithelial lesions develop, COX-2 expression is elevated and the use of COX-2 inhibitors can delay the progression of the disease in preclinical KRas-driven mouse models. Indeed, inhibition of COX-2 can reduce tumor growth in several experimental models of pancreatic cancer (15, 16). Importantly, prostaglandins secreted from stromal fibroblasts facilitate tumor cell proliferation and survival even in tumors and cell lines where COX-2 is not expressed by tumor cells, suggesting that COX-2 is an important signaling molecule in the tumor microenvironment (17). Collectively, these findings make COX-2 an attractive target for anticancer therapy.
Despite advances in diagnostics and treatment, the prognosis for inoperable pancreatic cancer remains poor, largely because of local invasion and metastatic progression at early stages (18). The current standard-of-care for pancreatic cancer is gemcitabine but it affords minimal survival benefit to patients (19). Addition of the COX-2 inhibitor celecoxib to gemcitabine-containing regimens have yielded mixed results; indeed, strategies to augment gemcitabine activity have largely failed to improve overall survival in phase III clinical studies, excepting combination with erlotinib, an inhibitor of EGF receptor (EGFR). However, while statistically significant, erlotinib-based improvement remains modest (20, 21). Rationale exists for the combined targeting of EGFR and COX-2 as significant overlap and interaction occur between these pathways. PGE2 can transactivate EGFR, which can subsequently increase expression of COX-2. In addition, PGE2, via promotion of epithelial-to-mesenchymal transition. Apricoxib is a novel COX-2 inhibitor in phase II clinical trials in pancreatic cancer being investigated as a strategy to augment the efficacy of gemcitabine and erlotinib. Here, in preclinical models of pancreatic cancer, apricoxib significantly enhanced the efficacy of gemcitabine plus erlotinib in reducing primary tumor burden and the occurrence of metastases in orthotopic tumor models with elevated COX-2 activity. Strikingly, apricoxib treatment also robustly prevented tumor cells from adopting a mesenchymal phenotype in vivo, regardless of COX-2 expression levels by the primary tumor. Enhancing an epithelial phenotype via COX-2 inhibition may improve efficacy of chemotherapy.

**Translational Relevance**

Current standard treatment regimens in pancreatic cancer are minimally effective in prolonging overall survival, highlighting the need for novel treatment strategies. The precise function of COX-2 in the tumor microenvironment remains incompletely understood but it has been implicated in tumor angiogenesis and epithelial-to-mesenchymal transition. Apricoxib is a novel COX-2 inhibitor in phase II clinical trials in pancreatic cancer being investigated as a strategy to augment the efficacy of gemcitabine and erlotinib. Here, in preclinical models of pancreatic cancer, apricoxib significantly enhanced the efficacy of gemcitabine plus erlotinib in reducing primary tumor burden and the occurrence of metastases in orthotopic tumor models with elevated COX-2 activity. Strikingly, apricoxib treatment also robustly prevented tumor cells from adopting a mesenchymal phenotype in vivo, regardless of COX-2 expression levels by the primary tumor. Enhancing an epithelial phenotype via COX-2 inhibition may improve efficacy of chemotherapy.

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The mechanistic basis of COX-2 inhibitor therapy in pancreatic cancer remains ill-defined, although in the HT29 model of colorectal cancer reversion of the EMT phenotype has been reported to be a key mechanism of action (26). Apricoxib is a novel, selective COX-2 inhibitor currently in phase II studies for NSCLC and pancreatic cancer. Apricoxib has shown significant antitumor effects in xenograft models of lung and colorectal cancer and seems more potent than previous COX-2 inhibitors (23, 27, 28). In this study, we characterized the baseline expression and activity of EGFR and COX-2 in commonly employed pancreatic cancer cell lines as well as functional responses to inhibition of each pathway. In vivo studies with COX-2–positive and -negative cell lines showed that addition of apricoxib enhanced the antitumor efficacy of gemcitabine and erlotinib in COX-2-dependent models. We present evidence that apricoxib adds significantly to the treatment effect of standard therapy in COX-2 expressing cell lines and also promotes vascular stabilization while reversing EMT regardless of the COX-2 status of the primary tumor.

**Materials and Methods**

**Cell lines**

Human pancreatic cancer cell lines AsPC-1, Su.86.86, HPAF-II, PL45, and CFPAC-1 were obtained from ATCC (Manassas, VA); Colo357 was obtained from MD Anderson Cancer Center. AsPC-1 and PL45 were grown in DMEM, CFPAC-1 in IMDM, Colo357 and HPAF-II in MEM, and Su.86.86 in RPMI (Fisher). All cell lines were grown in a humidified atmosphere with 5% CO2 at 37°C, and have been DNA fingerprinted for provenance using the PowerPlex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained by ATCC and confirmed to be free of mycoplasma by the e-Myco kit (Boca Scientific).

**Baseline expression status**

For Western blot analysis, cell lysates were produced using MPER (Pierce) with added protease and phosphatase inhibitors (Pierce) and protein concentration was determined by BCA assay (Pierce). Immunodetection was conducted by electrophoretic transfer of SDS-PAGE separated proteins to PVDF membranes. Antibodies used for Western blot analysis included EGFR (Upstate), Tyr 1069 phospho-EGFR, and Cox-2 (Santa Cruz).

For PCR analysis, RNA was prepared using TRIzol (Invitrogen) per manufacturer instructions and concentration was determined by spectrophotometry. The cDNA used for subsequent for PCR was made using iScript (Bio-Rad Laboratories) and Choice DNA Taq polymerase (Denville Scientific). The expression of COX-2 and EGFR was analyzed by quantitative real-time PCR using β-actin as an internal reference gene. Each reaction was conducted in triplicate with RNA harvested from 3 independent cell cultures. The comparative Ct method was used to compute relative expression values (29).

**In vitro cytotoxicity and drug response assay**

MTS assays were conducted in 96-well plates; cells were plated on day 0 and drug was added on day 1 in 4-fold dilutions. Drugs were evaluated as single agents with maximum concentration of 2,000 nmol/L for gemcitabine and 400 µmol/L for erlotinib and apricoxib. For combination studies gemcitabine was added with a fixed concentration of 1 µmol/L erlotinib, 0.1 µmol/L apricoxib, or 1 µmol/L apricoxib, and triple combination with 1 µmol/L erlotinib and 1 µmol/L apricoxib. Relative cell number was determined by adding MTS (Promega; final concentration...
incubating for 1 to 3 hours at 37°C, and reading absorbance. Drug sensitivity curves and IC_{50} were calculated using in-house software (30).

Phospho-EGFR ELISA (R&D Systems) was conducted after incubating cells overnight in a 96-well plate, serum starving for 4 hours, and stimulating for 15 minutes with 10 ng/mL EGF. The plate was processed per manufacturer instructions. PGE\textsubscript{2} ELISA (Cayman) was used to evaluate PGE\textsubscript{2} levels in conditioned media. Cells were plated in 24-well plates overnight and incubated for 24 hours with increasing concentrations of apricoxib in low serum medium. Assays were conducted in triplicate and were carried out minimum of 3 times.

**Animal studies**

All animals were housed in a pathogen-free facility with 24-hour access to food and water. Experiments were approved by, and conducted in accordance with, the IACUC at UT Southwestern (Dallas, TX). Four- to 6-week-old female NOD/SCID mice were obtained from a campus supplier. A total of 1 x 10\textsuperscript{6} AsPC-1, Colo357, and HPAF-II cells were injected orthotopically as described (30) and tumor growth monitored by ultrasound. Mice with established tumors were randomized to receive either gemcitabine 25 mg/kg twice weekly plus erlotinib 100 μg daily (standard-of-care), or standard-of-care plus 10 or 30 mg/kg apricoxib daily by oral gavage. Mice bearing Colo357 and AsPC-1 tumors received 3 weeks of therapy prior to sacrifice. Animals bearing HPAF-II tumors were divided into an early sacrifice at 3 weeks and a late sacrifice at 7 weeks of therapy. Primary tumor burden was established by weighing pancreas and tumor en bloc. Metastatic incidence was determined by visual inspection of the liver and abdominal cavity as well as by quantitation of H&E liver sections. Tissues were fixed in 10% formalin or snap-frozen in liquid nitrogen for further studies.

**Histology and tissue analysis**

Formalin-fixed tissues were embedded in paraffin and cut in 10-μm sections. Sections were evaluated by H&E and immunohistochemical analysis using antibodies CD31 (Dianova), NG2 (Millipore), vimentin (Phosphosolutions), endomucin, Zeb1, E-cadherin, PCNA (Santa Cruz), phospho-histone H3 (Upstate), TUNEL (Promega), cleaved caspase-3 (Cell Signaling), VEGF, and COX-2 (Abcam). Negative controls included omission of primary antibody and immunofluorescent evaluation was conducted as described (31). Human and mouse VEGF levels in plasma and tumor lysates were determined by ELISA (R&D Systems) per manufacturer instructions.

**Statistics**

Data were analyzed using GraphPad software (GraphPad Prism version 4.00 for Windows; GraphPad Software; www.graphpad.com). Results are expressed as mean ± SEM. Data were analyzed by t test or ANOVA and results are considered significant at P < 0.05.
Figure 1. Baseline expression and functional activity of EGFR and COX-2 in human pancreatic cancer cell lines. A, RNA was harvested from human pancreatic cancer cell lines and evaluated by qPCR for expression of COX-2 and EGFR; levels were normalized to β-actin internal loading control. B, the expression of EGFR, p-EGFR at ty1068, and COX-2 was determined by Western blot analysis. Expression of β-actin was used as a loading control. C, the IC50 of erlotinib for inhibition of phosphorylation of EGFR after stimulation with 10 ng/mL EGF was determined by ELISA. D, PGE2 levels in conditioned media measured by ELISA after 24-hour incubation with apricoxib.

Table 1. Apricoxib enhances in vitro sensitivity to gemcitabine

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Apricoxib, μmol/L</th>
<th>Erlotinib, μmol/L</th>
<th>Gemcitabine, nmol/L</th>
<th>1 μmol/L E</th>
<th>0.1 μmol/L A</th>
<th>1 μmol/L A</th>
<th>1 μmol/L A/E</th>
<th>Fold change</th>
</tr>
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<tbody>
<tr>
<td>ASPC-1</td>
<td>70 (16)</td>
<td>375 (15)</td>
<td>2,000 (0)</td>
<td>1,630 (319)</td>
<td>2,000 (3)</td>
<td>830 (216)</td>
<td>537 (200)</td>
<td>−3.7</td>
</tr>
<tr>
<td>Colo357</td>
<td>30 (14)</td>
<td>57 (10)</td>
<td>158 (51)</td>
<td>92 (7)</td>
<td>116 (31)</td>
<td>98 (16)</td>
<td>52 (8)</td>
<td>−2.9</td>
</tr>
<tr>
<td>HPAF-II</td>
<td>80 (1)</td>
<td>400 (0)</td>
<td>2,000 (0)</td>
<td>172 (32)</td>
<td>2,000 (0)</td>
<td>400 (23)</td>
<td>13 (2)</td>
<td>−13.2</td>
</tr>
<tr>
<td>CFPAC-1</td>
<td>19 (1)</td>
<td>4.3 (1.3)</td>
<td>2.7 (1)</td>
<td>ND</td>
<td>2.1 (0.1)</td>
<td>2 (0.3)</td>
<td>0.72 (0.5)</td>
<td>−3.75</td>
</tr>
<tr>
<td>PL45</td>
<td>26 (15)</td>
<td>400 (0)</td>
<td>12.8 (6)</td>
<td>ND</td>
<td>9.6 (1)</td>
<td>9.1 (0.5)</td>
<td>7 (0.6)</td>
<td>−1.8</td>
</tr>
<tr>
<td>Su.86.86</td>
<td>77 (8)</td>
<td>400 (0)</td>
<td>2,000 (0)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>630 (1)</td>
<td>−3.17</td>
</tr>
</tbody>
</table>

NOTE: The mean IC50 (SD) for gemcitabine, erlotinib, and apricoxib was determined by MTS assay as single agents or in combination. Means represent a minimum of 3 independent assays with conditions conducted in octuplicate per assay. The fold change in the IC50 for gemcitabine in the presence of apricoxib (A) and erlotinib (E) each at 1 μmol/L is displayed. SD = 0 indicates that an IC50 value was not achieved and the maximum concentration of drug used is displayed. Abbreviation: ND, not determined.
sensitivity to apricoxib as a single agent, with IC_{50} values of 70 to 80 μmol/L. Cell lines insensitive to gemcitabine (IC_{50} not achieved with maximum dose 2000 nmol/L) included AsPC-1, HPAF-II, and Su.86.86. Highly gemcitabine sensitive lines included PL45 and CFPAC-1. Colo357 showed an intermediate antiproliferative response with an IC_{50} of 150 μmol/L. Addition of 0.1 μmol/L apricoxib did not significantly alter response to gemcitabine in any cell line other than Colo357, which showed 1.3-fold reduction in gemcitabine IC_{50}. Apricoxib at 1 μmol/L enhanced the response of AsPC-1, HPAF-II, and Colo357 to gemcitabine but had little effect on gemcitabine activity in the remaining lines. Erlotinib augmented response to gemcitabine in all lines evaluated. Triple combination of gemcitabine, 1 μmol/L erlotinib, and 1 μmol/L apricoxib showed measurable reduction of gemcitabine IC_{50} in all cell lines (Table 1); sensitization was particularly marked in HPAF-II cells, where the gemcitabine IC_{50} was reduced by over 3 orders of magnitude by the combination of apricoxib and erlotinib. Colo357 and HPAF-II, as lines expressing COX-2 and showing sensitization to gemcitabine with COX-2 inhibition, were selected for further in vivo studies in addition to AsPC-1, a COX-2–negative cell line.

**Inhibition of COX-2 significantly reduces primary tumor growth in vivo and metastatic incidence in cell lines with high COX-2 expression**

From the in vitro data we selected a COX-2–negative cell line, AsPC-1, and 2 COX-2–positive cell lines, one with minimal erlotinib response, Colo357, and a highly erlotinib sensitive line, HPAF-II for in vivo studies. Mice began therapy 10 to 14 days postorthotopic tumor cell injection. Therapy consisted of control (vehicle alone), standard therapy, that is 25 mg/kg gemcitabine and the effect was further enhanced by apricoxib treatment (P < 0.05; ANOVA) and was not statistically significant in either group. Apricoxib treatment virtually eliminated COX-2 expression in tumors from both cell lines. PGE_{2} is rapidly metabolized in plasma and therefore difficult to assess in vivo. However, PGE_{2} stimulates COX-2 expression, forming a positive feedback loop, so reduction in overall COX-2 levels shows that apricoxib effectively inhibits PGE_{2} production in vivo (32, 33). In COX-2 expressing tumors, this resulted in increased efficacy of standard therapy (Fig. 2A and B). It has been reported that a COX-2 nonexpressing cell line can upregulate COX-2 under conditions of stress. To recapitulate in vivo changes resulting from tumor–host interactions, PGE_{2} production was evaluated after forced EMT (26). In Colo357, PGE_{2} production was not significantly different between normal and EMT conditions at baseline. At 24 hours after a single dose of apricoxib (0.5 μmol/L), representing the lowest range of in vivo levels (26), PGE_{2} levels had decreased by 50% for both conditions. AsPC-1 cells, which produce no measurable amount of PGE_{2} at baseline, showed a significant increase in PGE_{2} levels after induction of EMT. In contrast to Colo357, this was not affected by apricoxib (Fig. 2E).

**Inhibition of COX-2 attenuates VEGF and promotes vascular normalization in vivo**

COX-2 expression correlates with VEGF levels in patient tumor samples and COX-2 inhibition can modulate VEGF production (27, 34). VEGF levels in plasma and tumor samples collected from mice tumor-bearing mice were
measured by ELISA. In AsPC-1 tumor-bearing mice, plasma VEGF levels were decreased in all treatment groups, but these differences were not significantly different from control-treated animals. In Colo357 tumor-bearing mice, VEGF levels were unaffected by G+E treatment but addition of apricoxib resulted in depletion of human VEGF from plasma samples (Fig. 3A). Interestingly, these changes did not correlate with intratumoral levels of human VEGF. In fact, the lowest levels of tumor-associated VEGF were found in G+E-treated animals (NS for AsPC-1 tumors, \( P < 0.005 \) in Colo357 tumors, Fig. 3B).

To assess the ultimate effect of COX-2 inhibition on angiogenesis, tumor microvessel density was evaluated by immunofluorescence for endomucin and CD31 (CD31 shown; Fig. 3C and D). Vessel density was decreased moderately by G+E alone (NS for Colo357 but trending downward, \( P < 0.05 \) for AsPC-1); however, addition of apricoxib to the treatment regimen did not significantly alter the effect of G+E on vessel density (Fig. 3C). To determine if therapy affected pericyte–endothelial cell interaction, NG2, a pericyte marker, was colocalized with endomucin or CD31 to assess pericyte coverage of vessels. Pericyte coverage index (%NG2 positive vessels) increased significantly in apricoxib-treated AsPC-1 and Colo357 tumors (\( P < 0.001 \) AsPC-1, \( <0.05 \) Colo357, ANOVA; Fig. 3C, D). In summary, COX-2 inhibition did alter plasma levels of VEGF but this did not translate to a change in levels of tumor-associated VEGF or neovascularization, instead COX-2 inhibition promoted vascular stabilization, which may improve drug delivery as well as contribute to the reduction of metastases (35).

**Inhibition of COX-2 reduces proliferation and increases apoptosis**

Inhibition of COX-2 activity with apricoxib enhanced the antitumor effect of G+E. To determine if this was because...
of changes in cell proliferation or survival, we evaluated markers of apoptosis and cell proliferation in AsPC-1 and Colo357 tumors. The level of apoptosis in tumors from control and treated animals was determined by TUNEL (Fig. 4A and B). Apricoxib significantly increased the number of apoptotic cells in AsPC-1 and Colo357 tumors. The increase in TUNEL was most evident at the 10 mg/kg dose (Fig. 4A), but animals treated with 30 mg/kg of apricoxib showed the highest levels of cleaved caspase-3 levels (data not shown). In AsPC-1 tumors, proliferative markers (phospho-histone H3 and PCNA) were unchanged (data not shown and Fig. 4C). By contrast, apricoxib strongly enhanced the modest anti-proliferative effect of G+E in Colo357 tumors, especially at the 30 mg/kg dose (P < 0.01; Fig. 4C and D). To substantiate the effect of apricoxib on the efficacy of standard therapy, we evaluated γH2AX levels, which have been shown to mark gemcitabine-induced stalled replication forks (36). γH2AX expression was evaluated by immunofluorescence in Colo357 tumor sections and found to be significantly increased in apricoxib treated tumors compared with either control or G+E treatment alone (P < 0.05; Fig. 4E).

**COX-2 inhibition reverses EMT**

We have recently found that apricoxib reverses EMT in HT29 xenografts (26); thus, we assessed tumor sections in this study for epithelial (E-cadherin) and mesenchymal (vimentin, and Zeb-1) markers by immunofluorescence. In Colo357 tumors, vimentin and Zeb1 expression decreased in a dose-dependent fashion in apricoxib-treated animals compared with control and G+E groups, although changes in vimentin expression did not reach statistical significance (P < 0.05 vs. control, G+E for Zeb1; Fig. 5A). Conversely, E-cadherin was strongly induced by apricoxib treatment (P < 0.001 vs. control, G+E; Fig. 5A). Representative double-stained images are shown for vimentin and E-cadherin in Colo357 tumors (Fig. 5B). Untreated Colo357 tumors showed a mesenchymal phenotype with strong vimentin positive staining and negligible E-Cadherin expression. By comparison, Colo357 cells in vitro show an epithelial phenotype that seems preserved (or reacquired)
after apricoxib treatment (Fig. 5C). Treatment of cells in culture with G+E alone actually increased Zeb1 expression, but combination with apricoxib reversed this effect and dramatically upregulated E-Cadherin expression (Fig. 5C).

Inhibition of COX-2 resulted in a shift toward a more epithelial phenotype regardless of baseline COX-2 status of the tumor; although this effect was more dramatic in high COX-2 expressing Colo357 tumors and correlated with the overall reduction of metastatic incidence. AsPC-1 cells, which increased COX-2 expression in vivo and following EMT in vitro (Fig. 2E), show this shift toward epithelial phenotype by apricoxib treatment with decreased vimentin and Zeb1 and dramatically increased E-Cadherin expression (Fig. 5D). In vitro, Zeb1 expression by AsPC-1 cells is unchanged after G+E or combination with apricoxib. As these cells have undetectable COX-2 expression and PGE2 production under normal culture conditions, EMT was induced in these cells to recapitulate in vivo changes. Following EMT, apricoxib treatment either in combination with G+E or as a single agent at 1 μmol/L significantly diminished Zeb1 expression, this shift did not occur with G+E alone, suggesting that COX-2 is intimately involved with the occurrence of EMT and that transformed cells that undergo EMT show increased sensitivity to apricoxib.

Discussion

Resistance to chemotherapy remains a major challenge in the treatment of pancreatic cancer, combination strategies to augment gemcitabine failed to show improved overall survival in phase III clinical trials before addition of erlotinib (20). Early results of small phase II studies combining the COX-2 inhibitor celecoxib with gemcitabine or gemcitabine plus irinotecan showed promise with prolonged survival compared with the historic average survival of 6 months for gemcitabine (23). Apricoxib is currently in phase II clinical trials in NSCLC and pancreatic cancer. Our studies aimed to determine the antitumor activity of apricoxib in molecularly defined preclinical pancreatic cancer models using colorectal and pancreatic cancer cell lines.

Figure 4. COX-2 inhibition enhances therapy-induced effects on cell survival and proliferation in pancreatic cancer xenografts. A, apoptosis was evaluated through TUNEL analysis in sections of AsPC-1 and Colo357 tumors. Data are displayed as mean% area fraction ± SEM and represent 5 images per tumor with 5 tumors per group evaluated. B, representative images of TUNEL (green) reactivity in Colo357 tumors are shown. Total magnification, 200×; scale bar, 100 μm. C, paraffin-embedded sections of AsPC-1 and Colo357 tumors were analyzed for cell proliferation (PCNA) by immunofluorescence. Data are displayed as mean% area fraction ± SEM and represent 5 images per tumor with 5 tumors per group evaluated. D, representative images of PCNA (green) immunofluorescence in sections of Colo357 tumors are displayed. Total magnification, 400×; scale bar, 50 μm. E, Colo357 tumors were analyzed for H2AX by immunofluorescence. Data are displayed as mean% area fraction and represent 5 images per tumor with 5 tumors per group evaluated; representative images are shown (H2AX, red; DAPI, blue). Total magnification 400×; **, P < 0.05, *** P < 0.005 vs. control; #, P < 0.05, ###, P < 0.005 vs. G+E.
models with the hope of identifying subsets of pancreatic cancer patients who would benefit from the addition of COX-2 inhibition to current therapy.

In vitro, we determined the mRNA and protein expression of EGFR and COX-2 in cancer cell lines and measured how these targets contribute to cellular response to gemcitabine. We found in all cell lines that concentrations of apricoxib required to exert direct antitumor activity far exceeded those needed to eliminate PGE2 production (0.5–2 μmol/L). However, apricoxib at pharmacologically achievable concentrations sensitized cells to standard therapy. This suggests that antitumor effects of COX-2 inhibition in these models in vivo resulted primarily from sensitization to gemcitabine/erlotinib therapy or modification of tumor-host interactions as opposed to direct antitumor cell effects, although it is possible that COX-2-dependent lines would have responded under anchorage-independent growth conditions (26). This is also supported by the increased production of PGE2 in vitro by AsPC-1 following forced EMT corresponding to increased COX-2 expression by cells in vivo which have adopted an increasingly mesenchymal phenotype.

In vivo, in mice bearing tumors derived from AsPC-1, a COX-2 low-expressing cell line, addition of apricoxib to standard therapy did not improve antitumor activity nor dramatically effect cell proliferation as measured by PCNA expression. By contrast, in cell lines that express moderate to high levels of COX-2 and produce high levels of PGE2, significantly increased efficacy was achieved by the addition of apricoxib. These results suggest that clinical studies should consider COX-2 activity, not simply expression, when evaluating apricoxib or other COX-2 inhibitors in cancer patients. Assaying the level of PGEM, a PGE2 metabolite, is a potential strategy for patient stratification.

COX-2 and PGE2 are strongly linked to angiogenesis via promotion of VEGF and bFGF production stimulating growth, migration, and survival of endothelial cells (27, 34). Reciprocally, these factors form a positive feedback
loop amplifying the production of COX-2. In our studies, apricoxib did modulate VEGF levels in plasma but this did not result in a reduction in microvessel density. In all tumor groups, VEGF levels were observed to be lowest in tumors that received standard (G + E) therapy. High expression of COX-2 corresponds to high VEGF levels in tumor specimens; however, studies have reported that although VEGF production is reduced initially by COX-2 inhibition, its production is not exclusively COX-2 dependent. Recovery and amplification of VEGF levels may occur as a compensatory response to loss of COX-2 activity and levels of VEGF continue to increase with higher doses of COX-2 inhibitors (15, 37, 38). This may provide a plausible explanation for the paradoxical dose–response in our models, as well as other preclinical studies. Furthermore, these data might also help explain the failure of COX-2 inhibitors to show significant improvement in clinical studies that previously employed doses twice that used for analgesic and anti-inflammatory effect (39, 40). Reciprocally, mechanisms of anti-VEGF resistance may involve induction of other proangiogenic cytokines, such as IL-1β and IL-8 that are associated with increased COX-2 production in the tumor microenvironment. This collateral pathway may explain why COX-2 inhibition alone failed to be significantly antiangiogenic in this model.

Microvessel density was reduced in all therapy groups receiving standard therapy and was unchanged by the addition of apricoxib. The decrease in microvessel density by treatment with gemcitabine has been reported previously (41) and may be a result of the dosing schedule employed. However, apricoxib treatment resulted in increased pericyte coverage of blood vessels. Pericyte attachment is critical for stabilizing vascular structures (35). Absent or loose pericyte attachment results from imbalanced angiogenic signaling in the tumor leading to dysfunctional vessels that are hallmark by hyperpermeability. These vessels display abnormal blood flow than can increase tumor hypoxia, reduce delivery of chemotherapy and facilitate extravasation and hematogenous spread of metastatic cells (35). COX-2 inhibition impeded this process, suggesting that COX-2 participates in vascular remodeling in the tumor microenvironment.

The COX-2 product PGE2 can impact tumor progression and cancer cell proliferation by activating the Ras-MAPK signaling cascade, which subsequently increases expression of PGE2 synthase, forming a positive feedback loop (42, 43). In addition, exogenous PGE2 can stimulate proliferation of COX-2–negative cells in vitro (17). PGE2 can mediate cell survival by inducing expression of antiapoptotic proteins such as Bcl-2 and increasing NF-κB transcriptional activity (44). Chemotherapy can increase COX-2 protein expression and PGE2 production, driving tumor cell survival and resistance to therapy. In this study, proliferative activity in vivo was not affected by COX-2 inhibition in AsPC-1 tumors; however, apricoxib significantly reduced proliferation in Colo357 tumors. Apoptosis resulting from COX-2 inhibition, alternatively, was not specific to COX-2 status of the tumor and in each group was most profound in the 10 mg/kg group; indicating that adaptation of AsPC-1 cells in vivo can lead to increased dependency on COX-2. γH2AX has been implicated as a marker of gemcitabine specific DNA damage and was found to be significantly elevated in apricoxib treated tumors. This finding correlated with improvement in apoptotic activity and vessel maturity, indicating that improved chemotherapeutic response may be related in part to improved drug delivery.

COX-2 and PGE2 are implicated in driving EMT (45, 46), which contributes to metastasis and resistance to chemotherapy (47, 48). We found that untreated Colo357 and AsPC-1 tumors displayed a robust EMT phenotype. In contrast, tumor-bearing mice that received apricoxib had a noticeable shift to an epithelial phenotype. These observations suggest that COX-2 inhibition can reverse EMT in pancreatic tumors. PGE2 directly induces the transcription factor Zeb1 and enhances its binding to the proximal e-box of the E-cadherin promoter, resulting in downregulation of E-cadherin. E-cadherin is essential for intercellular adhesion, such that disruption facilitates migration, invasion, and metastasis (5, 49). Clinical tumor samples have shown an inverse relationship between COX-2 expression/high-grade tumor type and E-cadherin expression (49). Vimentin, a marker of mesenchymal differentiation, is also highly expressed in pancreatic cancers and correlates with poor prognosis (47). Notably, gemcitabine resistance has been linked to vimentin expression in pancreatic cancer cells (50). In vitro, apricoxib dramatically increased E-cadherin expression in Colo357 cells as well as reversed the increased Zeb1 production observed after G + E treatment. Unsurprisingly, no affect was seen in AsPC-1 cells at baseline as they have minimal baseline COX-2 activity. However, the increased COX-2 production with forced EMT greatly sensitized these cells to apricoxib and showed apricoxib effectively reversed EMT. This suggests that the maintenance of the mesenchymal phenotype in these cells relies in part on the activity of COX-2. In vivo, minimal change was seen in the mesenchymal phenotype of Colo357 tumors with standard therapy, although G + E increased Zeb-1 and vimentin in AsPC-1 tumors. Addition of apricoxib significantly shifted AsPC-1 tumors toward a more epithelial phenotype, although it should be noted that AsPC-1 tumors are typically epithelial at baseline. This may explain why these shifts, while present, may not have an overwhelming impact on overall tumor response. Importantly, Colo357 tumors showed a dose-dependent mesenchymal-to-epithelial transition (MET). Although this did not translate into a reduction in metastatic burden for AsPC-1 tumors, the effect was dramatic in Colo357 tumor-bearing mice, implicating COX-2 as important in governing EMT and sensitization to standard therapy in pancreatic cancer.

In summary, our findings show that the clinical COX-2 inhibitor, apricoxib, enhances the efficacy of standard chemotherapy in preclinical models of pancreatic cancer. Inhibition of COX-2-mediated PGE2 production enhanced the antiproliferative and apoptotic effect of standard therapy in COX-2-dependent tumors and affected the tumor microenvironment by promoting vascular normalization and
attenuating EMT. Further clinical evaluation of apricoxib in a molecularly selected patient population is warranted in the development of strategies to improve treatment for pancreatic cancer.

Disclosure of Potential Conflicts of Interest

R.A. Brekken has received a commercial research grant from Tragara Pharmaceuticals, Inc. F. J. Burrows and S. Zaknoen are employees of Tragara Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


References


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Kirane, R. E. Schwarz, F. J. Burrows. Study supervision: A. Kirane, R. A. Brekken.

Acknowledgments

The authors thank Surya Vadrevu for technical assistance and Drs. Joan Schiller, David H. Johnson, and John Minna and members of the Brekken laboratory for advice and thoughtful discussion.

Grant Support

This work was supported in part by a sponsored research agreement from Tragara Pharmaceuticals, Inc. and the Effie Marie Cain Scholarship in Angiogenesis Research (to R. A. Brekken) and an institutional training grant from the NCI (T32 CA136515, to A. Kirane).

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Received February 21, 2012; revised July 7, 2012; accepted July 14, 2012; published OnlineFirst June 24, 2012.


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Amanda Kirane, Jason E. Toombs, Katherine Ostapoff, et al.


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