Apricoxib, a novel inhibitor of COX-2 markedly improves standard therapy response in molecularly defined models of pancreatic cancer

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Statement of 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.
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 post-treatment analysis for cell proliferation, viability and EMT phenotype. Vascular parameters were also determined.

Results: COX-2 inhibition reduced the IC_{50} of gemcitabine +/- erlotinib in six pancreatic cancer cell lines tested in vitro. Furthermore, apricoxib increased the anti-tumor 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.
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 anti-cancer therapy.

Despite advances in diagnostics and treatment, the prognosis for inoperable pancreatic cancer remains poor, largely due to 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 epidermal growth factor 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 (EMT), can increase resistance to EGFR inhibitors (22). Furthermore, short-circuiting both pathways simultaneously can have synergistic effects in preclinical cancer models (23-25).

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 demonstrated significant antitumor effects in xenograft models of lung and colorectal cancer and appears more potent than previous COX-2 inhibitors (23, 27, 28). In the present 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 demonstrated that addition of apricoxib enhanced the anti-tumor 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 performed 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 performed 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 performed in 96-well plates; cells were plated on day 0 and drug was added on day 1 in four-fold dilutions. Drugs were evaluated as single agents with maximum concentration of 2000 nM for gemcitabine and 400 μM for erlotinib and apricoxid. For combination studies gemcitabine was added with a fixed concentration of 1 μM erlotinib, 0.1 μM apricoxid, or 1 μM
apricoxib, and triple combination with 1 μM erlotinib and 1 μM apricoxib. Relative cell number was determined by adding MTS (Promega, final concentration 333 μg/ml), incubating for 1 to 3 hours at 37°C, and reading absorbance. Drug sensitivity curves and IC50s were calculated using in-house software (30).

Phospho-EGFR ELISA (R&D Systems) was performed 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. PGE2 ELISA (Cayman) was used to evaluate PGE2 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 performed in triplicate and were performed a minimum of three 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 performed in accordance with, the IACUC at UT Southwestern (Dallas, TX). 4-6 weeks old female NOD/SCID mice were obtained from a campus supplier. 1x10^6 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 mg/kg 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 micron 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 performed 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, San Diego, CA, www.graphpad.com). Results are expressed as mean ± SEM. Data was analyzed by t-test or ANOVA and results are considered significant at p < 0.05.
Results

Human pancreatic cancer cell lines vary in expression of COX-2 and EGFR and show differential response to inhibition.

Five human pancreatic cancer cell lines were screened for baseline expression of COX-2 and EGFR by qPCR. All cell lines expressed detectable levels of mRNA for EGFR and COX-2. There was generally close accordance between message and protein, except COX-2 levels in AsPC-1 cells that were detectable (albeit barely) by qPCR (Fig. 1A) but not at the protein level (Fig. 1B). Colo357 cells demonstrated moderate to high expression of COX-2 at the mRNA and protein level (Fig. 1A, B). PL45, HPAF-II, and CFPAC-1 cells exhibited low to moderate expression of COX-2 at the mRNA and protein level (Fig. 1A, B). EGFR expression was detectable in all cell lines by qPCR. The majority of cells displayed low to moderate expression with the exception of AsPC-1, which demonstrated relatively high expression (Fig. 1A). Expression of EGFR at the protein level was assessed by Western blot and confirmed robust expression of EGFR in AsPC-1 cells. However, it was notable that phosphorylated EGFR (p-EGFR) was weak or not detected in the majority of cell lines under basal conditions. In contrast there was a strong p-EGFR signal in Colo357 cells (Fig. 1B). The efficacy of erlotinib inhibition of p-EGFR after stimulation with EGF in each cell line was determined by ELISA (Fig. 1C). AsPC-1 and HPAF-II cells were most sensitive with IC_{50}s of 0.1 μM and 0.2 μM, respectively. All cells demonstrated an IC_{50} below the pharmacologic range (2.2 μM) with the exception of PL45 cells, which were resistant to erlotinib at maximum dose (500 μM, Fig. 1C). To determine the effect of apricoxib, conditioned media of cells incubated for 24 hours with apricoxib was collected and PGE_{2} levels determined. Baseline levels of PGE_{2} production by AsPC-1 and PL45 cells was minimal at <10 pg/ml (below detectable range) and remained unchanged by apricoxib treatment (AsPC-1 shown, Fig. 1D). Apricoxib effectively prevented PGE_{2} production by Colo357 and HPAF-II cells with IC_{50}s within the pharmacologic range (0.5-2 μM, Colo357, shown in Fig. 1D). CFPAC-1 displayed a dose-dependent reduction but did not achieve an IC_{50} within the tested range (max 1 μM, data not shown). From this we concluded that all cell lines except PL45 demonstrate inhibition of EGFR activity in vitro at
pharmacologically relevant concentrations of drug; however, while most cell lines express COX-2, this did not directly correlate to level of PGE₂ production. For cell lines with detectable PGE₂ expression, apricoxib inhibited its production at nanomolar concentrations in vitro.

**Erlotinib and apricoxib combination therapy augments efficacy of gemcitabine in vitro.**

MTS assays were used to evaluate the antiproliferative effect of each drug as a single agent and in combination. As shown in Table 1, the IC₅₀ for erlotinib and apricoxib as single agents was above pharmacologic range for all cell lines. AsPC-1, Su.86.86 and HPAF-II demonstrated the least sensitivity to apricoxib as a single agent, with IC₅₀ values of 70-80 μM. Cell lines insensitive to gemcitabine (IC₅₀ not achieved with maximum dose 2000 nM) included AsPC-1, HPAF-II, and Su.86.86. Highly gemcitabine sensitive lines included PL45 and CFPAC-1. Colo357 demonstrated an intermediate antiproliferative response with an IC₅₀ of 150 μM. Addition of 0.1 μM apricoxib did not significantly alter response to gemcitabine in any cell line other than Colo357, which demonstrated 1.3-fold reduction in gemcitabine IC₅₀. Apricoxib at 1 μM 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 μM erlotinib, and 1 μM apricoxib showed measurable reduction of gemcitabine IC₅₀ in all cell lines (Table 1); sensitization was particularly marked in HPAF-II cells, where the gemcitabine IC₅₀ was reduced by over three orders of magnitude by the combination of apricoxib and erlotinib. Colo357 and HPAF-II, as lines expressing COX-2 and demonstrating 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 two 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 10-14 days post orthotopic tumor cell injection. Therapy consisted of control (vehicle alone), standard therapy, i.e. 25 mg/kg gemcitabine + 100 μg erlotinib (G+E), or G+E + apricoxib at 10 or 30 mg/kg. AsPC-1 and Colo357 tumor bearing mice were sacrificed after 3 weeks of therapy. Tumor and pancreas were dissected en bloc to determine tumor weight. Metastatic burden was initially surveyed by inspection of the abdominal cavity, diaphragm, and liver and later quantified by H&E staining of the left lobe of the liver. For AsPC-1 tumors, addition of apricoxib resulted in a significant decrease in primary tumor weight compared to control treated animals (p<0.05, two tailed t-test, Mann-Whitney); however, the effect was equivalent to the standard of care, demonstrating that the antitumor activity was due to gemcitabine and erlotinib in this model (Fig. 2A). Metastatic burden in AsPC-1 bearing mice was highly variable with no significant differences between groups (Fig. 2B). Mice bearing Colo357 tumors demonstrated a significant reduction in primary tumor size with the standard of care alone compared to saline (p<0.05, ANOVA) and the effect was further enhanced by apricoxib treatment (p<0.05, ANOVA). Metastases in animals bearing Colo357 were virtually eliminated in apricoxib treated groups, with 10 mg/kg showing greatest response (p<0.005 compared to G+E, Dunn MCT, Fig. 2B). Mice bearing HPAF-II tumors received therapy for 3 weeks, at which point saline controls were moribund and required sacrifice. HPAF-II tumors were highly sensitive to erlotinib treatment and at this time all treatment groups had virtually no tumor growth, thus it was elected to sacrifice only 3 animals /group for comparison to control and allow remaining mice to continue on therapy. There were no differences in tumor burden amongst treatment groups at the 3 week time point with all final weights being equivalent to normal pancreas weight. The remaining animals continued on therapy for an additional 4 weeks, at which point apricoxib-treated animals showed superior tumor control rate compared to G+E (p<0.001, ANOVA). Metastases in the HPAF-II model were suppressed significantly in all treatment groups compared to control animals, but again apricoxib enhanced the activity of G+E, abolishing metastatic spread altogether (Fig. 2A, B).
Given the small primary tumor burden of HPAF-II, which largely consisted of normal pancreas, only Colo357 and AsPC-1 tumors were used for further evaluation. Paraffin embedded tumor sections were used to analyze COX-2 expression by immunofluorescence.

**COX-2 expression can be induced in vivo and in vitro upon EMT in a COX-2 negative cell line**

Notably, while AsPC-1 cells did not express measurable levels of COX-2 in vitro, untreated AsPC-1 xenografts stained positive for COX-2, although to a far lesser extent than Colo357 tumors. Interaction of EGFR and COX-2 signaling (24) is supported by a clear reduction in COX-2 expression by G+E treatment, although this value 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 demonstrates 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 2. A, B). It has been reported that a COX-2 non-expressing 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 following a single dose of apricoxib (0.5 μM), 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, demonstrated a significant increase in PGE$_2$ levels following induction of EMT. In contrast to Colo357, this was not affected by apricoxib (Fig 2. E).

**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<.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, 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 anti-tumor effect of G+E. To determine if this was due to 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, 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, 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 to 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 xenograft (26); thus we assessed tumor sections in the current 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 to 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 demonstrated a mesenchymal phenotype with strong vimentin positive staining and negligible E-Cadherin expression. By comparison, Colo357 cells in vitro demonstrate an epithelial phenotype which appears 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), demonstrate 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 μM 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 demonstrate 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 demonstrate improved overall survival in phase III clinical trials prior to 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 to 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 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 μM). 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 anti-tumor 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 anti-tumor 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 while 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. Further these data might also help explain the failure of COX-2 inhibitors to demonstrate 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 pro-angiogenic 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 hallmarked 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 PGE$_2$ can impact tumor progression and cancer cell proliferation by activating the Ras-MAPK signaling cascade, which subsequently increases expression of PGE$_2$ synthase, forming a positive feedback loop (42, 43). Additionally, exogenous PGE$_2$ can stimulate proliferation of COX-2 negative cells in vitro (17). PGE$_2$ can mediate cell survival by inducing expression of antiapoptotic proteins such as Bcl-2 and increasing NFkB transcriptional activity (44). Chemotherapy can increase COX-2 protein expression and PGE$_2$ production, driving tumor cell survival and resistance to therapy. In the present 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 PGE$_2$ 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. PGE$_2$ directly induces the transcription factor Zeb1 and enhances its binding to the proximal e-box of the E-cadherin promoter, resulting in down regulation 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 effect 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 demonstrated that 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, while G+E increased Zeb-1 and vimentin in AsPC-1 tumors. Addition of apricoxib significantly shifted AsPC-1 tumors towards 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 demonstrated a dose-dependent mesenchymal to epithelial transition (MET). While 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 demonstrate 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: commercial research grant from Tragara Pharmaceuticals, Inc

F.J. Burrows and S. Zaknoen: employees of Tragara Pharmaceuticals, Inc

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REFERENCES


Table 1. Apricoxib enhances in vitro sensitivity to gemcitabine

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Apricoxib (µM)</th>
<th>Erlotinib (µM)</th>
<th>Gemcitabine (nM)</th>
<th>Gemcitabine (nM)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 µM E</td>
<td>0.1 µM A</td>
</tr>
<tr>
<td>ASPC-1</td>
<td>70 (16)</td>
<td>375 (15)</td>
<td>2000 (0)</td>
<td>1630 (319)</td>
<td>2000 (0)</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>
</tr>
<tr>
<td>HPAF-II</td>
<td>80 (1)</td>
<td>400 (0)</td>
<td>2000 (0)</td>
<td>172 (32)</td>
<td>2000 (0)</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 (0.1)</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>
</tr>
<tr>
<td>Su.86.86</td>
<td>77 (8)</td>
<td>400 (0)</td>
<td>2000 (0)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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 performed in octuplicate per assay. The fold change in the IC₅₀ for gemcitabine in the presence of apricoxib (A) and erlotinib (E) each at 1 µM is displayed. SD = 0 indicates that an IC₅₀ value was not achieved and the maximum concentration of drug used is displayed. ND, not determined.
Figure Legends

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 tyr1068, and COX-2 was determined by Western blot analysis. Expression of β-actin was used as a loading control. C, The IC_{50} 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.

Figure 2. COX-2 inhibition results in decreased primary tumor growth and metastatic burden. A & B, AsPC-1 (n=6-8 animals/group), Colo357 (n=7-8 animals/group), and HPAF-II (n=7-10 animals/group) human pancreatic cancer cells (1x10^6) were injected orthotopically into the pancreas of SCID mice. Treatment began when established tumor was visible by ultrasound (~10 mm^3) and consisted of control (vehicle alone, delivered ip), standard therapy of gemcitabine 25 mg/kg twice weekly plus erlotinib 100 μg daily (G+E) or standard therapy plus apricoxib at 10 mg/kg (10A) or 30 mg/kg (30A) daily and continued for 3 weeks or 7 weeks (HPAF-II tumor bearing mice only). Upon sacrifice mean tumor weight (A) and metastatic burden (B) were compared. C, D, Paraffin embedded tumor sections were analyzed for COX-2 expression by immunofluorescence. Data are displayed as mean ± SEM and represents 5 images per tumor with all tumors evaluated. *p<0.05 vs. control, #p<0.05 vs. G+E. D, Representative images (COX-2, red; DAPI, blue) are shown. Total magnification, 200X; scale bar, 100 μM (C). E, AsPC-1 and Colo357 cells were plated either under normal conditions or under conditions
of forced EMT. PGE$_2$ levels were measured by ELISA in conditioned media at baseline and 24 hours following one time apricoxib dosing at 0.5 μM.

**Figure 3.** COX-2 inhibition does not alter intratumoral levels of VEGF or microvessel density but facilitates vascular normalization. A & B, Levels of human VEGF in plasma (A) and tumors (B) collected at time of sacrifice were determined by ELISA or immunofluorescence. Mean plasma levels of VEGF are expressed as pg/ml +/- SEM and were assayed in triplicate from each animal under study. The level of VEGF in tumor tissue was determined by ELISA (AsPC-1, pg/50 μg protein) or immunofluorescence (Colo357, % area fraction), the mean +/- SEM are displayed. C & D, Paraffin embedded sections of AsPC-1 and Colo357 tumors were analyzed by immunofluorescence for endomucin, CD31 and NG2 expression. C, Data are displayed as mean ± SEM and represent 5 images per tumor with five tumors per group evaluated. D, Representative images of CD31 (green) and NG2 (red) are shown for Colo357 tumors. Total magnification, 200X; scale bar, 100 μM. Images were analyzed using Elements software. *p<0.05, **p<0.01 vs. control, #p<0.05, ##p<0.005, #p<0.0005 vs. G+E by one way ANOVA.

**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 represents 5 images per tumor with five tumors per group evaluated. B, Representative images of TUNEL (green) reactivity in Colo357 tumors are shown. Total magnification, 200X; 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 represents 5 images per tumor with five tumors per group evaluated. D, Representative images of PCNA (green) immunofluorescence in sections of Colo357 tumors are displayed. Total magnification, 400X; scale bar, 50 μM. E, Colo357 tumors were analyzed for H2AX by immunofluorescence. Data is displayed as mean % area fraction and represents 5 images per tumor with five tumors per group evaluated, representative images are shown (H2AX, red; DAPI, blue). Total magnification 400X. *p<0.05, **p<0.005 vs. control, #p<0.05, ##p<0.005 vs. G+E.

**Figure 5: COX-2 inhibition results in reversal of EMT.** A, Paraffin embedded sections of Colo357 tumors were analyzed for E-cadherin, vimentin, and Zeb1 expression by immunofluorescence. Data are displayed as mean % area fraction per high power field ± SEM and represents 5 images per tumor with five tumors per group evaluated. Images were analyzed using Elements software. B, Representative images of vimentin (red) and E-cadherin (green) levels in treated Colo357 tumors. Total magnification, 200X; scale bar, 100 μM. C, Colo357 cells in vitro were treated with either 100 nM gemcitabine (G) plus 1 μM erlotinib (E) or G+E 1 μM apricoxib and Zeb1 and E-Cadherin expression was evaluated by western blot. D, Paraffin embedded sections of AsPC-1 tumors were analyzed for E-cadherin, vimentin, Zeb1 expression by immunofluorescence. E, Western blot analysis of Zeb1 expression by AsPC-1 cells under normal culture conditions or following induction of EMT and treatment with G+E, G+E+apricoxib, or apricoxib single agent. *p<0.05, **p<0.005 vs. control, #p<0.05, ##p<0.005, ###p<0.0005 vs. G+E.
Fig 1

A

COX-2

Fold Increase

AsPC-1
CFPAC-1
HPAF-II
PL45
Colo357

AsPC-1
CFPAC-1
HPAF-II
PL45
Colo357

EGFR

Fold Increase

B

EGFR
p-EGFR
COX-2
Actin

Colo357

C

% of Control

Erlotinib (uM)

Colo357

AsPC-1

% of Control

Erlotinib (uM)

Colo357

D

Table: IC50 uM

Cell Line  | IC50 uM | Erlotinib
--- | --- | ---
AsPC-1 | 0.1 |
PL45 | 500 |
CFPAC-1 | 1.1 |
Colo357 | 0.7 |
HPAF-II | 0.2 |

AsPC-1

PGE2 (pg/ml)

No Drug  | 0.1 | 1 | 10 | 100 | 1000
--- | --- | --- | --- | --- | ---
Aprinoxib (uM)

Colo357

PGE2 (pg/ml)

No Drug  | 0.1 | 1 | 10 | 100 | 1000
--- | --- | --- | --- | --- | ---
Aprinoxib (nM)

IC50 1.1 nM
Fig 2
Fig 3

A

**AsPC-1 Plasma**

**Colo357 Plasma**

B

**AsPC-1 Tumor**

**Colo357 Tumor**

C

**AsPC-1 Endomucin**

**Colo357 Endomucin**

D

**AsPC-1 Pericyte Coverage**

**Colo357 Pericyte Coverage**

**NG2/CD31**

Control  
G+E  
G+E 10A  
G+E 30A

100μM

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Fig 4

A. AsPC-1 TUNEL and Colo357 TUNEL

B. TUNEL images for AsPC-1 and Colo357

C. AsPC-1 PCNA and Colo357 PCNA

D. PCNA images for AsPC-1 and Colo357

E. γH2AX

% Area Fraction

Control G+E G+E 10A G+E 30A

Control G+E G+E 10A G+E 30A

Control G+E G+E 10A G+E 30A

% Area Fraction

Control G+E G+E 10A G+E 30A

Control G+E G+E 10A G+E 30A

Control G+E G+E 10A G+E 30A

Fig 4
Clinical Cancer Research

Apricoxib, a novel inhibitor of COX-2 markedly improves standard therapy response in molecularly defined models of pancreatic cancer

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

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