Cell Cycle Effects of Nonsteroidal Anti-Inflammatory Drugs and Enhanced Growth Inhibition in Combination with Gemcitabine in Pancreatic Carcinoma Cells

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ABSTRACT

Increased cyclooxygenase-2 (COX-2) expression in human pancreatic adenocarcinomas, as well as the growth-inhibitory effect of nonsteroidal anti-inflammatory drugs (NSAIDs) in vitro, suggests that NSAIDs may be an effective treatment for pancreatic cancer. Gemcitabine is currently the most effective chemotherapeutic drug available for patients with pancreatic cancer, but is only minimally effective against this aggressive disease. Clearly, other treatment options must be identified. To design successful therapeutic strategies involving compounds either alone or in combination with others, it is necessary to understand their mechanism of action. In the present study, we evaluated the effects of three NSAIDs (sulindac, indomethacin, and NS-398) or gemcitabine in two human pancreatic carcinoma cell lines, BxPC-3 (COX-2-positive) and PaCa-2 (COX-2-negative), previously shown to be growth-inhibited by these NSAIDs. Effects on cell cycle and apoptosis were investigated by flow cytometry or Western blotting. Treatment with NSAIDs or gemcitabine altered the cell cycle phase distribution as well as the expression of multiple cell cycle regulatory proteins in both cell lines, but did not induce substantial levels of apoptosis. Furthermore, we demonstrated that the combination of the NSAID sulindac or NS-398 with gemcitabine inhibited cell growth to a greater degree than either compound alone. These results indicate that the antiproliferative effects of NSAIDs and gemcitabine in pancreatic tumor cells are primarily due to inhibition of cell cycle progression rather than direct induction of apoptotic cell death, regardless of COX-2 expression. In addition, NSAIDs in combination with gemcitabine may hold promise in the clinic for the treatment of pancreatic cancer.

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the enzyme cyclooxygenase (COX), which catalyzes the conversion of arachidonic acid to prostaglandins and plays a key role in the inflammatory response. Recent studies have revealed a link between COX expression and carcinogenesis. Specifically, over-expression of the isoform COX-2 in cultured cells resulted in inhibition of apoptosis, increased invasiveness, and promotion of angiogenesis, thereby potentially enhancing tumorigenic potential (Tsujii and DuBois, 1995; Tsujii et al., 1997, 1998). Direct genetic evidence for COX-2 involvement in colorectal tumorigenesis was obtained in a mouse model system for human familial adenomatous polyposis, an inherited condition leading to colorectal cancer; COX-2 gene knockouts and treatment with a specific COX-2 inhibitor reduced the number of intestinal polyps formed (Oshima et al., 1996). Recent genetic disruption studies suggest that COX-1 may also contribute to intestinal tumorigen-

esis (Chulada et al., 2000). Furthermore, epidemiological studies showed an association between prolonged NSAID use in humans and reduced risk of colon cancer (Thun, 1994). The NSAIDs sulindac and celecoxib were also effective in the treatment of familial adenomatous polyposis patients, demonstrating the chemopreventative potential of NSAIDs (Giardiello et al., 1993; Steinbach et al., 2000). More recently, COX-2 expression was reported to be up-regulated in several types of human cancers, including colon and pancreatic, implicating COX-2 in the development of cancer (Eberhart et al., 1994; Yip-Schneider et al., 2000).

The antiproliferative and antineoplastic properties of NSAIDs have been evaluated both in vitro and in vivo to determine their mechanism of action. Treatment of HT-29 colon cancer cells with sulindac or sulindac sulfide, the active metabolite of sulindac, was shown to inhibit proliferation, alter cell cycle distribution, and induce apoptosis (Shiff et al., 1995). Whether the known ability of NSAIDs to inhibit COX and therefore prostaglandin production, which mediates

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PI, propidium iodide; CI, combination index; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; TdT, terminal deoxynucleotidyl transferase; FITC, fluorescein isothiocyanate.

their anti-inflammatory properties, is required for their antineoplastic and antiproliferative effects is not completely understood. Studies demonstrating inhibition of colon cancer growth by selective COX-2 inhibition as well as the reversal of NSAID-induced inhibitory effects by the addition of prostaglandins confirm the importance of the COX pathway in mediating the effects of these compounds (Sheng et al., 1997, 1998). On the other hand, the role of COX inhibition as the sole mechanism has been brought into question by studies with sulindac metabolites. Specifically, sulindac sulfone, which does not inhibit COX activity, reduced tumor growth as effectively as the prodrug sulindac in rodent mammary tumor model systems (Thompson et al., 1995). Furthermore, neither COX expression nor activity was required for the antiproliferative and antineoplastic actions of NSAIDs in COX-null embryo fibroblasts (Zhang et al., 1999).

We and others previously reported elevated COX-2 expression in human pancreatic adenocarcinomas and inhibition of pancreatic cancer cell growth by NSAIDs, providing preclinical support for the use of NSAIDs in the treatment of pancreatic cancer patients (Molina et al., 1999; Tucker et al., 1999; Yip-Schneider et al., 2000). Currently the most effective treatment for pancreatic cancer is the chemotherapeutic drug gemcitabine. Gemcitabine (2′,2′-difluorodeoxycytidine, Gemzar) is a deoxycytidine analog that, after conversion to gemcitabine triphosphate, is incorporated into DNA, thereby inhibiting DNA synthesis. Gemcitabine results in a modest prolongation of survival compared to 5-fluorouracil alone (Burris et al., 1997). However, most patients with pancreatic cancer treated with gemcitabine succumb to their disease in less than 6 months. Clearly, alternative treatments, such as NSAIDs and gemcitabine in combination with other agents, should be explored for pancreatic cancer, the fifth leading cause of cancer-related deaths in the United States (Kroep et al., 1999).

We previously demonstrated that the NSAIDs sulindac, indomethacin, and NS-398 inhibited cell growth of the COX-2-positive BxPC-3 and COX-2-negative PaCa-2 human pancreatic tumor cell lines; the status of COX-2 expression was confirmed by the presence or absence, respectively, of prostaglandin E$_2$ production (Yip-Schneider et al., 2000). Since NSAID treatment effectively reduced cell proliferation in both pancreatic cell lines regardless of COX-2 expression, we concluded that the NSAID-induced growth inhibition was at least in part COX-2-independent (Yip-Schneider et al., 2000). In this study, we evaluated the mechanism of gemcitabine- and NSAID-induced growth inhibition in the BxPC-3 and PaCa-2 cell lines. Exposure of the pancreatic tumor cells to NSAIDs resulted in cell cycle alterations, as well as changes in the expression of several cell cycle regulatory proteins in a COX-2-independent manner, but did not induce substantial apoptosis. Furthermore, we found that the combination of gemcitabine and the NSAIDs sulindac or NS-398 inhibited cell growth to a greater extent than either compound alone.

Materials and Methods

Cell Culture and Drug Treatment. Human pancreatic cancer cell lines BxPC-3 and MIAPaCa-2 were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended. NSAIDs sulindac (Sigma, St. Louis, MO), indomethacin (Sigma), and NS-398 (Biomol, Plymouth Meeting, PA) were dissolved in DMSO; gemcitabine (Eli Lilly, Indianapolis, IN) was dissolved in H$_2$O. For single-drug and combination treatment studies, compounds at the indicated concentrations or the solvent (DMSO) were added to cells plated in duplicate the previous day. Three days after drug addition, cells were harvested by trypsinization, stained with trypan blue, and counted manually with a hemacytometer. Cell growth was determined by averaging the cell counts and expressed as a percentage of the number of cells in the DMSO solvent control sample (set to 100%). Drug additivity or synergy was determined by data analysis using CalcuSyn software (Biosoft, Cambridge, UK) based on the method of Chou and Talalay (1984) for dose-effect analysis. Combination index (CI) values were determined as a quantitative measure of drug interaction indicating either an additive (CI = 1), synergistic (CI < 1), or antagonistic (CI > 1) effect.

Cell Cycle Analysis. Cells were plated in six-well plates, and the following day, NSAIDs or gemcitabine was added for either 24 h or 3 days. Cells floating in the medium were combined with the adherent cell layer, which was trypsinized. Cells (5 × 10^5) were washed, pelleted, and then incubated with 2 mg/ml RNase A in PBS (200 μl) and 0.1 mg/ml propidium iodide (PI) in 0.6% Nonidet P-40 in PBS (200 μl) on ice for 30 min. Samples were immediately analyzed by flow cytometry. Cell cycle phase distribution was determined using Modfit software (Verity Software House, Inc., Topsham, ME) to analyze DNA content histograms.

Western Blotting. Cells were lysed in radioimmune precipitation buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM Na$_3$VO$_4$), and the supernatants were obtained. Cell lysates (10 μg of total protein) were resolved by SDS-polyacrylamide gel electrophoresis on 10% or 4 to 20% gradient gels (Invitrogen, San Diego, CA) and transferred to Immobilon P membranes (Millipore Corporation, Bedford, MA). The blots were probed with primary antibodies specific for the following proteins: cyclins A (NeoMarkers, Inc.); B1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); D1, E, and p21 (NeoMarkers, Inc., Fremont, CA); p27, actin (C-11), COX-1, and COX-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); bax and bcl-xl (Trevigen, Inc., Gaithersburg, MD); bak (CalBiochem, San Diego, CA); and bcl-2 (Transduction Laboratories, San Diego, CA) according to the manufacturers' protocol followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ).

Apoptosis Assays. Following the indicated treatments, apoptosis was measured by annexin V binding (detection kit I) or by a DNA fragmentation assay (Apo-Direct) as recommended by the manufacturer (PharMingen, San Diego, CA). Briefly, cells floating in the supernatant were combined with the adherent fraction, which was trypsinized and then washed. An aliquot of 10^5 cells was incubated with annexin V-FITC and PI for 15 min at room temperature in the dark. Cells were immediately analyzed by flow cytometry. Viable cells exclude both annexin V-FITC and PI. Early apoptotic cells are annexin V-FITC-positive and PI-negative, whereas cells that are no longer viable due to apoptotic or necrotic cell death are positively stained by both annexin V and PI. Percentage of stained cells in each quadrant was quantified using CellQuest software (BD Biosciences, Franklin Lakes, NJ).

The apoptotic assay based on DNA fragmentation was performed as follows. Treated cells (adherent and floating) were fixed in 1% formaldehyde in PBS overnight. After washing, 10^6 fixed cells were incubated with terminal deoxynucleotidyl transferase enzyme (TdT) and FITC-dUTP for 90 min at 37°C to label DNA breaks. Cells were rinsed, incubated in RNase A/propidium iodide in the dark for 30 min at room temperature to stain total DNA, then analyzed by flow cytometry. Cell doublets and clumps were eliminated from the analysis by gating.

Results

NSAIDs Alter Cell Cycle Progression of Pancreatic Tumor Cells Independently of COX-2 Expression. To elucidate potential COX-2-independent mechanisms of NSAID-induced growth inhibition previously shown by our laboratory, we examined the effect of NSAID treatment on cell cycle distribution in two human pancreatic tumor cell lines, BxPC-3 (COX-2 positive) and PaCa-2 (COX-2 negative). For these experiments, two different doses of the NSAIDs were used. The lower concentration of sulindac (250 μM), indomethacin (100 μM), and NS-398 (50 μM) inhibited BxPC-3 and PaCa-2 cell growth by approximately 50 to 60% after 3 days of exposure (IC_{50}); the higher concentration of sulindac (500 μM), indomethacin (200 μM), and NS-398 (100 μM) inhibited cell growth by approximately 80 to 90% after 3 days (IC_{80}) (Yip-Schneider et al., 2000). Sulindac and indomethacin are nonselective COX inhibitors (Meade et al., 1993), whereas NS-398 is a more specific inhibitor of COX-2 (Futaki et al., 1994). For cell cycle analysis, the cells were treated with the NSAIDs for 24 h followed by staining with propidium iodide and analysis by flow cytometry (Table 1). Both concentrations of sulindac led to the accumulation of PaCa-2 cells in G_2/M phase. In BxPC-3 cells, the lower concentration of sulindac increased the proportion of cells in G_0/G_1 phase, while at the higher concentration, cells accumulated in G_2/M phase. In contrast, both concentrations of indomethacin and NS-398 caused cell accumulation in G_0/G_1. Similar results were obtained after NSAID treatment for 3 days (Table 1). The cell cycle alterations were achieved at NSAID concentrations that repressed pancreatic cell growth (Yip-Schneider et al., 2000), indicating that cell cycle arrest is one of the primary mechanisms responsible for the antiproliferative action of NSAIDS in pancreatic tumor cells in vitro.

Effect of Gemcitabine Treatment on Pancreatic Cell Growth and Cell Cycle. Combining drugs with different mechanisms of action is the cornerstone of combination chemotherapy. The biological effects and cellular targets of the chemotherapeutic drug gemcitabine were therefore compared with NSAIDs. We first confirmed that gemcitabine inhibited cell growth of the two pancreatic cancer cell lines, BxPC-3 and PaCa-2, in a dose-dependent manner following treatment for 3 days (Fig. 1). Gemcitabine treatment for 24 h or 3 days at two different concentrations also altered the cell cycle distribution of both pancreatic cell lines. In contrast to the NSAIDs and as previously reported (Li et al., 1999; Ng et al., 2000), there was an increased proportion of cells in S phase consistent with inhibition of DNA synthesis (Table 2).

Expression of Cell Cycle Regulatory Proteins in Pancreatic Tumor Cells Treated with NSAIDs or Gemcitabine. Since both NSAIDs and gemcitabine were found to have distinct effects on the cell cycle, we evaluated the effects of these compounds on the expression of cell cycle regulatory proteins including cyclins and the cyclin-dependent kinase inhibitors, p21 and p27. Total cell lysates were prepared from BxPC-3 and PaCa-2 cells treated with the two concentrations of the compounds for 24 h and analyzed by Western blotting (Fig. 2). Sulindac treatment of PaCa-2 cells at the higher dose (500 μM) decreased the expression of the G_1 phase cyclin D1 and slightly decreased the level of cyclin E (G_1/S phase). The levels of cyclins D1, B1 (G_2/M phase), and A (S/G_2 phase) were decreased by indomethacin and NS-398 in PaCa-2 cells. In BxPC-3 cells, sulindac decreased expression of cyclins D1 and A. Indomethacin and NS-398 inhibited the expression of cyclins D1, E, and A in BxPC-3 cells. In both cell lines, the expression of p21 was increased following exposure to sulindac and indomethacin. The expression of p27 was increased by indomethacin and NS-398 in PaCa-2 cells as well as by

TABLE 1

Cell cycle phase distribution of pancreatic tumor cells treated with NSAIDs

PaCa-2 or BxPC-3 cells were treated with the NSAIDs at the indicated concentrations for 24 h or 3 days, followed by staining with propidium iodide and flow cytometric analysis of DNA content. The percentage of cells in each cell cycle phase was determined by analysis of the DNA content histograms using Modfit software. Numbers highlighted in bold are statistically different from the corresponding control values ($P < 0.001$, Student's t test). Values from a representative experiment are presented, and similar results were obtained in at least two independent experiments.

| Treatment	24 h			3 days		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
	%			%		
PaCa-2 cells						
Control (DMSO)	51	29	19	57	23	19
Sulindac						
250 μM	42	27	**30**	55	24	20
500 μM	15	27	**57**	52	18	**29**
Indomethacin						
100 μM	**60**	22	17	**71**	11	16
200 μM	**73**	8	19	**83**	2	15
NS-398						
50 μM	**61**	22	17	**70**	10	19
100 μM	**72**	12	16	**70**	4	25
BxPC-3 cells						
Control (DMSO)	50	33	17	48	37	15
Sulindac						
250 μM	**73**	12	14	53	36	10
500 μM	36	37	**27**	43	17	**39**
Indomethacin						
100 μM	**71**	18	10	**62**	28	9
200 μM	**77**	7	16	**86**	4	9
NS-398						
50 μM	**59**	31	9	**71**	21	7
100 μM	**71**	18	10	**82**	10	6
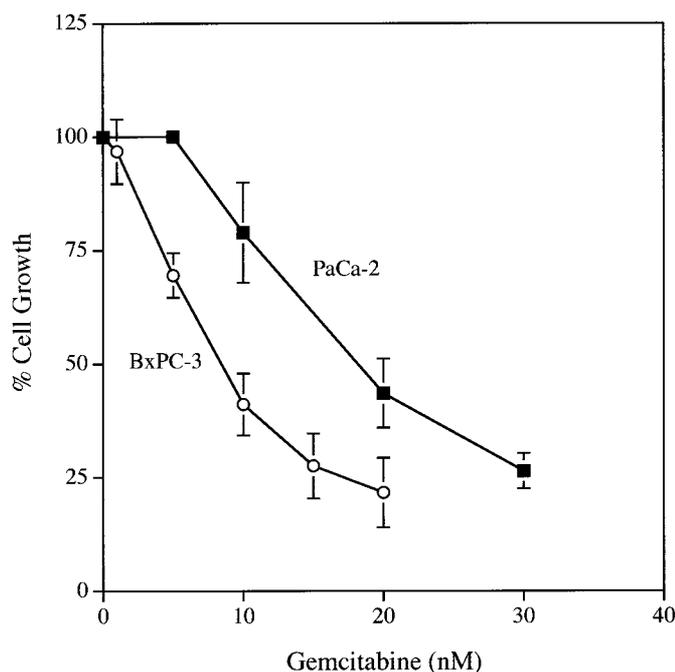
Fig. 1. Inhibition of pancreatic tumor cell growth by gemcitabine. The pancreatic tumor cell lines BxPC-3 (open circles) and PaCa-2 (solid squares) were treated with the indicated concentrations of gemcitabine for 3 days. Cells were harvested and counted manually with a hemacytometer. Cell growth was calculated by averaging the cell counts and expressed as percentage of cell growth relative to the control (set to 100%). The mean ± S.D. from at least three separate experiments performed in duplicate is shown.

levels of many cell cycle regulatory proteins, correlating with their effects on the cell cycle in pancreatic tumor cells.

Treatment with NSAIDs or Gemcitabine Does Not Significantly Induce Apoptosis. To determine whether the NSAIDs or gemcitabine mediated their inhibitory effects in part by inducing apoptosis, BxPC-3 or PaCa-2 cells were treated with the various agents for 3 days before analysis. The extent of apoptosis was measured by the incorporation of FITC-dUTP in the presence of TdT enzyme to detect DNA fragmentation (Fig. 3A and Table 3). Apoptosis was induced in BxPC-3 cells only after treatment with the highest concentration of sulindac for 3 days. The other compounds had negligible effects. Similar results were observed in PaCa-2 cells treated with the various agents (Table 3). To confirm these results, we employed a second, more sensitive assay based on the ability of annexin V to bind phosphatidylserine on the outer membrane surface as an early indicator of apoptosis. Simultaneous staining with PI was performed to measure cell viability. After treatment of BxPC-3 cells with sulindac for 24 h, the higher concentration of sulindac resulted in a slight, reproducible increase in annexin V-positive and PI-negative cells (lower right quadrant), indicative of early apoptotic cells (Fig. 3B). Similarly, following drug treatment for 3 days, only BxPC-3 cells treated with the higher concentration of sulindac were induced to undergo apoptosis as evidenced by an increased percentage of annexin V-positive and PI-positive cells (Fig. 3C, upper right quadrant, late apoptotic cells). Similar effects were observed following treatment of PaCa-2 cells (data not shown). These results suggest that pancreatic tumor cells are relatively resistant to apoptosis induced by NSAIDs or gemcitabine. Apoptosis was only apparent in cells treated with the higher concentration of sulindac and was not detectable at the lower IC_{50} concentration of sulindac, which substantially inhibited cell growth. Furthermore, the IC_{80} concentration of the other NSAIDs and gemcitabine did not induce apoptosis despite their ability to significantly suppress cell growth.

The expression of several bcl-2 family members involved in regulating apoptosis was also determined in the pancreatic cell lines following treatment with the compounds for 24 h (Fig. 4). No change in the expression of two inducers of apoptosis, bax or bak, or an inhibitor of apoptosis, bcl-2, was

sulindac and indomethacin in BxPC-3 cells. Clearly, common cell cycle targets of NSAIDs exist in the two cell lines. The effect of NSAIDs on COX-2 expression was also evaluated and found to be decreased by sulindac and indomethacin treatment of the COX-2-positive cell line BxPC-3; COX-1 expression was not changed by the treatments (Fig. 2). In PaCa-2 cells, COX-1 and COX-2 proteins were not expressed or induced by the various compounds. Treatment with the chemotherapeutic drug gemcitabine affected expression of cell cycle proteins in the BxPC-3 cell line with increases in the levels of cyclins A, E, and B1. Gemcitabine also upregulated the expression of cyclin E in PaCa-2 cells. Thus, both NSAIDs and gemcitabine induced global changes in the

TABLE 2

Effect of gemcitabine on the cell cycle phase distribution of pancreatic tumor cells

PaCa-2 or BxPC-3 cells were treated with the indicated concentrations of gemcitabine for 24 h or 3 days, followed by staining with propidium iodide and flow cytometric analysis of DNA content. Cell cycle phase distributions were calculated using Modfit software. Numbers in bold are significantly different from the control values ($P < 0.001$, Student's t test). Values from a representative experiment are presented, and similar results were obtained in at least two independent experiments.

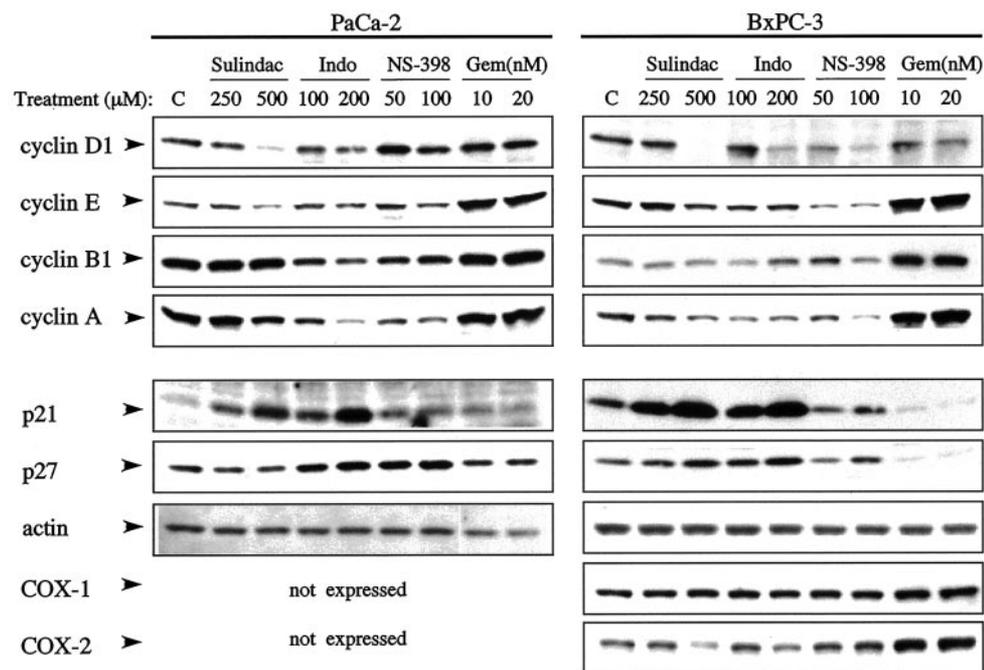| Treatment	24 h			3 days		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
		%			%	
PaCa-2						
Control	47	31	21	56	26	18
Gemcitabine						
10 nM	43	**38**	18	49	**33**	17
20 nM	36	**49**	13	19	**81**	0
BxPC-3						
Control	59	28	12	45	40	14
Gemcitabine						
10 nM	57	**39**	2	24	**52**	24
20 nM	63	**35**	1	20	**79**	0

Fig. 2. Effect of NSAIDs or gemcitabine on the expression of cell cycle regulatory proteins and COX. Cell lysates were prepared from PaCa-2 (COX-2-negative) and BxPC-3 (COX-2-positive) cells treated with the solvent DMSO (C, control) or with different concentrations of NSAIDs or gemcitabine for 24 h followed by Western blotting analysis to detect the indicated proteins. Actin levels confirm similar protein loading of the PaCa-2 and BxPC-3 cell lysates, respectively. Representative Western blots are shown.

detected following exposure of BxPC-3 and PaCa-2 cells to the compounds. In contrast, the expression level of bcl-xl, a close homolog of bcl-2, was decreased by sulindac and, slightly, by NS-398 in BxPC-3 cells and may be involved in inducing apoptosis observed in response to high doses of sulindac. PaCa-2 cells did not express detectable levels of bcl-xl. These results generally confirm the apoptosis results described above. Namely, NSAIDs and gemcitabine had only limited effects on the expression of apoptotic regulatory proteins in pancreatic cells. Taken together, these observations suggest that growth inhibition by NSAIDs and gemcitabine in pancreatic tumor cells is predominantly mediated by cell cycle arrest, not by apoptosis.

Antiproliferative Effect of NSAIDs in Combination with Gemcitabine. Since the NSAIDs and gemcitabine both inhibited cell growth in the pancreatic tumor cell lines but appeared to have different mechanisms of action, it was of interest to determine whether they could complement each other when used in combination. To address this question, BxPC-3 and PaCa-2 cells were grown in the presence of sulindac and gemcitabine alone or in combination at the indicated concentrations for 3 days. Cells were subsequently harvested and counted to measure cell growth (Fig. 5). The combination of sulindac and gemcitabine resulted in greater growth inhibition than either compound alone. In both cell lines, treatment with the combination (gemcitabine + sulindac 100 μM in BxPC-3 or gemcitabine + sulindac 200 μM in PaCa-2 cells) reduced the IC_{50} of gemcitabine approximately 2-fold. The inhibitory effect of the combination was determined to be additive following data analysis by the Chou and Talalay method (1984). Similarly, the combination of NS-398 and gemcitabine also inhibited cell growth more effectively than either compound alone in the two pancreatic tumor cell lines (Fig. 6). Taken together, these results provide in vitro support for evaluating the effectiveness of gemcitabine together with NSAIDs in patients with pancreatic cancer.

Discussion

NSAIDs have the potential for use as chemopreventative and chemotherapeutic agents in the treatment of cancer patients due to their antineoplastic and antiproliferative effects. In cultured colon cancer cells, NSAIDs have been found to mediate their inhibitory effects by arresting the cell cycle and inducing apoptosis, effects often accompanied by changes in the expression of critical proteins regulating the progression of these events (Goldberg et al., 1996; Qiao et al., 1998). In the present study using two pancreatic tumor cells lines with differential COX-2 expression, we aimed to determine how NSAIDs inhibit pancreatic cell growth independent of COX-2 expression. Treatment with the NSAIDs (sulindac, indomethacin, or NS-398) for 24 h altered the cell cycle phase distribution of both pancreatic cell lines. The NSAID-induced cell cycle alterations were also associated with changes in the expression of cell cycle regulatory proteins, such as cyclins, which bind to and activate cyclin-dependent kinases. In particular, cyclin D1 expression was decreased in both cell lines following treatment with each of the NSAIDs, suggesting that cyclin D1 may be the critical common determinant of cell growth and cell cycle progression targeted by NSAIDs in pancreatic cells. Inhibition of cyclin D1 expression by cyclin D1 antisense was previously shown to suppress pancreatic cell growth and tumorigenicity, identifying cyclin D1 as an important growth regulator in these cells (Kornmann et al., 1998). We also observed changes in the expression of cyclins A, E, and B1 as well as the cyclin-dependent kinase inhibitors p21 and p27, depending upon the cell line and the NSAID (summarized in Table 4). Each NSAID tested altered the expression of at least three cell cycle proteins that control progression through critical cell cycle transition points, correlating with cell cycle arrest and NSAID-induced growth inhibition in pancreatic tumor cells.

We also evaluated the effects and mechanism of the chemotherapeutic drug gemcitabine in the two pancreatic tumor
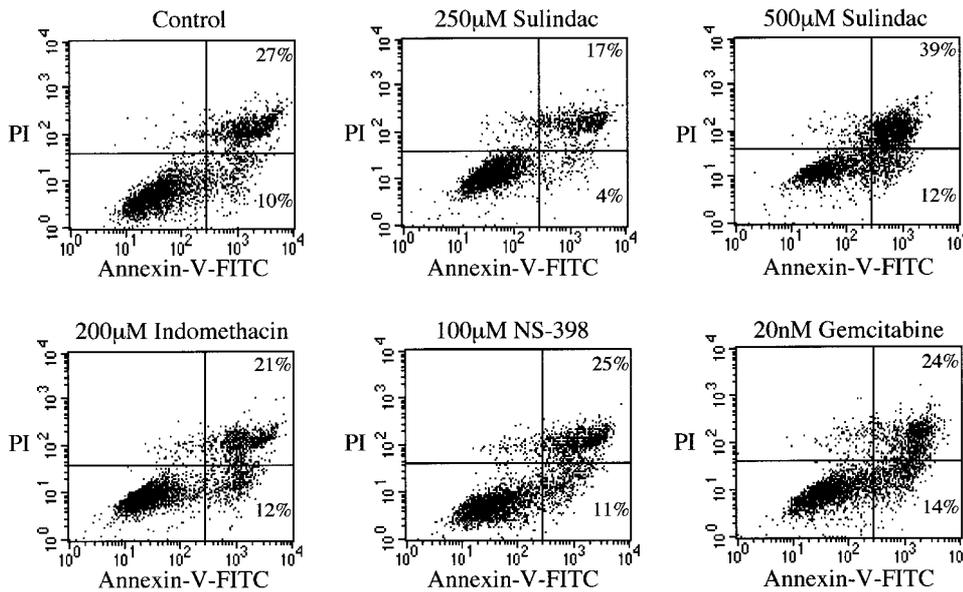
A.
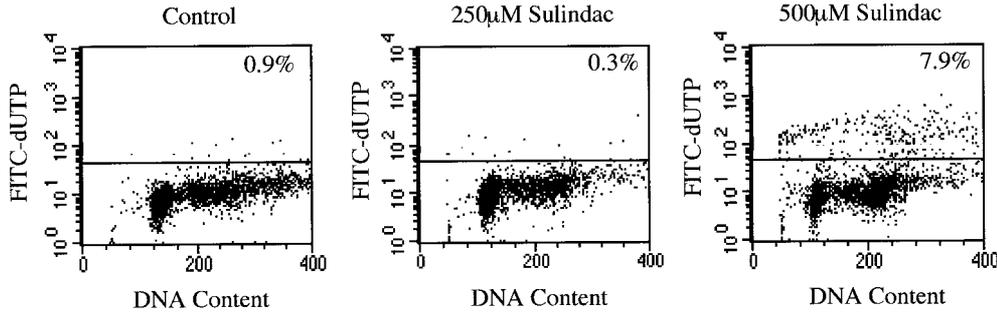
B. 24 hours
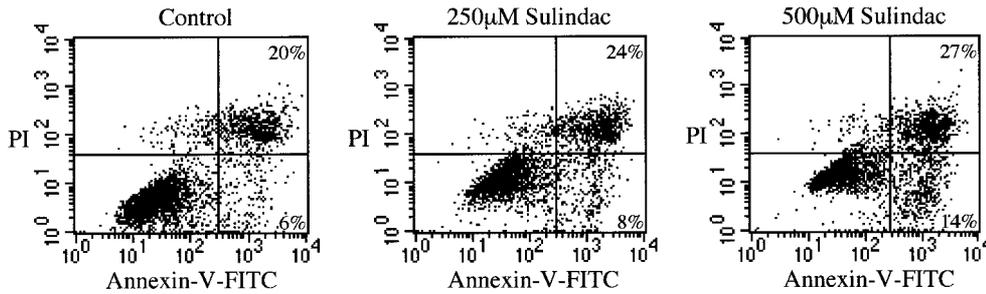
C. 3 days

Fig. 3. Apoptosis induced by NSAIDs or gemcitabine in BxPC-3 cells. A, BxPC-3 cells were treated with sulindac or solvent (control) for 3 days, followed by incorporation of FITC-dUTP in the presence of TdT enzyme and staining with RNase/PI as described under *Materials and Methods*. Percentage of FITC-positive apoptotic cells (top section) was quantified using CellQuest software. B, extent of apoptosis induced in BxPC-3 cells by a 24-h sulindac treatment was measured by staining with annexin V-FITC/PI and analysis by flow cytometry. Percentage of stained cells in each quadrant was quantified using CellQuest software. C, effect of NSAID or gemcitabine treatment for 3 days on apoptosis was measured by annexin V-FITC/PI staining followed by flow cytometric analysis. Results from a representative experiment are shown; similar results were obtained in at least two independent experiments.

cell lines. The inhibition of pancreatic cell growth by gemcitabine was accompanied by cell cycle arrest in S phase and increased expression of cyclins A, E, and B1 in the BxPC-3 cell line. Elevated cyclin E expression was also observed in PaCa-2 cells. The unscheduled increase in the levels of these cyclins is similar to that described previously in which growth imbalance induced by DNA synthesis inhibitors dramatically increased the expression of cyclins E, A, and B1 in human MOLT-4, K562, and THP-1 cells (Gong et al., 1995; Hatse et al., 1999). Such growth imbalance is thought to be

TABLE 3
Effect of NSAIDs or gemcitabine on apoptosis in pancreatic tumor cells

Extent of apoptosis following treatment of BxPC-3 and PaCa-2 cells with the indicated NSAIDs or gemcitabine for 3 days was measured by the incorporation of FITC-dUTP in the presence of TdT enzyme to detect DNA fragmentation. Cells were stained with RNase A/PI and analyzed by flow cytometry. The results from a representative experiment are presented, and similar results were obtained in at least two separate experiments.

Treatment	Apoptosis	
	BxPC-3	PaCa-2
	%	
Control (DMSO)	0.9	0.3
Sulindac		
250 μM	0.3	0.8
500 μM	7.9	8.2
Indomethacin, 100 μM	1.1	2.2
NS-398, 100 μM	1.5	0.2
Gemcitabine, 20 nM	0.4	0.5

caused by the uncoupling of DNA replication from RNA and protein synthesis, thus perturbing the regulated, periodic expression of cell cycle regulatory proteins such as cyclins.

We next investigated the ability of the NSAIDs and gemcitabine to induce apoptosis in the pancreatic tumor cells. Sulindac induced a slight increase in the percentage of apoptotic cells but only at a high concentration (500 μM). At the IC$_{50}$ concentration (250 μM), sulindac did not induce a significant increase in apoptosis even after 3 days. Failure of the cells to undergo apoptosis may be explained in part by our observation of increased levels of activated extracellular signal-regulated kinase and protein kinase B/Akt following NSAID treatment, which may provide survival signals (data not shown). In addition, resistance to sulindac-induced apoptosis was previously observed in rat enterocytes transformed with oncogenic K-*ras* (Arber et al., 1997). Since activating K-*ras* mutations occur in a high percentage of pancreatic cancers, similar resistance to NSAID-induced apoptosis would be expected to occur in this type of cancer. In agreement, the other NSAIDs tested (indomethacin and NS-398) and gemcitabine failed to induce detectable apoptosis measured by two independent methods, even after treatment for 3 days at concentrations that suppressed cell growth by up to 90%. This suggests that the in vitro antiproliferative effects of the NSAIDs and gemcitabine in pancreatic tumor cells are primarily mediated by altering the normal cell cycle phase distribution, not by inducing apoptotic cell death. Nevertheless, the primary effect on the cell cycle may lead to senescence, ultimately resulting in cell death indirectly or a "slow cell death" (Blagosklonny, 2000). Our findings are consistent

with the apoptosis-resistant phenotype characteristic of pancreatic tumor cells that are resistant to undergoing apoptosis induced by chemotherapeutic agents, activation of surface receptors such as CD95 or tumor necrosis factor receptor, or by serum and growth factor withdrawal (Raitano et al., 1990; Ungefroren et al., 1998). In contrast to our results, indomethacin and NS-398 were recently reported to induce substantial apoptosis in serum-starved pancreatic tumor cell lines (Ding et al., 2000). Serum starvation of the cells before NSAID treatment would predispose the cells to undergoing apoptosis, in contrast to treatment of exponentially growing cells, a more physiologically relevant situation, as described in the current study.

Since similar effects on cell cycle progression and expression of cell cycle regulatory proteins were observed in both pancreatic tumor cell lines regardless of COX-2 expression, we conclude that NSAIDs mediate their inhibitory effects in part by targeting the cell cycle independently of COX-2 expression. Others have also demonstrated NSAID-induced inhibition that did not depend on COX expression or activity. For example, the COX-2-specific NSAID NS-398 was found to inhibit cell growth and induce apoptosis in two colon cancer cell lines, HT29 and S/KS, that were COX-2-positive and -negative, respectively (Elder et al., 1997). In COX-null embryo fibroblasts, the NSAIDs NS-398, sulindac, indomethacin, piroxicam, and ibuprofen suppressed colony formation in a soft agar assay and were also effective at inducing apoptosis independent of COX-1 or COX-2 inhibition (Zhang et al., 1999). Furthermore, when a series of NSAIDs were compared, COX inhibitory activity did not correlate with their ability to inhibit cell growth or induce apoptosis (Piazza et al., 1997). COX-independent effects of NSAIDs may be mediated by inhibition of alternative targets, including Ras, nuclear factor-κB or cGMP phosphodiesterase (reviewed in Shiff and Rigas, 1999). Taken together, these findings suggest that although the COX-2-dependent inhibitory effects of NSAIDs are clearly important, COX-2-independent effects also play a role in mediating the antiproliferative and antineoplastic properties of NSAIDs. This has important implications for the utility of NSAIDs in the treatment of COX-2-positive and -negative human cancers, including pancreatic cancer. Furthermore, these observations encourage the development of a new class of compounds that retain the chemopreventative properties of NSAIDs but fail to inhibit COX, therefore increasing their therapeutic benefit. These compounds have the potential additional
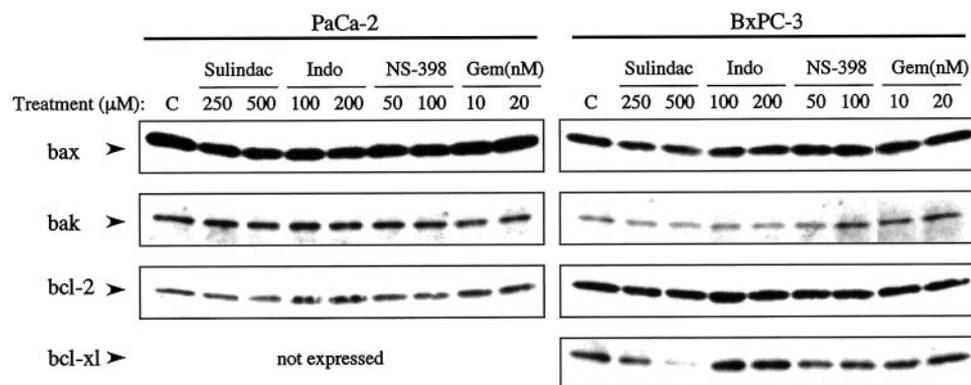
Fig. 4. Expression of apoptotic proteins in cells treated with NSAIDs and gemcitabine. BxPC-3 and PaCa-2 cells were treated with different concentrations of NSAIDs and gemcitabine for 24 h. Cell lysates were prepared and analyzed by Western blotting using antibodies specific for the indicated proteins.
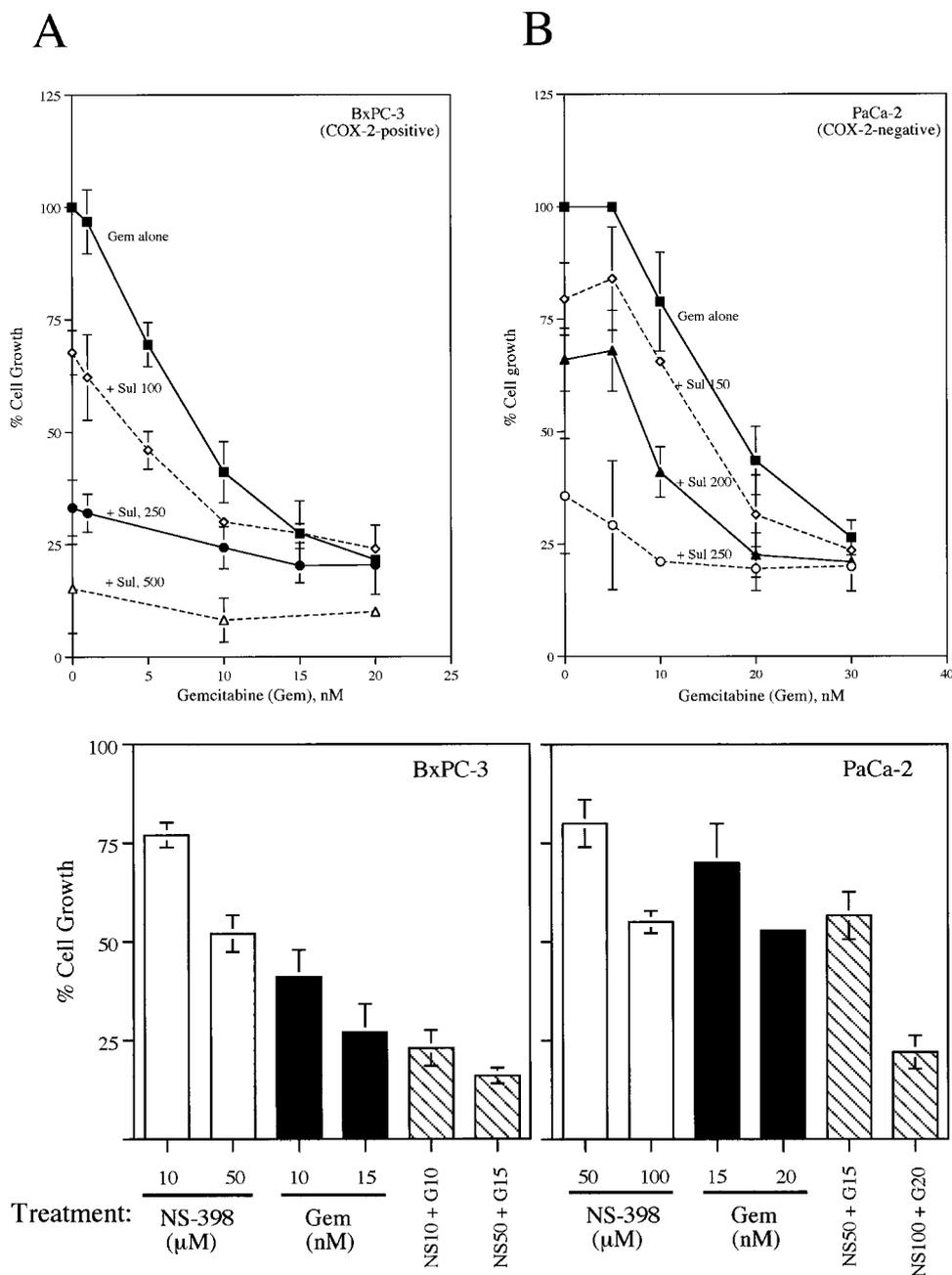
A

B

Fig. 5. Combined effects of sulindac and gemcitabine on cell growth. A, BxPC-3 cells were treated with sulindac (Sul) and gemcitabine (Gem), either alone or in combination at the indicated sulindac concentrations with increasing concentrations of gemcitabine. After 3 days, cells were harvested and counted manually with a hemacytometer. Percentage of cell growth was calculated by averaging cell counts and expressed relative to the control sample (set to 100%). Gemcitabine alone (solid squares); Gem + 100 μM sulindac (open diamonds); Gem + 250 μM sulindac (solid circles); Gem + 500 μM sulindac (open triangles). B, PaCa-2 cells treated with sulindac and gemcitabine, alone or in combination. Gem + 150 μM sulindac (open diamonds); Gem + 200 μM sulindac (solid triangles); Gem + 250 μM sulindac (open circles). The mean ± S.D. from at least two independent experiments performed in duplicate are shown.

Fig. 6. Growth inhibition by NS-398 and gemcitabine. BxPC-3 and PaCa-2 cells were treated with NS-398 (NS, white bars) and gemcitabine (Gem or G, black bars), either alone or in combination (hatched bars) for 3 days. Cells were harvested and counted. Cell growth was calculated by averaging cell counts and expressed as a percentage of the solvent control (100%). The mean ± S.D. from at least two independent experiments performed in duplicate are presented.

advantage of having no gastrointestinal or renal toxicity, the side effects associated with prolonged use of conventional COX inhibitors, and to a lesser extent with selective COX-2 inhibitors, which currently discourage their widespread use in the treatment of cancer. Also, the knowledge that these compounds interfere with the cell cycle at high doses has the potential to lead to the development of more potent and specific cell cycle inhibitors for the treatment of pancreatic cancer.

The drug concentrations tested in our study were comparable to those shown to be growth-inhibitory in vitro by other investigators. In patients, peak plasma concentrations of approximately 15 μM sulindac and 25 μM gemcitabine can be reached in vivo (Swanson et al., 1982; Grunewald et al., 1992). Moreover, gemcitabine has been shown to accumulate in leukemia blasts (Grunewald et al.,

1992). Since the NSAID concentrations we used were higher than those achievable in vivo, our results cannot be directly extrapolated to humans but can provide insight into potential mechanisms of NSAID action in pancreatic cancer cells. In a complex environment such as that found in vivo, lower NSAID concentrations may be effective due to effects on other cell types, thereby influencing cellular interactions and inhibiting processes such as angiogenesis.

Finally, we demonstrated that the combination of sulindac and gemcitabine was more effective at inhibiting cell growth than either compound alone in both pancreatic tumor cell lines. An increase in the potency of gemcitabine was observed, and the effects of the combination were found to be additive, possibly due to the combined effect of the individual agents targeting different cellular proteins and pathways. Treatment with the combination did not

TABLE 4
Summary of NSAID-induced effects on cell cycle and protein expression

Treatment	Cell Cycle Accumulation	Cyclin D1	Cyclin E	Cyclin B1	Cyclin A	p21	p27
PaCa-2							
Sulindac	G_2/M	↓	↓	—	—	↑	—
Indomethacin	G_0/G_1	↓	—	↓	↓	↑	↑
NS-398	G_0/G_1	↓	—	↓	↓	—	↑
Gemcitabine	S	—	↑				
BxPC-3							
Sulindac	G_0/G_1, G_2/M	↓	—	—	↓	↑	↑
Indomethacin	G_0/G_1	↓	↓	—	↓	↑	↑
NS-398	G_0/G_1	↓	↓	—	↓	—	—
Gemcitabine	S	↓	↑	↑	↑	↓	↓

↓, ↑, or — indicates a decrease, increase, or no change, respectively, in protein expression relative to control cells.

result in greater effects on the cell cycle or increased apoptosis relative to treatment with the single agents (data not shown). In addition, we found that the combination of NS-398, a more specific COX-2 inhibitor, and gemcitabine inhibited cell growth more effectively than either compound alone. Increased sensitivity to various chemotherapeutic drugs was recently reported following inhibition of cyclin D1 expression by stable antisense transfection into pancreatic tumor cells (Kornmann et al., 1999). Here, we describe pharmacological inhibition of cyclin D1 by both COX-2-specific and nonspecific COX inhibitors that was associated with increased sensitivity to gemcitabine. Taken together, the effectiveness of sulindac or NS-398 in combination with gemcitabine in vitro suggests that gemcitabine and NSAIDs or future NSAID derivatives may have potential for the treatment of pancreatic cancer in the clinic.

Acknowledgments

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