Tolfenamic Acid and Pancreatic Cancer Growth, Angiogenesis, and Sp Protein Degradation

Maen Abdelrahim, Cheryl H. Baker, James L. Abbruzzese, Stephen Safe

Background: Sp1, Sp3, and Sp4 are transcription factors that regulate cell proliferation and vascular endothelial growth factor (VEGF) expression and are overexpressed in many cancer cell lines. For some cancers, Sp1 overexpression is associated with poor survival. Cyclooxygenase inhibitors decrease Sp1 expression in cancer cells, and therefore different structural classes of nonsteroidal anti-inflammatory drugs (NSAIDs) were screened for their ability to decrease levels of Sp1, Sp3, and Sp4 and to decrease pancreatic tumor growth and metastasis in an in vivo model. Methods: Levels of Sp1, Sp3, Sp4, and VEGF proteins in pancreatic cancer cell lines were assessed by immunoblot analysis. mRNA was assessed by reverse transcription–polymerase chain reaction. Panc-1 pancreatic cancer cells transfected with VEGF promoter constructs were used to assess VEGF promoter activation. Pancreatic tumor weight and size and liver metastasis were assessed immunohistochemically. Results: Tolfenamic acid and structurally related biaryl derivatives induced degradation of Sp1, Sp3, and Sp4 in pancreatic cancer cells. Tolfenamic acid also inhibited VEGF mRNA and protein expression in pancreatic cancer cells; this inhibition was associated with the decreased Sp-dependent activation of the VEGF promoter. In the mouse model for pancreatic cancer, treatment with tolfenamic acid (50 mg/kg of body weight), compared with control treatment, statistically significantly decreased tumor growth and weight (P = .005), liver metastasis (P = .027), and levels of Sp3 and VEGF (P = .009) and Sp1 and Sp4 (P = .006) proteins in tumors. For example, tumors from mice treated with tolfenamic acid (50 mg/kg) had statistically significantly lower VEGF levels (45%, 95% confidence interval = 39% to 51%; P = .009) than tumors from control mice. Conclusions: Tolfenamic acid is a new antipancreatic cancer NSAID that activates degradation of transcription factors Sp1, Sp3, and Sp4; reduces VEGF expression; and decreases tumor growth and metastasis. [J Natl Cancer Inst 2006;98:855–68]

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX)-dependent synthesis of prostaglandins that mediate inflammatory responses in multiple tissues (1–3). NSAIDs also induce cell growth inhibition, apoptosis, and antiangiogenesis; activation of the pathways involved in these effects is critical for the reported anticarcinogenic activities of NSAIDs and related COX-2 inhibitors, such as celecoxib (3–7). Extensive epidemiologic studies of the role of NSAIDs in the prevention and treatment of colon cancer indicate that the use of NSAIDs, such as aspirin and some COX-2 inhibitors, is associated with a decrease in the incidence and/or mortality of colon cancer (8–13). Patients with familial adenomatous polyposis coli are highly susceptible to development of colon cancer, and these individuals have been successfully treated with the COX-1 and COX-2 inhibitor sulindac to repress colonic polyp formation (11,13). Laboratory animal and cell culture studies also confirm the efficacy of NSAIDs for inhibiting growth of colon cancers and tumors derived from other tissues (3–7,14–17).

Although aspirin use has not been associated with decreased incidence of pancreatic cancer (16–19), several studies have found that NSAIDs and COX-2 inhibitors alone and in

Affiliations of authors: Institute of Biosciences and Technology, Health Science Center, Texas A&M University, Houston, TX (MA, SS); Department of Cancer Biology (CHB), Department of Gastrointestinal Medical Oncology (JLA), University of Texas M. D. Anderson Cancer Center, Houston, TX; Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX (SS).

Correspondence to: Stephen Safe, DPhil, Department of Veterinary Physiology and Pharmacology, Texas A&M University, 4466 TAMU, Vet. Res. Bldg. 410, College Station, TX 77843-4466 (e-mail: ssafe@cvm.tamu.edu).

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combination with chemotherapeutic drugs can inhibit pancreatic cancer cell and tumor growth (20–28). For example, celecoxib statistically significantly increased gemcitabine-induced growth inhibition and apoptosis in BxPC-3 pancreatic cancer cells, which express high levels of COX-2 (27). Other studies (26–28) reported growth inhibition with combinations of NSAIDs and chemotherapeutic drugs, through both COX–2–dependent and –independent mechanisms.

Growth and metastasis of pancreatic cancer depend on activation of vascular endothelial growth factor (VEGF) and other angiogenic factors. Sp1, Sp3, and Sp4 are transcription factors that are overexpressed in many cancer cell lines. For some cancers, Sp1 overexpression is associated with poor survival. There is strong evidence that Sp1 and other Sp proteins regulate VEGF expression in pancreatic cancer cells (29,30). The COX-2 inhibitor celecoxib decreases the expression of Sp1 and VEGF by inducing degradation of Sp1 in pancreatic cancer cells (31), and studies from this laboratory (32) show that COX-2 inhibitors decrease VEGF expression in colon cancer cells by decreasing the level of Sp1 and Sp3.

Because VEGF expression in pancreatic cancer cells depends on expression of Sp1, Sp3, and Sp4 proteins (30), an ideal antiangiogenic agent for pancreatic cancer would induce degradation of all three Sp proteins. In this study, we screened 14 NSAIDs or inhibitors of COX-1 and/or COX-2 for their ability to reduce the levels of Sp1, Sp3, and Sp4 proteins in pancreatic cancer cells. We also investigated the mechanism of action of the most active NSAID in Panc-1, L3.6pl, and Panc-28 pancreatic cancer cell lines and in an orthotopic model for pancreatic cancer. The purpose of this study was to identify NSAIDs that decrease levels of Sp1, Sp3, and Sp4, resulting in decreased pancreatic tumor growth and metastasis in an in vivo model.

**Materials and Methods**

**Cell Lines, Cell Culture, Constructs, Antibodies, NSAIDs, and Oligonucleotides**

The human pancreatic cell line Panc-1 was obtained from the American Type Culture Collection (Manassas, VA). The human pancreatic cell lines Panc-28 and L3.6pl (33,34) were obtained from the University of Texas M. D. Anderson Cancer Center (Houston). DME/F12 medium with or without phenol red, a 100× antibiotic/antimycotic solution, and lactacystin were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was purchased from Intergen (Purchase, NY). γ-32P-ATP (300 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA). Poly(dI-dC) and T4 polynucleotide kinase were obtained from the University of Texas M. D. Anderson Cancer Center. The NSAIDs valeryl salsylate, acetamin, ampicam, diclofenac, diflunisal, naproxen, fenbufen, ibuprofen, ketoprofen, tolmetin, loretzole, and tolenamic acid (i.e., Clotam) were purchased from LKT Laboratories, Inc. (St. Paul, MN). Lysis buffer and luciferase reagent were obtained from Promega Corp. (Madison, WI). VEGF promoter constructs have previously been described (30,32).

**Transfection of Pancreatic Cancer Cells, Proteasome Inhibitor Experiments, and Preparation of Nuclear Extracts**

Panc-1 and L3.6pl cells were cultured in six-well plates, with each well containing 2 mL of DME/F12 medium supplemented with 5% fetal bovine serum. After 16–20 hours of incubation when cells were 50%–60% confluent, reporter gene constructs were transfected by use of the Lipofectamine reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). The pVEGF1 and pVEGF2 constructs contain VEGF promoter inserts (positions −2018 to +50 and positions −131 to +54, respectively) linked to luciferase (a reporter gene), as previously described (30,32). The effects of NSAIDs that induced Sp protein degradation (tolafenamic acid and diclofenac) and did not affect Sp protein levels (ampicam) on luciferase activity were investigated in Panc-1 and L3.6pl cells cotransfected with 500 ng of the pVEGF1 or pVEGF2 constructs. Cells were treated with 0.1% dimethyl sulfoxide (DMSO; control vehicle) or with the indicated concentration of NSAIDs for 24 or 48 hours, and then the effects on transactivation (luciferase activity, which reflects the activity of the VEGF promoter insert) in whole cell lysates (relative to β-galactosidase activity) was determined. For nuclear extracts, cells were washed twice in phosphate-buffered saline (PBS), scraped into 1 mL of 1× lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% [vol/vol] glycerol, 1% Triton X-100, and 5 μL of protease inhibitor cocktail [product P8340; Sigma] per 1 mL of buffer), incubated at 4 °C for 15 minutes, and centrifuged at 14,000g for 1 minute at 20 °C. Cell pellets were then washed three times in 1 mL of lysis buffer; lysis buffer supplemented with 500 mM KCl was then added to the cell pellet and incubated with it for 45 minutes at 4 °C with frequent vortex mixing. Nuclei were pelleted by centrifugation at 14,000g for 1 minute at 4 °C, and aliquots of supernatant were stored at −80 °C for use in Western blot analyses and gel shift assays. For proteasome inhibitor experiments, cells were cotreated with 2 μM lactacystin, a proteasome inhibitor that would inhibit NSAID-induced activation of this protein breakdown pathway. For the electrophoretic mobility shift assay (see below), nuclear extracts from Panc-1 and L3.6pl cells were isolated as previously described (30,32), and aliquots were stored at −80 °C.

**Immunoblot Analysis and Laser Scanning Densitometry**

Panc-1 cells were washed once with PBS and collected by scraping cells from the culture plate in 200 μL of lysis buffer. The cell lysates were incubated on ice for 1 hour with intermittent vortex mixing and then centrifuged at 40,000g for 10 minutes at 4 °C. Equal amounts of protein (60 μg per lane) from cells treated with 0.1% DMSO or individual NSAIDs, at the concentrations indicated, were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10%–12.5% gels. For VEGF immunoblots, 100 μg of protein per lane was used. Samples were electrophoresed and transferred to a membrane (30,32), and proteins were detected by incubation with primary polyclonal antibodies against Sp1, Sp3, Sp4, histone deacetylase, VEGF, or β-tubulin, followed by incubation with an appropriate horseradish peroxidase–conjugated goat anti–rabbit immunoglobulin G F secondary antibody, as previously described (30). Band intensities were determined by scanning laser densitometry (Sharp Electronics Corporation, Mahwah, NJ).
and Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA).

**Electrophoretic Mobility Shift Assay**

Sense and antisense VEGF oligonucleotides derived from the VEGF promoter (positions –66 to –47) were synthesized and annealed to each other, and 5-pmol aliquots were 5’ end-labeled by use of T4 kinase and [γ-32P]ATP. A 30-μL electrophoretic mobility shift assay reaction mixture contained approximately 100 mM KCl, 3 μg of crude nuclear protein from Panc-1 or L3.6p1 cells treated with 0.1% DMSO, 50 μM amiprixom, or 50 μM tolfenamic acid for 48 hours, 1 μg of poly(dI-dC) and 10 fmol of radiolabeled probe. After incubation for 20 minutes on ice, antibodies against Sp1, Sp3, or Sp4 protein were added, and the mixture was incubated another 20 minutes on ice. Protein–DNA complexes were resolved by polyacrylamide gel electrophoresis on 5% gels, as previously described (30,32). Specific DNA–protein and antibody-supershifted complexes were observed as retarded bands in the gel by autoradiography.

**Cell Proliferation Assay**

Panc-1, Panc-28, and L3.6p1 cells were plated in DME/F12 medium with 5% fetal bovine serum and treated on the next day with vehicle (0.1% DMSO) or various concentrations of amiprixom, tolfenamic acid, diclofenac, or naproxen, as indicated. Cells were counted at the indicated times with a Coulter Z1 cell counter (Beckman-Coulter, Fullerton, CA). Each experiment was done in triplicate, and results are expressed as means, with error bars representing 95% confidence intervals (CIs).

**Immunocytochemistry and Immunohistochemistry**

Panc1 cells were cultured on Lab-Tek Chamber slides (Nalge Nunc International, Naperville, IL) at 100,000 cells per well in DME/F12 medium supplemented with 5% fetal bovine serum. Cells were then treated with the indicated NSAID for 48 hours, the medium chamber was detached from the slide, and the glass slide was washed in PBS. VEGF was detected by immunostaining essentially as previously described (30). In brief, cells on the glass slides were fixed in methanol at –20 °C for 10 minutes and then washed for 2 minutes in 0.3% PBS–TWEEN 20 before blocking with 5% goat serum in antibody dilution buffer (stock solution = 100 mL of PBS–TWEEN, 1 g of bovine serum albumin, and 45 mL of glycerol, pH 8.0) for 1 hour at 20 °C. After removal of the blocking solution, rabbit anti-VEGF polyclonal antibodies (1:200 dilution) were added in antibody dilution buffer and incubated for 12 hours at 4 °C. Slides were washed for three 10-minute periods with 0.3% Tween–PBS and incubated with fluorescein isothiocyanate–conjugated goat anti-rabbit antibodies (1:1000 dilution) for 2 hours at 20 °C. Slides were then washed for four 10-minute periods in 0.3% Tween–PBS. Slides were mounted in ProLong antifading medium with 4’, 6-deamidino-2-phenylindole for nuclear counterstaining (Molecular Probes, Eugene, OR), and cover slips were sealed with NailSlicks nail polish (Noxell Corp., Hunt Valley, MD). Fluorescence imaging was performed with a Carl Zeiss Axiophoto 2 (Carl Zeiss, Thornwood, NY), and Adobe Photoshop version 5.5 was used to capture the images.

For immunohistochemistry and histologic staining procedures using mouse tumors, one part of the tumor tissue was fixed in formalin and embedded in paraffin and the other part of the tumor was embedded in OCT compound (Miles, Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at –70 °C. Paraffin-embedded tissues were used for identification of VEGF and CD31. Sections (4–6 μm thick) were mounted on positively charged Superfrost slides (Fischer Scientific, Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene, treated with a graded series of alcohol (100%, 95%, and 80% ethanol [vol/vol] in double distilled H2O), and rehydrated in PBS (pH 7.5). Tissues were then treated with pepsin (Biomedia Corp., Foster City, CA) for 15 minutes at 37 °C and washed with PBS (35). A positive reaction was visualized by incubating the slides with stable 3,3’-diaminobenzidine (Research Genetics, Huntsville, AL) for 10–20 minutes. The sections were rinsed with distilled water, counter-stained with Gill’s hematoxylin (Sigma) for 1 minute, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining.

**Semiquantitative Reverse Transcription–Polymerase Chain Reaction Analysis**

Panc-1 cells were treated with 0.1% DMSO (control) or the NSAID as indicated for 48 hours, and then total RNA was collected (32) for reverse transcription-polymerase chain reaction (RT-PCR). For VEGF mRNA stability studies, Panc-1 cells were treated with actinomycin D (5 mg/mL) alone or in combination with 50 μM tolfenamic acid, as indicated. Total RNA was obtained with RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. The RNA concentration was determined by use of the 260 nm/280 nm absorption ratio, and RNA at 200 ng/μL was used in each reaction mixture for RT-PCR. RNA was reverse transcribed at 42 °C for 25 minutes with oligo(dT) primers (Promega), and then the reverse transcription product was amplified by PCR in a reaction mixture containing MgCl2 (2 mmol/L), each gene-specific primer at 1 μmol/L, all four deoxynucleoside triphosphates (each at 1 mmol/L), and 2.5 units of AmpliTag DNA polymerase (Promega). Gene products were amplified for 22–25 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds. Sequences of the oligonucleotide primers used in this study were as follows: VEGF forward = 5′-CCATGAACTTTCTCGTCCTTT-3′; VEGF reverse = 5′-ATCTGATCAAGGCGACACAG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward = 5′-ATCTCCATCACCATCTTCCA-3′; and GAPDH reverse = 5′-CTGTACATATTTGGCAGTGGT-3′. After amplification in a Peltier thermal cycler (Hybaid US, Franklin, MA), 20 μL of each reaction mixture was loaded on a 2% agarose gel containing ethidium bromide. Electrophoresis was performed at 80 V in 1x TAE buffer (48.4 g of Tris base, 11.4 mL of acetic acid, and 20 mL of 0.5 M EDTA in 1 L of water; diluted 1:10) for 1 hour, and the gel was photographed by UV transillumination on Polaroid film (Waltham, MA). The intensity of VEGF and GAPDH bands was obtained by scanning the Polaroid film with a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ); and a densitometric analysis was performed on the inverted image with Zero-D software (Scanalytics). Results for VEGF band intensity were normalized to GAPDH band intensity values and then the normalized values from three determinations for each treatment group were averaged.

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Animals and Orthotopic Implantation of Tumor Cells

Male athymic nude mice (NCr-nu/nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8–12 weeks old.

To produce tumors, L3.6pl cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with minimal essential medium containing 10% fetal bovine serum, and the harvested cells were washed once in serum-free medium and resuspended in Hanks’ balanced salt solution. Only single-cell suspensions with greater than 90% viability were used for the injections. Injection of 1×10⁶ cells into the pancreas was performed as described previously (33,34). Each treatment group contained 10 mice. The mice were killed when moribund (5–6 weeks after injection). The size and weight of each primary pancreatic tumor and the incidence of liver metastases were recorded. Histopathologic studies confirmed the nature of the disease. For immunohistochemical and histologic staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, the other part of the tumor was embedded in OCT compound (Miles), snap-frozen in liquid nitrogen, and stored at −70 °C.

Treatment of Established Human Pancreatic Carcinoma Tumors Growing in the Pancreas of Nude Mice

Seven days after implantation of 10⁶ L3.6pl tumor cells into the pancreases of each mouse (10 mice per treatment group, for a total of 40 mice); five mice were killed to confirm the presence of tumor lesions. Tumor volumes were calculated by using the following formula: 0.5 × length × width². At 7 days after injection of tumor cells, the median tumor volume was 21 mm³ (interquartile range of 18–25 mm³). Histologic examination confirmed that the lesions were actively growing pancreatic cancers. Mice were randomly assigned to receive one of the following treatments: 1) thrice weekly oral administrations of vehicle solution for tolfenamic acid in corn oil (control group); 2) thrice weekly oral administrations of tolfenamic acid (25 mg/kg of body weight) in corn oil; 3) thrice weekly oral administrations of tolfenamic acid (50 mg/kg) in corn oil; or 4) twice weekly intraperitoneal injections of gemcitabine (50 mg/kg) alone, which is the most commonly used drug for treatment of pancreatic cancer (33,34). Treatments were continued for 4 weeks, and the mice were killed by CO₂ asphyxiation on day 35, weighed, and subjected to necropsy. The volume of pancreatic tumors and the incidence of liver metastases were recorded (33,34).

Necropsy Procedures and Histologic Studies

Mice were killed and body weight was recorded. Primary tumors in the pancreas were excised, measured, and weighed. For immunohistochemical and hematoxylin–eosin staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, and another part was embedded in OCT compound, rapidly frozen in liquid nitrogen, and stored at −70 °C. Visible liver metastases were counted with the aid of a dissecting microscope, and the tissues were processed for hematoxylin–eosin staining.

Statistical Analysis

Statistical significance of differences in protein levels, luciferase activity, cell and tumor growth, and liver metastasis between NSAID-treated cells or mice and solvent control cells or mice, respectively, was determined by an analysis of variance and Scheffe’s test, and the levels of probability were noted. The results of cell culture studies are expressed as means (95% confidence intervals) for at least three separate (replicate) experiments for each treatment, and statistical significance of differences between values in the treated and solvent control cells (P<0.05) was determined by an analysis of variance and Scheffe’s tests. All statistical tests were two-sided.

Results

Structurally Diverse NSAIDs and the Inhibition of Sp1, Sp3, and Sp4 Expression in Pancreatic Cancer Cells

Previous RNA interference studies have reported that Sp1, Sp3, and Sp4 proteins regulate VEGF expression in pancreatic cancer cells and that celecoxib decreased the expression of Sp1, Sp3, and VEGF in both in vitro and in vivo models (30,31). To investigate the effects of NSAID structure on expression of Sp proteins, we screened biphenyl or biphenylamine carboxylic acids (tolfenamic acid, diclofenac, and diflunisal), oxicas (amproxicam), acetic acid derivatives (acemetacin and tolmetin), and propionic acid (ibuprofen, naproxen, fenbufen, and ketoprofen) derivatives. We initially screened compounds at 50 and 100 μM, including compounds from different structural classes of NSAIDs, celecoxib (a COX-2 inhibitor), and valeryl salicylate (a COX-1 inhibitor), because most studies (20–30) found that growth was inhibited at COX-1 or COX-2 inhibitor concentrations between 25 μM and 100 μM. We used Panc-1 cells and immunoblot analysis to screen COX-1 or COX-2 inhibitor compounds for changes in the expression of Sp1, Sp3, and Sp4 proteins after 48 hours of treatment. Treatment with 50 μM celecoxib decreased levels of Sp1 and Sp4 proteins (Fig. 1, A), as previously described in colon cancer cells (32). Treatment with acemetacin, amproxicam, naproxen, fenbufen ibuprofen, tolmetin, letrozole, or valeryl salicylate (each at 50 μM) had no effect. Treatment with diphenyl- and diphenylamine-derived carboxylic acid compounds (each at 50 μM) decreased expression of Sp1, Sp3, and Sp4 proteins in Panc-1 cells. The most potent compound of the third group was tolfenamic acid (Fig. 1, B). Similar data for each compound were obtained at 100 μM (data not shown). A 48-hour treatment with 50 μM tolfenamic acid also induced a time-dependent decrease in the expression of Sp1, Sp3, and Sp4 proteins in Panc-1 cells (a >80% decrease in levels of all three proteins), compared with those in solvent (0.1% DMSO)-treated cells (Fig. 1, C) and in highly metastatic L3.6pl pancreatic cancer cells (a >65% decrease in the levels of all three proteins) (Fig. 1, D). In contrast, treatment with 50 μM of the NSAID amproxicam (used as a negative control) did not affect the expression of Sp proteins. Thus, the effects of
Fig. 1. Nonsteroidal antiinflammatory drugs (NSAIDs) and Sp1, Sp3, and Sp4 protein expression in pancreatic cancer cells. A) Screen of NSAIDs and cyclooxygenase 1 (COX-1) and Cox-2 inhibitors for Sp protein degradation in Panc-1 cells. Cells were treated with 50 μM of the indicated compounds for 48 hours, and whole-cell lysates were examined by immunoblot analysis for the expression of Sp1, Sp3, Sp4, tubulin, and histone deacetylase (HDAC). B) Quantitation of Sp proteins expressed by Panc-1 cells after treatment with an NSAID or COX-1 or Cox-2 inhibitors, shown in panel A. The relative percentage of Sp1, Sp3, and Sp4 levels in selected treated cells compared with control cells (0.1% dimethyl sulfoxide [DMSO]); all values set at 100%) are presented as averages of two separate determinations from different gels and the duplicate values differed by less than 20%. Protein band intensities were normalized to that of β-tubulin protein, which was used as a loading control. HDAC was also unaltered by the treatments. C and D) Effects of selected NSAIDs on Sp protein in Panc-1 and L3.6p1 cells, respectively. Panc-1 cells were treated with 0.1% DMSO, 50 μM ampiroxicam, or 50 μM tolfenamic acid for 24 or 48 hours, and Sp1, Sp3, and Sp4 protein levels were determined in whole-cell lysates by immunoblot analysis (left panels). Protein band intensities were normalized to β-tubulin, and protein levels are presented as means with error bars representing 95% confidential intervals for three replicate determinations for each treatment group (right panels). Statistically significantly (P = .003) decreased protein levels, compared with those of control cells, are indicated by an asterisk.
tolfenamic acid on VEGF expression were investigated further in Panc-1 cells transfected with constructs (pVEGF1 and pVEGF2) containing VEGF promoter inserts.

**Tolfenamic Acid and Transactivation of Luciferase Activity in Pancreatic Cancer Cells Transfected With VEGF Promoter Constructs**

Because previous studies (29–32) showed that COX-2 inhibitors or Sp small inhibitory RNAs decreased VEGF expression in colon and pancreatic cancer cells, we investigated the effect of 0.1% DMSO, 50 μM tolfenamic acid, or 50 μM ampiroxicam on transactivation in Panc-1 cells transfected with pVEGF1 or pVEGF2 (Fig. 2, A and B). pVEGF1 contains a VEGF promoter insert from positions −2018 to +50, and pVEGF2 contains a VEGF promoter insert from positions −131 to +54. These experiments were intended to determine the effects of tolfenamic acid on the full-length VEGF promoter (pVEGF1) and on the Sp-binding, GC-rich proximal region of the VEGF promoter (pVEGF2). Tolfenamic acid, but not ampiroxicam, statistically significantly decreased the expression of luciferase by at least 50% in cells transfected with either construct. For example, in Panc1 cells, relative luciferase activity in cells treated with 0.1% DMSO and transfected with pVEGF1 (Fig. 2, A) or pVEGF2 (Fig. 2, B) was statistically significantly higher (0.214, 95% CI = 0.200 to 0.228, or 0.037, 95% CI = 0.023 to 0.051, respectively) than that in transfected cells treated with 50 μM tolfenamic acid (0.070, 95% CI = 0.061 to 0.079, and 0.010, 95% CI = 0.0004 to 0.0196, respectively) (P = .003). In parallel experiments in Panc-1 cells, we investigated the effect of diclofenac, which also induces Sp protein degradation (Fig. 1, A) and found that treatment with diclofenac decreased transactivation of luciferase activity in Panc-1 cells transfected with pVEGF1 (Fig. 2, C) or with pVEGF2 (Fig. 2, D). Decreased luciferase activity after treatment with tolfenamic acid or diclofenac is consistent with previous studies showing that activation of these constructs depended on interactions of Sp1, Sp3, and Sp4 proteins with proximal GC-rich motifs (29–32). A comparable experiment was carried out in L3.6pl cells (Fig. 2, E and F), and the results were similar to those observed in Panc-1 cells. These data are consistent with the decreased levels of Sp1, Sp3, and Sp4 protein in L3.6pl and Panc-1 cells treated with tolfenamic acid, as shown in Fig. 1. A concentration-dependent decrease in transactivation of luciferase activity was detected in Panc-1 cells transfected with pVEGF2 and treated with 20–80 μM tolfenamic acid (Fig. 2, G), but ampiroxicam (Fig. 2, G) and the COX-1 inhibitor valeryl salicylate (data not shown) had no effect.

**Tolfenamic Acid and Sp Protein Binding to GC-rich VEGF Promoter Oligonucleotides**

VEGF is regulated by the binding of Sp proteins to proximal GC-rich promoter sequences (29–32). To determine the effects of tolfenamic acid and ampiroxicam on the binding of Sp proteins to such DNA sequences, we used gel electrophoretic mobility shift assays (Fig. 3, A and B). Panc-1 or L3.6pl cells were treated with 0.1% DMSO, 50 μM tolfenamic acid, or 50 μM ampiroxicam for 48 hours. Nuclear extracts from these cells were isolated and incubated with 32P-labeled VEGF oligonucleotide, and then the presence of Sp–oligonucleotide complexes was assessed in gel mobility shift assays. The 32P-labeled oligonucleotide contained a GC-rich region (positions −66 to −47) from the VEGF promoter that binds Sp proteins. In extracts from Panc-1 cells or from L3.6pl cells treated with 0.1% DMSO or ampiroxicam, the retarded bands containing Sp3 or Sp1–Sp4 (overlapping) had similar intensities; however, in extracts from Panc-1 cells or from L3.6pl cells treated with tolfenamic acid, intensities of both retarded bands decreased compared with bands from extracts of cells treated with 0.1% DMSO. No retarded bands were observed in reaction mixtures containing 32P-labeled VEGF oligonucleotides alone. To confirm that the complexes contained the indicated Sp protein, antibodies against Sp1, Sp3, or Sp4 proteins were added to the reaction mixtures described above, so that the band of the corresponding Sp–oligonucleotide–antibody complex would be retarded even more or supershifted, as shown in Fig. 3, B. Thus, tolfenamic acid decreased the binding of Sp1, Sp3, and Sp4 to GC-rich oligonucleotides containing the VEGF promoter sequence between positions −66 and −47, a result that is consistent with decreased transactivation observed in transient transfection studies with the pVEGF1 and pVEGF2 constructs.

**Tolfenamic Acid, VEGF mRNA and Protein Expression, and Activation of Proteasome-Dependent Degradation of Sp1, Sp3, and Sp4**

We next investigated the effects of tolfenamic acid on the expression of VEGF mRNA and protein. We first treated Panc-1 cells with 0.1% DMSO, 50 μM ampiroxicam, or 50 μM tolfenamic acid for 48 hours and then determined VEGF protein levels (relative to that of β-tubulin) (Fig. 4, A). Treatment with tolfenamic acid decreased VEGF protein expression by more than 60%, compared with that of solvent (0.1% DMSO) control. The effects of tolfenamic acid on VEGF mRNA levels were also investigated in Panc-1 cells by RT-PCR and compared with levels in solvent (0.1% DMSO)- or ampiroxicam-treated cells. A 24-hour tolfenamic acid treatment decreased VEGF mRNA levels by more than 60%, compared with that of 0.1% DMSO treatment (Fig. 4, B). The possibility that tolfenamic acid decreased VEGF mRNA stability was ruled out because VEGF mRNA levels decreased at similar rates in Panc-1 cells treated with actinomycin D alone or with 50 μM tolfenamic acid. In Panc-1 cells treated with 0.1% DMSO or 50 μM ampiroxicam, VEGF protein was secreted, and extracellular VEGF staining was observed (Fig. 4, D). However, in Panc-1 cells treated for 48 hours with 50 μM tolfenamic acid, no secreted VEGF protein was detected, indicating that VEGF protein was not secreted. Thus, the tolfenamic acid–dependent decrease in the expression of Sp1, Sp3, and Sp4 proteins appears to lead to decreased levels of VEGF mRNA and protein, a result that is consistent with the reported Sp-dependent regulation of VEGF (29–32).

In a previous study (32), we reported that the COX-2 inhibitor nimesulide decreased levels of Sp1 and Sp4 (but not Sp3) in colon cancer cells by activating the proteasome pathway and that this response was blocked by the proteasome inhibitor gliotoxin. Treatment with tolfenamic acid decreased the levels of Sp1, Sp3, and Sp4 proteins (Fig. 4, E), in contrast to treatment with nimesulide (32) or celecoxib (Fig. 1, A), both of which induced degradation of Sp1 and Sp4 but not Sp3. However, when Panc-1 cells were treated with a combination of 50 μM tolfenamic acid and 2 μM lactacystin (a proteasome inhibitor), Sp1, Sp3, and Sp4 protein levels were not decreased. Thus, like the COX-2 inhibitors celecoxib and nimesulide, tolfenamic acid appeared to activate proteasome-dependent degradation of Sp1 and Sp4 in Panc-1 cells, but unlike those compounds, tolfenamic acid also...
Fig. 2. Decreased transactivation of the vascular endothelial growth factor (VEGF) promoter (luciferase activity) in pancreatic cancer cells transfected with pVEGF1 or pVEGF2 (contain VEGF promoter inserts positions −2018 to +50 and positions −131 to +54, respectively) and treated with nonsteroidal antiinflammatory drugs (NSAIDs). Panc-1 cells were transfected with pVEGF1 or pVEGF2 and treated with 0.1% dimethyl sulfoxide (DMSO), 50 μM ampiroxicam, or 50 μM tolfenamic acid (A and B) or with 0.1% DMSO, 50 μM ampiroxicam, or 50 μM diclofenac (C and D). Luciferase activity was determined as a reflection of the activation of the VEGF promoter. In a similar experiment, L3.6p1 cells were transfected with pVEGF1 (E) or pVEGF2 (F) and treated with 0.1% DMSO, 50 μM ampiroxicam, or 50 μM tolfenamic acid, and then luciferase activity was determined. G) NSAIDs and activation of VEGF promoter constructs. Panc-1 cells were transfected with pVEGF2 and treated with ampiroxicam or tolfenamic acid (each with 20, 40, 60, or 80 μM), and luciferase was determined. Results are expressed as the mean luciferase activity with error bars representing 95% confidence intervals for three separate determinations per treatment group. A statistically significant (P = .003) decrease in luciferase activity is indicated by an asterisk.
induced degradation of Sp3. Also, decreased transactivation of luciferase activity in Panc-1 cells transfected with pVEGF2 and treated with tolfenamic acid was also blocked with cotreatment of lactacystin (Fig. 4, F), and similar results were obtained for gliotoxin (data not shown). Ampiroxicam had no effect in the presence or absence of lactacystin. These results show that inhibition of tolfenamic acid-induced Sp protein degradation by proteasome inhibitors blocks decreased VEGF expression and also confirmed the role of Sp proteins as key mediators of VEGF expression.

**Tolfenamic Acid and the Proliferation of Pancreatic Cancer Cells**

Because Sp proteins regulate genes involved in cancer cell proliferation and angiogenesis, we investigated the mechanism by which tolfenamic acid and other NSAIDs inhibit the proliferation of pancreatic cancer cells. Ampiroxicam, naproxen, diclofenac, and tolfenamic acid decreased the proliferation of Panc-1 cells, with diclofenac and tolfenamic acid having the highest potencies (Fig. 5, A–D). Because the differences in the effects of NSAIDs at 50 μM observed for Sp protein degradation (Fig. 1, A) may be related, in part, to their relative growth-inhibitory activities, we compared the effects of tolfenamic acid, ampiroxicam, and naproxen on Sp protein expression by use of concentrations that induced comparable inhibition of Panc-1 cell proliferation. Thus, Panc-1 cells were treated with 50 μM tolfenamic acid, 150 μM naproxen, or 150 μM ampiroxicam for 48 hours and subjected to Western blot analysis. Tolfenamic acid was the only compound tested that induced protein degradation and poly(ADP-ribose)polymerase cleavage (Fig. 5). These data support results of the initial screening assay (Fig. 1, A) showing that only NSAIDs containing the diphenyl or diphenylamine carboxylic acid structure induced Sp protein degradation. When we used a similar range of concentrations in L3.6pl (Fig. 6, A and B) and Panc-28 (Fig. 6, C and D) cells, we also found that tolfenamic acid was a more potent inhibitor of pancreatic cancer cell proliferation than ampiroxicam. Thus, the growth inhibitory effects of tolfenamic acid in pancreatic cancer cells appear to be associated, in part, with degradation of Sp proteins.

**Tolfenamic Acid and Pancreatic Tumor Growth and Metastasis in an Orthotopic Model**

Potential antitumorigenic and antiangiogenic activities of tolfenamic acid against pancreatic tumors were next investigated in an orthotopic athymic nude mouse model. L3.6pl cells were used for this study because of their aggressive growth and production of liver metastases (33, 34). Treatment with tolfenamic acid (at 25 or 50 mg/kg/day) for 4 weeks decreased median tumor weights and volumes (Fig. 7, A and B) and also decreased the percent incidence of liver metastasis (Table 1). Moreover, at these doses, changes in body or organ weights or organ toxic effects were not observed. At comparable doses, tolfenamic acid appeared to be a more effective tumor growth inhibitor than gemcitabine; gemcitabine, however, did not affect the incidence of liver metastasis.

We also assessed levels of Sp1, Sp3, Sp4, and VEGF proteins in pancreatic tumors from tolfenamic acid–treated versus corn oil–treated mice. Tolfenamic acid treatment statistically significantly decreased expression of Sp1, Sp3, Sp4, and VEGF proteins in pancreatic tumors (Fig. 7, C), as observed in pancreatic cancer
Fig. 4. Nonsteroidal antiinflammatory drugs (NSAIDs), vascular endothelial growth factor (VEGF) and Sp protein expression in Panc-1 cells, and the proteasome inhibitor lactacystin. A) VEGF protein expression. Panc-1 cells were treated with 0.1% dimethyl sulfoxide (DMSO), 50 μM ampiroxicam, or 50 μM tolfenamic acid for 48 hours, and then VEGF protein levels were determined by western blot analysis. B) VEGF mRNA levels. Panc-1 cells were treated with 0.1% DMSO, 50 μM ampiroxicam, or 50 μM tolfenamic acid for 12 hours, and mRNA levels (relative to that of glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were determined by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). Results in panels A and B are presented as the means, with error bars representing 95% confidence intervals, for three separate determinations for each treatment group, and statistically significantly decreased VEGF expression is indicated by an asterisk (in panel A, P = .01, and in panel B, P = .007). C) VEGF mRNA stability. Panc-1 cells were treated with actinomycin D (ActD; 5 mg/mL) alone or in combination with 50 μM tolfenamic acid (TOL) for up to 4.5 hours, and VEGF mRNA levels were determined at time 0 and 1.5, 2.5, and 4.5 hours after treatment by semiquantitative RT-PCR. Results are the average of duplicate experiments, and VEGF mRNA levels are normalized to that of GADPH mRNA. D) Immunocytochemistry for VEGF. Panc-1 cells were treated with 0.1% DMSO, 50 μM ampiroxicam, and 50 μM tolfenamic acid for 48 hours and then immunostained for VEGF (green). The blue color represents nuclear staining that was obtained by use of mounting medium containing 4′,6-diamidino-2-phenylindole. Scale bar = 10 μm. E) Lactacystin and the degradation of Sp proteins. Panc-1 cells were treated with 0.1% DMSO, 50 μM ampiroxicam, or 50 μM tolfenamic acid alone or in combination with 2 μM lactacystin for 48 hours, and whole cell lysates were analyzed for Sp proteins by Western blot analysis. β-Tubulin was the loading control. Results are expressed as the mean percentage of control and error bars representing 95% confidence intervals. Statistically significantly decreased levels of Sp proteins after treatment with tolfenamic acid (*, P = .005) or inhibition of the response by treatment with lactacystin (**, P = .006) are indicated. F) Lactacystin and decreased luciferase activity in cells transfected with pVEGF2. Panc-1 cells were transfected with pVEGF2 (containing a VEGF promoter insert from position −131 to position +54) and then treated with 0.1% DMSO, 50 μM ampiroxicam, or 50 μM tolfenamic acid alone or in combination with 2 μM lactacystin. Luciferase activity was determined as a measure of activation of the VEGF promoter. Results are expressed as the mean relative luciferase activity, with error bars representing 95% confidence intervals for three separate determinations for each treatment group. Statistically significantly decreased activity by tolfenamic acid (*, P = .003) or inhibition of this response by lactacystin (**, P = .009) are indicated.
cells treated with tolfenamic acid (Figs. 1 and 4). For example, the VEGF protein level in tumors from mice treated with tolfenamic acid (50 mg/kg) was statistically significantly lower (45%, 95% confidence interval = 39% to 51%; \( P = .009 \)) than that in tumors from control mice treated with corn oil (i.e., 100%). Immunostaining for VEGF in tumor sections from control animals and animals treated with gemcitabine (50 mg/kg) or tolfenamic acid (25 and 50 mg/kg) also indicated relatively high concentrations of VEGF in tumors from control and gemcitabine-treated animals but relatively low concentrations of VEGF in pancreatic tumors from mice treated with tolfenamic acid (Fig. 7, D).

In parallel studies, staining with CD31 to determine microvessel density indicated lower microvessel density in tumors from mice treated with tolfenamic acid than in tumors from vehicle (corn oil)-treated or gemcitabine-treated animals (Fig. 7, E). Thus, tolfenamic acid had antitumorigenic and antiangiogenic activities in pancreatic cancer cells and tumors in vivo through the degradation of Sp proteins, which leads to decreased VEGF expression.

**DISCUSSION**

Tolfenamic acid induced degradation of Sp proteins and decreased expression of VEGF in pancreatic cancer cells and tumors, inhibited cell or tumor growth and decreased metastasis to the liver in a mouse orthotopic pancreatic tumor model. Sp proteins play a critical role in growth and metastasis of cancer (36–38), and Sp1 expression may be negatively associated with survival in some cancer patients (29,39–43). The important role of Sp proteins in tumor growth and metastasis is not surprising because Sp1 and other Sp proteins are transcription factors that regulate sets of genes responsible for cancer cell proliferation and angiogenesis (29–32,36–38). Development of anticancer drugs that specifically inhibit Sp-dependent transactivation have been reported, including oligonucleotides, peptide nucleic acid–DNA chimeras, drugs such as mithramycin that disrupt Sp protein–DNA binding (44–48), and agents such as COX-2 inhibitors that induce Sp protein degradation (31,32). Wei et al. (31) first reported that celecoxib decreased cell and tumor growth and the expression of Sp1 and VEGF in pancreatic cancer cells and in tumors from nude mice bearing FG pancreatic cancer cells (orthotopic and xenograft models). In colon cancer cells, the COX-2 inhibitors celecoxib, nimesulide, and NS-398 induced proteasome-dependent degradation of Sp1 and Sp4 proteins but not Sp3 protein, and this degradation also resulted in decreased VEGF expression (32).

In several pancreatic cancer cell lines, we used RNA interference to knock down the expression of Sp proteins (30) and showed that Sp1, Sp3, and Sp4 are important transcription factors for constitutive expression of VEGF. We have also found (Kelly Higgins, Maen Abdelrahim, and Stephen Safe, unpublished results) that Sp proteins are important for regulation of VEGF receptors in pancreatic cancer cells. Therefore, an optimal antiangiogenic
agent for treating pancreatic cancer should modulate expression of all three Sp proteins. In our initial screening of pancreatic cancer cells with COX-2 inhibitors, such as celecoxib (Fig. 1, A), we found that celecoxib induced degradation of Sp1 and Sp4, as previously reported in colon cancer cells (32). However, on the bases of previous reports of the antiangiogenic activity of NSAIDs (4–6), we further screened various NSAIDs as potential inducers of Sp protein degradation (Fig. 1, A). Our initial screening approach was to examine the effects of different structural classes of NSAIDs, including substituted diphenylamines (diclofenac and tolfenamic acid), diphenyls (diflunisal and fenbufen), diphenylmethanes (letrozole), indoles (acemetacin), 1,2-benzothiazines (ampiroxicam), benzophenones (ketoprofen), phenyl/pyrrole ketones (tolmetin), naphthyl and phenyl acetic acids (ibuprofen and naproxen), celecoxib, and valeryl salicylate, a COX-1 inhibitor. Only the substituted diphenyl and diphenylamine carboxylic acid derivatives induced degradation of Sp1, Sp3, and Sp4 proteins (Fig. 1, A), and the most active compound was tolfenamic acid. This is, to our knowledge, the first report of a compound that induces degradation of all three Sp proteins.

We then investigated the antiangiogenic and antitumorigenic activity of tolfenamic acid, which induced the parallel degradation of Sp1, Sp3, and Sp4 proteins. Ampiroxicam, an NSAID that does not affect Sp protein expression, was used as a negative control for these studies. Tolfenamic acid treatment reduced Sp protein levels by inducing degradation of Sp proteins (Fig. 1) by activation of the proteasome pathway (Fig. 5), as previously reported for COX-2 inhibitors (32), and the proteasome inhibitor lactacystin reversed the effects of tolfenamic acid. Degradation of Sp proteins after treatment of pancreatic cancer cells with tolfenamic acid was accompanied by decreased transactivation of luciferase activity in pancreatic cancer cells transfected with VEGF promoter constructs (Fig. 2), decreased expression of VEGF protein and mRNA in cells (Fig. 4), and decreased pancreatic cancer cell growth (Figs. 5–7).

A recent study (49) reported that prostaglandin E2 induced VEGF in smooth muscle cells and that this induction was accompanied by increased phosphorylation of Sp1. It is unlikely that tolfenamic acid acts by inhibiting COX–2 dependent prostaglandin E2 synthesis because most other COX inhibitors do not affect Sp protein expression (Fig. 1) and because Panc-1 cells do not express COX-2 (50). Thus, our results coupled with the decreased or nondetectable activity of the NSAID ampiroxicam in the same assays support the role of Sp protein degradation as a major pathway for the growth-inhibitory and/or antiangiogenic activity of tolfenamic acid and the direct linkage of this degradation of Sp protein to decreased VEGF expression. Our results, however, do not exclude a role for other Sp-dependent genes in mediating effects of tolfenamic acid.

Results of our in vitro studies in pancreatic cancer cells clearly demonstrate that tolfenamic acid exhibits growth-inhibitory and antiangiogenic activities through the activation of proteasome-dependent degradation of Sp1, Sp3, and Sp4. The in vivo effects of tolfenamic acid were further investigated in an orthotopic model of pancreatic cancer by use of L3.6pl cells, which not only form pancreatic tumors in mice but also metastasize...
Fig. 7. Inhibition of orthotopic pancreatic tumor growth and angiogenesis in mice by tolfenamic acid and gemcitabine. Median tumor volumes (A) and weights (B) in athymic nude mice treated orthotopically with L3.6p1 cells and then with corn oil (control), tolfenamic acid (25 or 50 mg/kg), or gemcitabine (50 mg/kg) were determined 4 weeks after initial treatment with these compounds. Results as indicated are expressed as means, with error bars representing 95% confidence intervals. Statistically significantly \((P = .005)\) decreased tumor volumes and weights, compared with the corn oil control, are indicated by an asterisk. C) Sp and vascular endothelial growth factor (VEGF) protein expression. Tumors from the various treatment groups were analyzed for Sp1, Sp3, Sp4, and VEGF protein expression by Western blot analysis. Results are expressed relative to the solvent (corn oil) control and normalized to \(\beta\)-tubulin within each group and are the means, with error bars representing 95% confidence intervals for at least three separate determinations for each treatment group. Pancreatic tumor sections from animals treated with solvent (control), gemcitabine (50 mg/kg), or tolfenamic acid (25 or 50 mg/kg) were immunostained for VEGF (D) and CD31 (E) with VEGF and CD31 antibodies, respectively. CD31 expression was obtained by immunostaining paraffin-embedded section, and the brown staining is a reflection of microvessel density in the tumor.

to the liver \((33, 34)\). Gemcitabine (50 mg/kg) was included as a control in this model because it is widely used for treatment of pancreatic cancer \((51)\). Treatment with gemcitabine at 50 mg/kg decreased tumor volume and weight but did not affect metastasis to the liver (Table 1). In contrast, tolfenamic acid (50 mg/kg) statistically significantly decreased the median tumor volume and
weight (Fig. 7, A, and Table 1) and liver metastasis (Table 1). The expression of Sp1, Sp3, Sp4, and VEGF proteins in individual tumors was decreased by tolfenamic acid treatment (Fig. 7, C). Moreover, the expression of VEGF was decreased in pancreatic tumors (Fig. 7, D) and cells (Fig. 4, D) by treatment with tolfenamic acid, and this decreased VEGF expression corresponded to decreased CD31 expression in tumors from mice treated with tolfenamic acid (Fig. 7, E). These effects of tolfenamic acid in pancreatic cells and tumors demonstrate that Sp transcription factors may be important targets for pancreatic cancer chemotherapy. Moreover, among intercalating agents, COX-2 inhibitors, and Sp oligonucleotide decoys used for blocking Sp-mediated transcription and downstream responses (30–32, 44–48), tolfenamic acid and structurally related NSAIDs appear to be among the most effective.

This study has several limitations. It is also possible that structural classes of NSAIDs other than tolfenamic acid may exhibit similar activities, and this possibility will require more extensive screening of these compounds. Sp protein degradation is accompanied by a parallel decrease in levels of VEGF mRNA and protein (Figs. 4 and 7); however, identity of other key growth regulatory and angiogenic factors that are also affected by these NSAIDs has not been determined. Moreover, although it is clear that tolfenamic acid has antipancreatic cancer activity in cells and in an orthotopic model for pancreatic cancer, the different effects of these NSAIDs on other tumor and nontumor tissues, from mice and other species (including humans), require further investigation.

In summary, two structurally related classes of NSAIDs (i.e., diphenyl and diphenylamine carboxylic acids) were potent inhibitors of pancreatic cell and tumor growth and angiogenesis. The mechanisms of action for tolfenamic acid appeared to result, at least in part, from activation of proteasome-dependent degradation of Sp1, Sp3, and Sp4. The use of tolfenamic acid for treatment of migraines can require daily doses of 7.5–10 mg/kg, which is less than the doses used in this study, and so development of more active analogs may be required for clinical treatment of pancreatic cancer. The prospect that tolfenamic acid, alone or in combination with other drugs, may be useful in the treatment of pancreatic cancer—which is often not diagnosed in humans until the tumors are well established and have already metastasized—is tantalizing. Future studies should focus on the development of more potent derivatives of tolfenamic acid, on determining the mechanism by which these compounds activate proteasomes to selectively degrade Sp transcription factors, and on investigating the effects of tolfenamic acid on expression of other key factors involved in tumor growth and angiogenesis.

### REFERENCES


NOTES

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Present address: Cheryl H. Baker, M. D. Anderson Cancer Center, Orlando Cancer Research Institute, Orlando, FL 32806.

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