Tolfenamic acid enhances pancreatic cancer cell and tumor response to radiation therapy by inhibiting survivin protein expression

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Abstract

Survivin is overexpressed in most human cancers, including pancreatic adenocarcinoma. Expression of survivin is regulated by specificity protein (Sp) proteins and related to resistance to radiation therapy. Tolfenamic acid induces Sp protein degradation in several cancer cell lines. The purpose of this study is to investigate whether tolfenamic acid inhibits survivin expression and sensitizes pancreatic cancer cells/tumor to radiotherapy. Panc1 and L3.6pl cells have been used to study the effect of radiation on survivin expression and to investigate the efficacy of tolfenamic acid in enhancing the response to radiation therapy. In addition, an orthotopic model for human pancreatic cancer has been used to confirm the efficacy of tolfenamic acid to enhance tumor response to radiation *in vivo*. Pancreatic cancer cell lines express variable levels of survivin mRNA/protein, which correlate with their radiosensitivity. Radiation increased survivin promoter activity and protein expression in Panc1 and L3.6pl cells and tolfenamic acid inhibited both constitutive and radiation-induced survivin protein expression and enhanced the response of pancreatic cancer cells to radiation therapy. *In vivo* studies show that tolfenamic acid enhanced the radiation-induced apoptosis associated with decreased survivin expression in tumors and this correlates with the enhanced response of these tumors to the radiation. Thus, tolfenamic acid significantly enhances pancreatic cancer cells/tumor response to radiation therapy. The underlying mechanism includes tolfenamic acid-induced degradation of Sp proteins, which in tumor decreases expression of the Sp-dependent antiapoptotic protein survivin. These preclinical data suggest that tolfenamic acid has the potential to increase the response of pancreatic adenocarcinoma to radiation therapy. [Mol Cancer Ther 2009;8(3):533–42]

Introduction

Survivin, a member of the inhibitor of apoptosis protein family, is a bifunctional protein that has been implicated in the control of cell division and inhibition of apoptosis (1). Survivin is usually undetectable in most normal adult tissues (2); however, it is widely expressed during fetal development (3) and is found in most human carcinomas, including pancreatic cancer (1, 4–6).

High expression of survivin is commonly associated with an enhanced proliferative index, reduced levels of apoptosis, and increased rates of tumor recurrence. Furthermore, elevated levels of survivin and also predominantly cytoplasmic survivin have been associated with aggressive clinicopathologic features and show a strong correlation with reduced disease-free and overall survival rates in most studies (7, 8).

In most retrospective trials, cancer patients with high levels of survivin exhibit increased resistance to radiotherapy. For example, in a rectal cancer trial, survivin expression was inversely correlated with the level of spontaneous apoptosis. Moreover, high survivin expression was significantly associated with the risk of local tumor recurrences (9). In cervical cancer, patients' expression of survivin after treatment with definitive radiotherapy showed that high survivin expression was correlated with decreased overall survival (10). In pancreatic cancer patients, survivin is a prognostic marker (6) and is thought to be involved in resistance of tumor cells to radiation therapy and the effect of other anticancer agents. Several reports show that inhibition of survivin expression in pancreatic cancer cells decreases growth and resistance to radiation therapy (11, 12).

Due to its high expression in cancer tissue and its potential for maintaining cancer cell viability (7), survivin is a suitable molecular target for radiosensitization. Different strategies to decrease survivin expression or activation in pancreatic and other tumor cells have shown that inhibition of

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survivin result in increased radiation-induced apoptosis and enhanced response to chemotherapy and radiotherapy (8, 13). These strategies include antisense oligonucleotides (11), ribozymes (12), small interfering RNA (14), and dominant-negative and small molecules (12, 15–18).

A major strategy for modulating survivin expression is control of its transcription. Previous reports show that, in some cancer cell lines, survivin expression is dependent in part on specificity protein (Sp) proteins (19–22) and Sp1 and other Sp proteins are overexpressed in cancer cells and tumors including the pancreatic tumors (23–27). Tolfenamic acid is a nonsteroidal anti-inflammatory drug that has been used previously in human to treat the symptoms of migraine (28–32). Recently, we reported that tolfenamic acid inhibited pancreatic cancer cell and tumor growth through proteasome-dependent degradation of Sp1, Sp3, and Sp4 proteins (33). Therefore, we investigated the effects of tolfenamic acid on inhibition of survivin protein expression and enhanced response of pancreatic cancer cells and tumors to radiation therapy.

Results of this study show for the first time that tolfenamic acid inhibits survivin protein/mRNA expression in Panc1 and L3.6p1 cells and this was dependent on induced degradation of Sp1, Sp3, and Sp4 proteins. Tolfenamic acid not only inhibited pancreatic cancer cell growth but also increased their sensitivity to radiation therapy. Basal and radiation-induced expression of survivin in Panc1 and L3.6pl is different and this could explain their different responses and sensitivity to radiation therapy. *In vivo* studies in an orthotopic pancreatic cancer model showed that radiation moderately inhibited tumor growth; however, the inhibition was more robust when tolfenamic acid was used in combination with radiation therapy. Furthermore, TUNEL staining of tumor tissue indicated that radiation-induced apoptosis significantly increased when mice were cotreated with tolfenamic acid. The results indicate that radiosensitizing effects of tolfenamic are associated with targeted degradation of Sp transcription factors and Sp-dependent survivin expression.

Materials and Methods

Cell Lines, Chemicals, and Biochemistry

Panc1 cells were obtained from the American Type Culture Collection. The L3.6pl cell line was developed at The University of Texas M. D. Anderson Cancer Center. DMEM/F-12 with and without phenol red, 100× antibiotic/antimycotic solution, and tolfenamic acid were purchased from Sigma. Rabbit IgG, antibodies for Sp1, Sp3, and Sp4, β-actin, and survivin proteins were obtained from Santa Cruz Biotechnology. Lysis buffer and luciferase reagent were obtained from Promega. Colorimetric TUNEL staining kits were purchased from Promega.

Irradiation

IC160 X-ray irradiation system (Kimtron) was employed to irradiate the cells and the orthotopic tumors. Irradiation was done at room temperature with the use of a 160 kV X-ray generator unit operating at 18.5 mA. Different assays were done at selected times after radiation.

Transfection of Pancreatic Cancer Cells and Preparation of Nuclear Extracts

Cells were cultured in six-well plates in 2 mL DMEM/F-12 supplemented with 5% fetal bovine serum. After 16 to 20 h, when cells were 50% to 60% confluent, reporter gene constructs were transfected using Lipofectamine reagent (Invitrogen). The effects of radiation and/or tolfenamic acid on transactivation were investigated in Panc1 and L3.6pl cells cotransfected with (500 ng) pSurvivin-269 construct. Cells were treated with DMSO (control), the indicated concentration of tolfenamic acid, and/or radiation (7 Gy); 48 h later, luciferase activity of lysates (relative to β-galactosidase activity) was determined.

Western Immunoblot

Cells were cultured in six-well plates in 2 mL DMEM/F-12 supplemented with 5% fetal bovine serum. After 16 to 20 h, cells were pretreated or treated with the indicated concentrations of tolfenamic acid, radiation (7 Gy), or their combination. At the indicated time point, cells were washed once with PBS and collected by scraping in 200 μL lysis buffer [50 mmol/L HEPES, 0.5 mol/L sodium chloride, 1.5 mmol/L magnesium chloride, 1 mmol/L EGTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μL/mL protease inhibitor cocktail (Sigma)]. Cell lysates were incubated on ice for 1 h with intermittent vortexing followed by centrifugation at $40,000 \times g$ for 10 min at 4°C. Equal amounts of protein (60 μg) from each treatment group were diluted with loading buffer, boiled, and loaded onto 10% SDS-polyacrylamide gel. Samples were electrophoresed and proteins were detected by incubation with polyclonal primary antibodies for Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), survivin, and β-actin followed by blotting with appropriate horseradish peroxidase-conjugated secondary antibody. After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics) using Zero-D Scanalytics software (Scanalytics).

Semiquantitative Reverse Transcription-PCR Analysis

Panc1 and L3.6pl cells were cultured in DMEM/F-12 supplemented with 5% fetal bovine serum. After 20 to 24 h, total RNA was collected. RNA concentration was measured by UV 260/280 nm absorption ratio, and 200 ng/μL RNA was used in each reaction for reverse transcription-PCR. RNA was reverse transcribed at 42°C for 25 min using oligo(dT) primer (Promega) and subsequently PCR amplified of reverse transcription product using 2 mmol/L MgCl$_2$, 1 μmol/L of each gene-specific primer, 1 mmol/L deoxynucleotriphosphates, and 2.5 units AmpliTaq DNA polymerase (Promega). The gene products were amplified using 22 to 25 cycles (95°C, 30 s; 56°C, 30 s; and 72°C, 30 s). The sequence of the oligonucleotide primers used in this study was as follows: survivin forward 5′-CACGGATC-CATGGGTGCCCCGACGTTGC-3′ and reverse 5′-CAG-GAATTCTCAATCCATGGCAGCCAGC-3′ and GAPDH forward 5′-AATCCCATCACCATCTTCCA-3′ and reverse 5′-GTCATCATATTTGGCAGGTT-3′.

Following amplification in a PCR express thermal cycler (Hybaid US), 20 μL of each sample were loaded on a 2% agarose gel containing ethidium bromide. Electrophoresis was done at 80 V in 1× TAE buffer for 1 h, and the gel was photographed by UV transillumination using Polaroid film. Survivin and GAPDH band intensity values were obtained by scanning the Polaroid on a Sharp JX-330 scanner (Sharp Electronics), the background signal was subtracted, and densitometric analysis was done on the inverted image using Zero-D software (Scanalytics). Results are expressed as survivin band intensity values normalized to GAPDH values and three separate determinations were carried out for each treatment group.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were done in Panc1 cells as described (34, 35). Panc1 cells were grown, treated, and radiated as described above for the Western blot. Cells were collected after washing with PBS containing protease inhibitor cocktail (2×) and cross-linked using 1.5% formaldehyde for 10 min at 37°C, and cells were lysed with SDS lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1)]. Cell lysates were then incubated on ice for 10 to 15 min and centrifuged at 2,000 rpm. Cell lysates (400 μL) were then sonicated (25×; Mesonix 3000 sonicator). After centrifugation, 50 μL of the supernatant were used for checking DNA fragmentation and also used as input and the remaining 350 μL were used for chromatin immunoprecipitation. The sample was diluted 10-fold with chromatin immunoprecipitation dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl (pH 8.1), 167 mmol/L NaCl]. To pre-clear the sample, 80 μL salmon sperm DNA/protein A agarose-50% slurry was added and rotated for 30 min at 4°C. Samples were centrifuged and supernatants were collected followed by immunoprecipitation with 5 μg each of Sp1, Sp3, and Sp4 antibodies overnight at 4°C with rotation. After 24 h, 60 μL salmon sperm DNA/protein A agarose-50% slurry was added and incubated for 1 h at 4°C with shaking to collect the antibody/antigen complex. Beads were pelleted by gentle centrifugation (1,000 rpm at 4°C for 1 min) and washed with the following buffers: low-salt buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 150 mmol/L NaCl], high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 500 mmol/L NaCl], LiCl buffer [0.25 mol/L LiCl, 1% NP-40, 1% deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8.1)], and twice with TE [10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0)]. The protein complex was eluted by adding 250 μL elution buffer (1% SDS, 0.1 mol/L NaHCO₃) to the pelleted protein A agarose/antibody/protein complex, and the mixture was vortexed and incubated at room temperature for 15 min with shaking. Beads were spun and the supernatant fraction (eluate) was carefully transferred to another tube and the elution was repeated. Eluates were combined (total volume of 500 μL) and 5 mol/L NaCl (20 μL) was added to reverse histone-DNA cross-linking by heating at 65°C for 5 h; then, 0.5 mol/L EDTA (10 μL), 1 mol/L Tris-HCl

(pH 6.5, 20 μL), and 10 mg/mL proteinase K (2 μL) was added to the elutes and incubated for 1 h at 45°C. DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation in the presence of 20 μg glycogen. The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 25 μL TE, and 5 μL were used as a template during PCR. PCRs were done with the following primers and PCR products were resolved on 2% agarose gels: proximal (Sp binding sites) forward 5′-CGCGTTCTTTG-AAAGCAGTCGAG-3′ and reverse 5′-AGCGCACGCCCT-CTTAGGCGGTC-3′ and distal (negative control) forward 5′-GCGGATATATTCAAGCTGGGCA-3′ and reverse 5′-CCCACAGCATCCAGGTGTAAGTTA-3′.

Animals and Orthotopic Implantation of Tumor Cells

Male athymic nude mice (NCI-*nu*) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center. 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 U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. Mice were used in accordance with institutional guidelines at age 8 to 12 weeks. 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 medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for the injections. Injection of cells into the pancreas was done as described previously (36). Mice were killed when moribund (4-5 weeks after injection). The size and weight of the primary pancreatic tumors were recorded. Histopathologic studies confirmed the nature of the disease. For immunohistochemistry and histologic staining procedures, tumor tissue were fixed in formalin and embedded in paraffin.

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

Seven days after implantation of tumor cells into the pancreas of each mouse, 5 mice were killed to confirm the presence of tumor lesions. Tumor volumes were calculated by using the following formula: $0.5 \times$ (length) $\times$ (width)^2. Tumor-bearing mice were randomized (7 per group) and treated with either 25 mg/kg tolfenamic acid or local tumor irradiation at a single dose of 5 Gy/wk or combination therapy of tolfenamic acid and radiation. Treatments were continued for 4 weeks and mice were sacrificed on day 35 and subjected to necropsy. An additional 5 mice were untreated and used as controls.

Histologic Studies

Mice were sacrificed and body weights were recorded. Primary tumors in the pancreas were excised, measured, and weighed. For immunohistochemistry and H&E staining procedures, tumor tissues were fixed in formalin and embedded in paraffin. For immunohistochemistry and histologic staining, paraffin-embedded tissues were
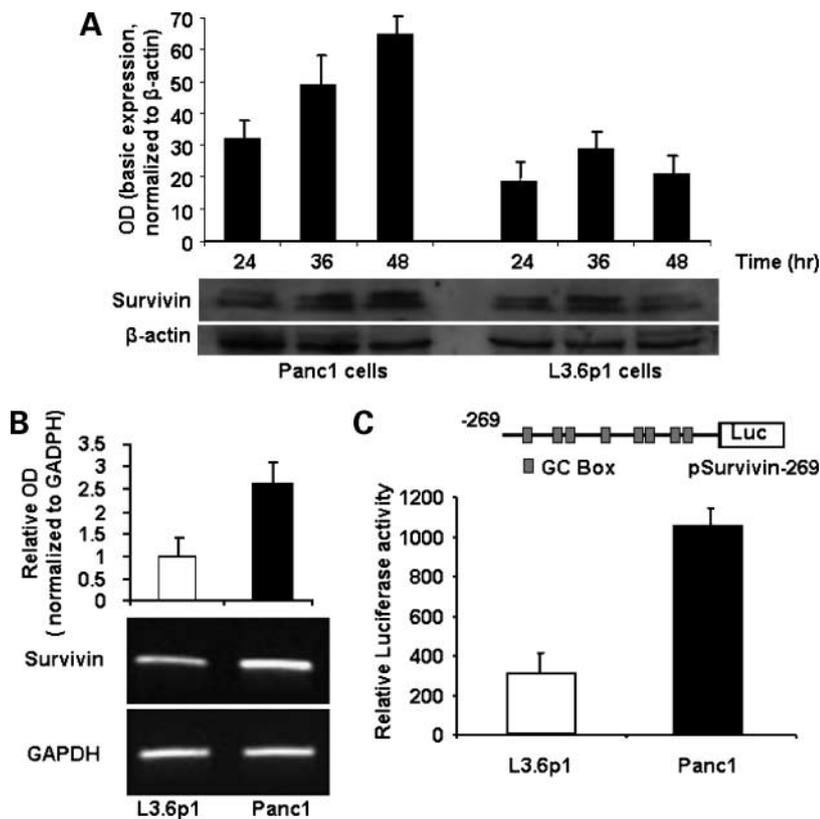
Figure 1. Survivin expression and promoter activity in pancreatic cancer cells. Survivin protein (**A**) and mRNA (**B**) levels and promoter activity in Panc1 and L3.6pl pancreatic cancer cells. Panc1 and L3.6pl cells were plated, and at the indicated time point, cells then harvested and whole-cell lysates were analyzed by Western blot analysis (**A**) as described in Materials and Methods. The experiment was replicated three times and average expression of survivin protein in Panc1 and L3.6pl cells was compared relative to β-actin. Survivin mRNA levels were determined in Panc1 and L3.6pl cells 24 h after seeding by semiquantitative reverse transcription-PCR (**B**) as described in Materials and Methods. Results are illustrated for replicate (three) experiments and relative expression of survivin mRNA in Panc1 cells was compared with survivin levels in L3.6pl cells (set at 1 unit). Transfection with pSurvivin-269 (**C**). Panc1 and L3.6pl cells were transfected with the pSurvivin-269 construct and relative luciferase activity was determined 48 h after transfection as described in Materials and Methods. All experiments were replicated at least three times. Mean ± SD.

used for identification of survivin. Sections (4-6 μm thick) were mounted on positively charged Superfrost slides (Fischer Scientific) and dried overnight. Sections were deparaffinized in xylene, treated with a graded series of alcohol [100%, 95%, and 80% ethanol (v/v) in double-distilled H_2O], and rehydrated in PBS (pH 7.5). Antigen retrieval occurred by placing slides in 97°C, 0.1 mol/L citrate buffer (pH 6.0) for 20 min. Slides were then washed with PBS that contained 0.1% Triton X-100. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. Nonspecific binding was blocked with 5% normal horse serum, 1% normal goat serum, and 0.1% Triton X-100. The slides were incubated at 4°C overnight in a moist chamber with a polyclonal anti-survivin antibody (Santa Cruz Biotechnology; 1:25 dilution). After incubation for 1 h at room temperature with a peroxidase-conjugated rabbit IgG secondary (Santa Cruz Biotechnology; 1:250 dilution), a positive reaction was visualized by incubating the slides with stable 3,3-diaminobenzidine (Invitrogen) for 8 to 10 min. The sections were rinsed with distilled water, counterstained with Gill's hematoxylin (Sigma) for 1 min, and mounted with Crystal Mount (Fischer Scientific). Control samples exposed to secondary antibody alone showed no specific staining.

TUNEL Assay

Paraffin-embedded tumor tissues were used for TUNEL staining, which was carried out using DeadEnd Colorimetric TUNEL System (Promega). Paraffin-embedded sections (4-6 μm thick) were processed per manufacturer's protocol.

Briefly, sections were deparaffinized in xylene and then treated with a graded series of alcohol [100%, 95%, 85%, 70%, and 50% ethanol (v/v) in double-distilled H_2O] and rehydrated in PBS (pH 7.5). Tissues were then treated proteinase K solution for permeabilization and then refixed with 4% paraformaldehyde solution. Slides were then treated with rTdT reaction mix and incubated at 37°C for 1 h and reactions were terminated by immersing the slides in 2× SSC solution for 15 min at room temperature. After blocking endogenous peroxidase activity (by 0.3% hydrogen peroxide), slides were washed with PBS and then incubated with streptavidin-horseradish peroxidase solution for 30 min at room temperature. After washing, slides were incubated with 3,3-diaminobenzidine (substrate) solution until a light brown background appears (10 min) and then rinsed several times in deionized water. After mounting, slides were observed by light microscope.

Statistical Analysis

Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. Results of cell culture studies are expressed as mean ± SD for at least three separate (replicate) experiments for each treatment.

Results

Survivin Protein Expression and Transcriptional Activity of Survivin Promoter in Pancreatic Cancer Cells

Basal expression of survivin mRNA/protein and transcriptional activity of its promoter were investigated in

highly metastatic L3.6p1 and chemoresistant and radio-resistant Panc1 pancreatic cancer cells. Results in Fig. 1A show that basal expression of survivin protein is 2- to 3-fold higher in Panc1 cells compared with L3.6p1 cells at different time points after cell seeding. We further asked whether differential expression of survivin in these cells is due to different transcriptional activity of survivin promoter. First, survivin mRNA levels in Panc1 and L3.6p1 were detected using reverse transcription-PCR. Figure 1B shows that survivin mRNA levels are 2.5-fold higher in Panc1 cells relative to L3.6p1 cells 24 h after cells were seeded. Differential transcriptional activity of survivin promoter was investigated using pSurvivin-269 reporter construct, which contains the -269 to +49 region of the survivin promoter. The construct was transfected into Panc1 and L3.6p1 cells, and after 48 h, luciferase activity was significantly higher (3 fold) in Panc1 compared with L3.6p1 cells (Fig. 1C).

Tolfenamic Acid Decreases Constitutive and Radiation-Induced Survivin mRNA/Protein Expression and Promoter Activity

Previous studies in pancreatic cancer cells have shown that both survivin promoter activity and mRNA expression correlated with radiosensitivity of the tumor cells and radiation significantly increased survivin mRNA expression (14). Expression of survivin in several cancer cell lines
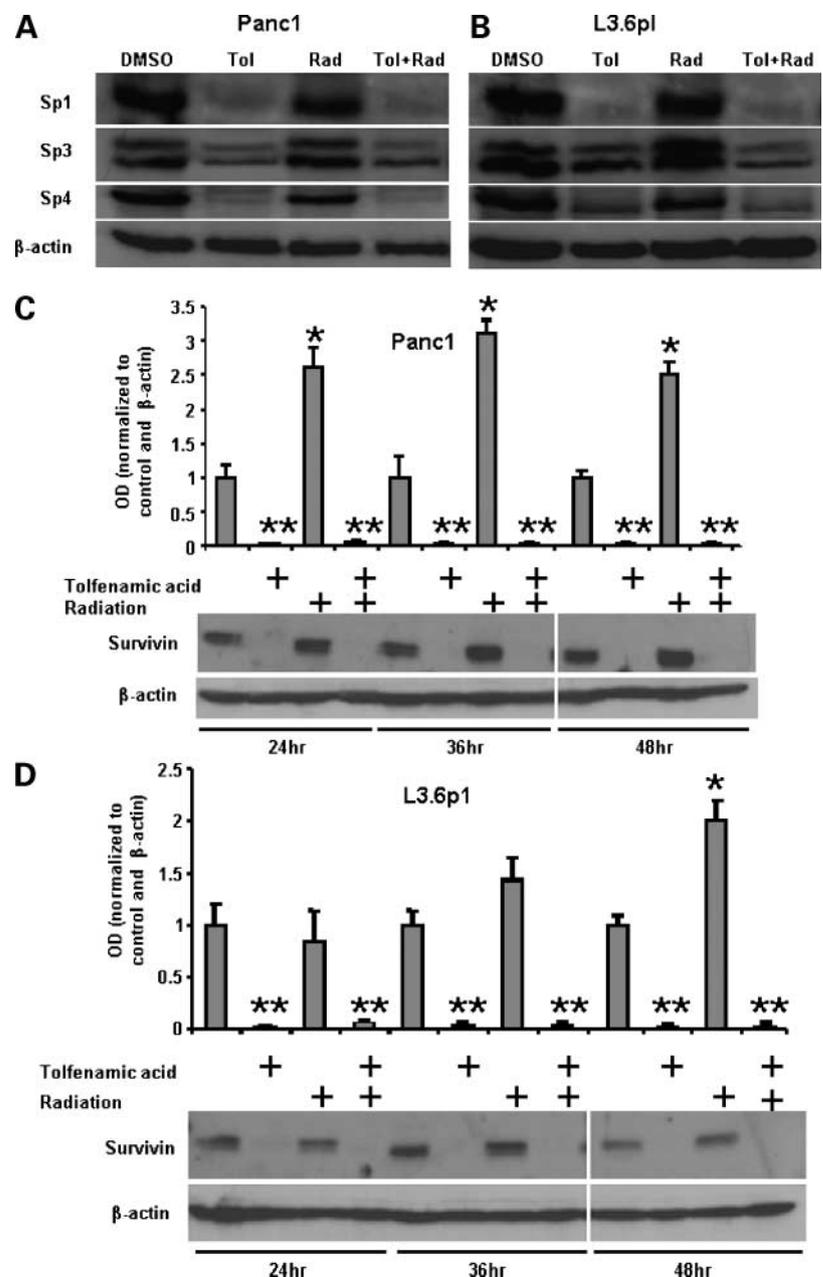

Figure 2. Tolfenamic acid decreases Sp and survivin proteins and radiation increases survivin protein in pancreatic cancer cells. Effects of tolfenamic acid and/or radiation in Panc1 (**A** and **C**) and L3.6p1 (**B** and **D**) cells. Cells were treated with DMSO, 50 μmol/L tolfenamic acid, 7 Gy radiation, or combination of tolfenamic acid and radiation for 48 h, and whole-cell lysates were analyzed by Western blot analysis. The experiment was replicated (three times) and Sp and survivin proteins levels were set at 1 unit in the control groups and significantly increased (*, $P < 0.05$) or decreased (**, $P < 0.005$) expression of survivin.
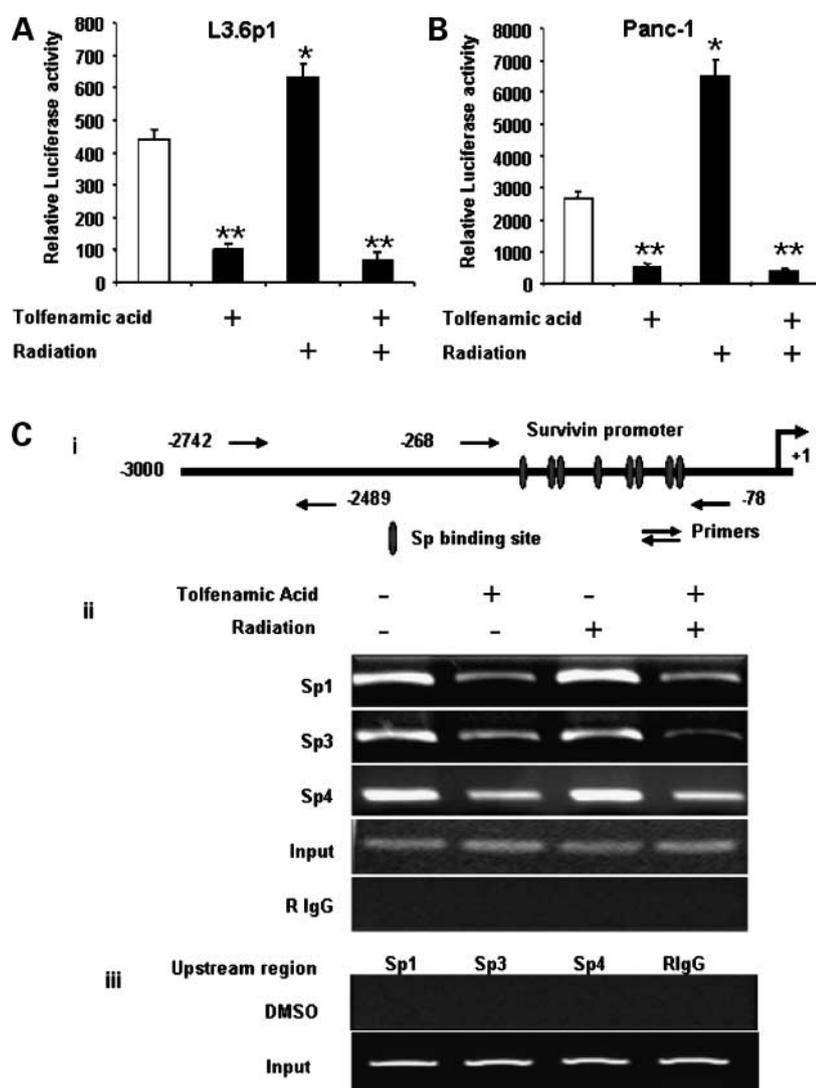
Figure 3. Effects of tolfenamic acid and/or radiation on survivin promoter activity and interactions of Sp proteins with survivin promoter sequences. Transfection with pSurvivin-269 in L3.6pl (**A**) and Panc1 (**B**) cells. L3.6pl and Panc1 cells were transfected with pSurvivin-269 construct and treated with DMSO, 50 μmol/L tolfenamic acid, 7 Gy radiation, or combination therapy of tolfenamic acid and radiation and luciferase activity was determined as described in Materials and Methods. Mean ± SD of replicate (three) experiments for each treatment group and significantly increased (*, $P < 0.05$) or decreased (**, $P < 0.005$) activity. **C,** chromatin immunoprecipitation assays. Primers designed for the proximal and distal (control) regions of the survivin promoter (*i*) were used for a chromatin immunoprecipitation assay in Panc1 cells (*ii*), which were treated with DMSO, 50 μmol/L tolfenamic acid, 7 Gy radiation, or combination therapy of tolfenamic acid and radiation for 24 h. Analysis of interactions of Sp1, Sp3, and Sp4 with the survivin promoter was carried out in the chromatin immunoprecipitation assay as described in Materials and Methods. Inputs shows equal chromatin loading and IgGs are used as negative controls. Chromatin immunoprecipitation assay done on the upstream region of survivin promoter shows no Sp proteins binding (*iii*) and used as a negative control as described in Materials and Methods.

is dependent on Sp proteins (19–22), which are overexpressed in many tumors and cancer cells (23–27). Sp proteins are associated with proliferation and antiapoptotic pathways; therefore, we investigated the effect of tolfenamic acid, a compound that induces Sp protein degradation on constitutive and radiation-induced survivin expression.

Results in Fig. 2A and B show that treatment of Panc1 and L3.6pl cells with 50 μmol/L tolfenamic acid for 48 h induced down-regulation of Sp1, Sp3, and Sp4 proteins and this was also accompanied by decreased basal expression of survivin protein (Fig. 2C). In contrast, radiation (7 Gy) significantly increased survivin expression; however, this expression was completely inhibited in Panc1 cells cotreated with 50 μmol/L tolfenamic acid. Interestingly, cotreatment with tolfenamic acid not only inhibited radiation-induced survivin expression but also decreased basal levels of this protein. The time-dependent increase of survivin protein in Panc1 cells 24, 36, and 48 h after irradiation with 7 Gy showed that higher expression of

survivin is first observed after 24 h and 2.5- to 3-fold increased expression of survivin protein was observed over the 24 to 48 h period (Fig. 2C). It is also worth noting that radiation (7 Gy) did not significantly affect Sp proteins expression after 48 h irradiation; however, the combination therapy of radiation plus tolfenamic acid resulted in Sp proteins levels similar to that observed for tolfenamic acid alone (Fig. 2A and B). Figure 2D illustrates that cotreatment of L3.6p1 cells, which express less survivin than Panc1, with tolfenamic acid also inhibiting basal and radiation-induced survivin expression. However, the time-dependent increased expression of survivin after radiation of L3.6pl cells was only significant after 48 h and only a 2-fold increase in survivin expression was observed.

The effect of tolfenamic acid on basic and radiation-induced survivin expression was further confirmed in promoter transfection studies. Panc1 (Fig. 3A) and L3.6pl (Fig. 3B) cells were transfected with pSurvivin-269 construct, which has multiple Sp binding sites and treated with tolfenamic acid (50 μmol/L), radiation (7 Gy), or their

combination. Forty-eight hours after treatment, luciferase activity was measured and results showed that tolfenamic acid decreased both basal and radiation-induced luciferase activity. However, the effects were more significant in Panc1 cells in which promoter activity was higher than in L3.6pl cells.

Previous studies showed activation of survivin in several cancer cells is dependent on interaction of Sp proteins with proximal GC-rich sites (19–22). The effect of tolfenamic acid on Sp protein binding to survivin promoter (Fig. 3C, *i*) was further investigated in a chromatin immunoprecipitation assay in Panc1 cells treated with the tolfenamic acid alone, radiation alone, or combination therapy for 48 h. The results (Fig. 3C, *ii*) show that tolfenamic acid decreased Sp1, Sp3, and Sp4 binding to the proximal GC-rich region of the survivin promoter. In contrast, radiation alone did not affect interactions of Sp proteins with the survivin promoter; however, there was a slight decrease in Sp3 binding compared with the control. In contrast, combination therapy decreased Sp binding to extent similar to that observed with tolfenamic acid treatment alone, except that the Sp3 binding to the promoter was further decreased. As a negative control for this experiment, chromatin immunoprecipitation assay was done on the upstream

region of survivin promoter and shows no Sp protein binding (Fig. 3C, *iii*). These results complement a recent study showing that the DNA-binding drug hedamycin also decreases survivin expression through inhibition of Sp protein interactions with the GC-rich survivin promoter (21). This is consistent with Sp-dependent activation of survivin by radiation and the mechanism of this response is currently being investigated.

Tolfenamic Acid Enhances Pancreatic Cancer Cells Response to Radiation Therapy

Because tolfenamic acid inhibits both basal and radiation-induced expression of survivin, we hypothesized that tolfenamic acid can be used to enhance the response of pancreatic cancer cells to radiation. Initially, we investigated the growth-inhibitory effects of radiation on Panc1 and L3.6pl cells because they exhibit different basal and radiation-induced expression of survivin. Figure 4 shows the survival curves for Panc1 and L3.6pl cells after treatment with 50 μmol/L tolfenamic acid, irradiation with 7 Gy, or their combination. Tolfenamic acid inhibited growth of both cell lines as reported (33). However, Panc1 cells (Fig. 4A) exhibited higher resistant to radiation than L3.6pl cells (Fig. 4B). Forty-eight hours after radiation, only 50% of the L3.6pl cells were alive, whereas >75% of Panc1 cells survived the radiation therapy. In contrast, cotreatment with tolfenamic acid enhanced responses of both cell lines to radiation therapy. In L3.6pl cells, addition of tolfenamic acid resulted in 30% increase in cell death compared with radiation treatment alone, whereas, in Panc1 cells, tolfenamic acid resulted in >60% increase in cell death when compared with radiation alone.

Tolfenamic Acid Enhances Radiosensitivity and Radiation-Induced Apoptosis in Pancreatic Tumor *In vivo*

We reported previously that tolfenamic acid inhibited pancreatic tumor growth and metastasis in the orthotopic athymic nude mouse model using L3.6pl cells (33). The effects of tolfenamic acid (25 mg/kg/d), radiation (5 Gy/ wk), and their combination on growth of pancreatic tumors were investigated in an orthotopic murine model for pancreatic cancer in which L3.6pl cells were injected directly into the pancreas and allowed to grow for 4 weeks after treatment started.

Results in Fig. 5 show that tolfenamic acid significantly enhanced radiation-induced tumor growth delay (inhibition). Radiation or tolfenamic acid monotherapy decreased tumor weight and volume by <40% (Fig. 5A and B). However, growth delay after the combined treatment was more than the sum of the growth delays caused by either treatment alone as evident from tumor weights and volumes.

In addition, the TUNEL assay (Fig. 5C) illustrates that radiation-induced apoptosis was much higher in tumor tissues from mice treated with both tolfenamic acid and radiation than tissues from mice treated with radiation only. Results in Fig. 5D show that survivin staining was decreased in tumor tissues from tolfenamic acid–treated mice compared with tissues from control untreated group.
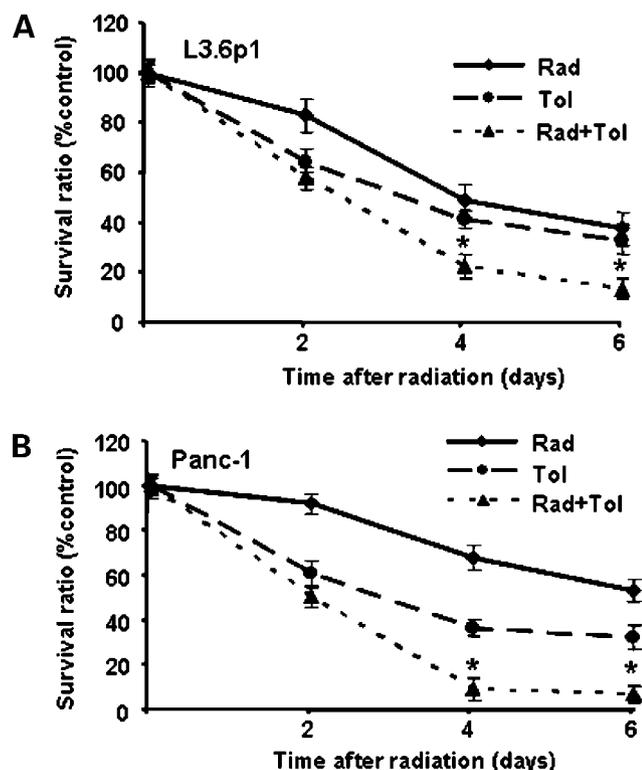

Figure 4. Effects of tolfenamic acid on radioresponse of L3.6pl and Panc1 cell *in vitro*. L3.6pl (**A**) and Panc1 (**B**) cells were treated with DMSO, 50 μmol/L tolfenamic acid, 7 Gy radiation, or combination of tolfenamic acid and radiation for 6 days, and the number of cells in each well was counted on days 2, 4, and 6 as described in Materials and Methods. Mean ± SD of replicate (three) experiments for each point and significantly ($P < 0.05$) decreased cell survival of the combination therapy compared with the monotherapy.
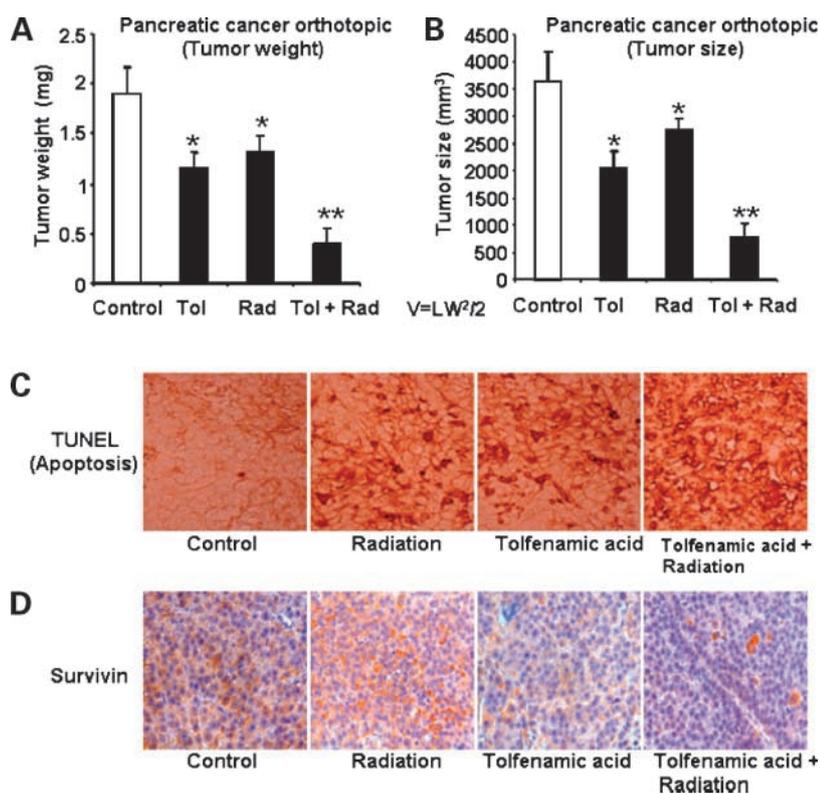
Figure 5. Tolfenamic acid enhances the radioresponse of orthotopic pancreatic tumors in mice. Tumor volumes (**A**) and weights (**B**). Athymic nude mice were injected orthotopically with L3.6p1 cells and then treated with corn oil (control), tolfenamic acid (25 mg/kg/d), radiation (5 Gy/wk single dose), or the combination of tolfenamic acid and radiation for 4 wk after the initial treatment. Mean $\pm$ SD for each treatment group ($n = 7$) and significantly decreased tumor volumes and weights compared with the corn oil control (*, $P < 0.05$) or with monotherapies (**, $P < 0.005$). Pancreatic tumor sections from control and treated animals were immunostained for apoptosis (TUNEL; **C**) and survivin (**D**) as described in Materials and Methods.

In contrast, in the radiation-treated group, there were several sections with clear overexpression or staining of survivin. Tissues from combined treatment group exhibited the least survivin staining with minimal to nondetectable staining.

Discussion

It has been reported previously that 68% of human pancreatic cancers are survivin positive and that normal pancreatic exocrine tissues are survivin negative (6). It was also reported that patients with survivin-positive pancreatic cancer show a significantly shorter survival time than those with survivin-negative cancer (6). Survivin is one of the radioresistant factors in a variety of human cancers, including pancreatic cancer, and it acts as a constitutive and inducible radioresistant factor in pancreatic cancer (37). Several strategies have been reported for targeting survivin and these include antisense oligonucleotides (38), ribozymes (12), dominant-negative molecules (11), an anticancer vaccine (39), small interfering RNA (18, 38, 40, 41), and small molecules (18).

One of the major strategies for modulating survivin expression is control of its transcription. It has been shown that the transcriptional activity of the survivin promoter was associated with the level of expression of survivin mRNA/protein in pancreatic cancer cell lines and that survivin gene expression in these cells is regulated at the transcriptional level by the 397-bp sequence upstream of the translation initiation site (14).

Previous studies show that, in several cancer cell lines, basic survivin expression is dependent, in part, on Sp1 and other Sp proteins; however, factors that regulate radiation-induced expression of survivin have not been determined (19–22). We have shown earlier that tolfenamic acid induces proteasome-dependent degradation of Sp1, Sp3, and Sp4 proteins in pancreatic cancer cells/tumor and this accompanied by inhibition of expression of several Sp-dependent angiogenic and growth factor genes (33, 36). Therefore, we examined the effects of tolfenamic acid on basal and radiation-induced expression of survivin protein in pancreatic cancer cells and tumors and we also explored the efficacy of tolfenamic acid to enhance the sensitivity of pancreatic cancer cells and tumors to radiation therapy.

Panc1 and L3.6p1 cells were selected for this study because they express different levels of survivin mRNA/protein (Fig. 1) and this might help to explain the differential responsiveness of cancer cells to radiation therapy. The results show that radiation induces survivin expression in pancreatic cancer cells as reported previously; however, in this study, the response was enhanced and observed more rapidly in Panc1 cells (Fig. 2A) compared with L3.6p1 cells (Fig. 2B), and for the first time, it was shown that tolfenamic acid inhibited both basal and radiation-induced expression of survivin protein both *in vitro* and *in vivo*.

The role of tolfenamic acid-induced degradation of Sp proteins in inhibiting of survivin expression was further investigated in Panc1 and L3.6p1 cells transfected with a construct containing the GC-rich proximal region from the

survivin gene promoter. Tolfenamic acid decreases basic and radiation-induced transactivation in both cell lines (Fig. 3A and B). Chromatin immunoprecipitation assay results also confirmed that tolfenamic acid inhibited activation of the survivin promoter by decreasing Sp protein binding to the proximal region of survivin promoter; however, radiation did not significantly affect Sp protein binding (Fig. 3C). These results confirm the linkage between the decreased basal and radiation-induced expression of survivin in Panc1 and L3.6p1 cells treated with tolfenamic acid and degradation of Sp1, Sp3, and Sp4 in these cells.

In this study, we explored the efficacy of tolfenamic acid to enhance the sensitivity of Panc1 and L3.6pl cells *in vitro* and the response of L3.6pl pancreatic tumor grown in mice to radiation. Tolfenamic acid significantly improved the radiosensitivity of Panc1 and L3.6pl pancreatic cancer cells in culture and that was evident from cell proliferation data (Fig. 4). This effect was more obvious in Panc1 cells, which are more radiation-resistant and express higher levels of survivin than L3.6pl cells. In addition, our *in vivo* data (Fig. 5) showed that tolfenamic acid significantly enhanced the orthotopic tumor response to radiation as evidenced by decreased final tumor volumes and weights. Moreover, examination of individual tumors clearly showed that combination therapy of tolfenamic acid plus radiation resulted in enhanced apoptosis than any of the mono-therapies alone and this was correlated, in part, to survivin expression.

In addition to enhancing pancreatic tumor response to radiation, tolfenamic acid may also increase the radio-response indirectly by inhibiting tumor angiogenesis. Inhibition of tumor angiogenesis has been reported to enhance tumor sensitivity to radiation therapy via several mechanisms including reduced levels of vascular endothelial growth factor (42). In a previous study, we have shown that tolfenamic acid inhibited vascular endothelial growth factor and vascular endothelial growth factor receptor 1 expression in pancreatic cancer cells and tumors (33, 36), suggesting that tumor growth delay observed in cells and tumors treated with tolfenamic acid and radiotherapy may also be due to tolfenamic acid-induced inhibition of angiogenesis.

Tolfenamic acid-induced radiosensitivity might also involve other cellular and molecular mechanisms in addition to decreased survivin expression. In a previous study (43), we have shown that decreasing Sp1 and Sp3 expression in pancreatic cancer cells by RNA interference resulted in accumulation of cells in G_0-G_1 phase of the cell cycle, which is relatively more sensitive to radiation-induced damage than the cells in S phase. This suggests that tolfenamic acid-induced radiosensitivity may also be due to modulation of the cell cycle and increase percentage of cells in G_0-G_1.

Moreover, it has been reported (33) that the cytotoxic activity of tolfenamic acid in Panc1 and L3.6pl cells was accompanied by induction of apoptosis as evident from poly(ADP-ribose) polymerase cleavage and other assays. This suggests that another mechanism by which tolfenamic acid modulates the response of pancreatic cancer cells to radiation may be due to enhanced apoptotic pathways.

In conclusion, the preclinical data presented in this report show that tolfenamic acid significantly enhanced pancreatic cancer cell and tumor response to radiation therapy and that this response may be due, in part, to decreased survivin expression. However, it is also possible that other cellular and molecular mechanisms such as tolfenamic acid-mediated induction of apoptosis and cell cycle redistribution may also enhance tumor/cell response to radiation therapy. Identification of other possible pathways associated with the action of tolfenamic acid has been investigated in a microarray screening assay in L3.6 cells using Illumina WG-6_v3 Expression Bead Chips (Supplementary Data). This array contained ∼38,000 genes, and after extensive data analysis, we have identified several proapoptotic genes that are altered after treatment with tolfenamic acid. Results of the microarray analysis have also been confirmed for a few apoptosis-related genes by determining effects on mRNA and protein levels using quantitative reverse transcription-PCR and Western blot analysis, respectively. The results show that tolfenamic acid up-regulates both mRNA and protein of proapoptotic genes such as BAD and caspase-9 and down-regulated antiapoptotic genes such as Bcl-2 and Bcl-xL (Supplementary Figs. S1-S3).[6] These results show that tolfenamic acid can affect multiple pathways associated with apoptosis and cell survival. These preclinical data suggest that tolfenamic acid has the potential to increase the radioresponse of unresectable or recurrent pancreatic cancer and current studies are focused on the mechanisms of tolfenamic acid-radiotherapy interactions and the enhanced therapeutic effectiveness of this treatment regimen.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[6] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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