

Curcumin Potentiates Antitumor Activity of Gemcitabine in an Orthotopic Model of Pancreatic Cancer through Suppression of Proliferation, Angiogenesis, and Inhibition of Nuclear Factor- κ B–Regulated Gene Products

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Abstract

Gemcitabine is currently the best treatment available for pancreatic cancer, but the disease develops resistance to the drug over time. Agents that can either enhance the effects of gemcitabine or overcome chemoresistance to the drug are needed for the treatment of pancreatic cancer. Curcumin, a component of turmeric (*Curcuma longa*), is one such agent that has been shown to suppress the transcription factor nuclear factor- κ B (NF- κ B), which is implicated in proliferation, survival, angiogenesis, and chemoresistance. In this study, we investigated whether curcumin can sensitize pancreatic cancer to gemcitabine *in vitro* and *in vivo*. *In vitro*, curcumin inhibited the proliferation of various pancreatic cancer cell lines, potentiated the apoptosis induced by gemcitabine, and inhibited constitutive NF- κ B activation in the cells. *In vivo*, tumors from nude mice injected with pancreatic cancer cells and treated with a combination of curcumin and gemcitabine showed significant reductions in volume ($P = 0.008$ versus control; $P = 0.036$ versus gemcitabine alone), Ki-67 proliferation index ($P = 0.030$ versus control), NF- κ B activation, and expression of NF- κ B–regulated gene products (cyclin D1, c-myc, Bcl-2, Bcl-xL, cellular inhibitor of apoptosis protein-1, cyclooxygenase-2, matrix metalloproteinase, and vascular endothelial growth factor) compared with tumors from control mice treated with olive oil only. The combination treatment was also highly effective in suppressing angiogenesis as indicated by a decrease in CD31⁺ microvessel density ($P = 0.018$ versus control). Overall, our results suggest that curcumin potentiates the antitumor effects of gemcitabine in pancreatic cancer by suppressing proliferation, angiogenesis, NF- κ B, and NF- κ B–regulated gene products. [Cancer Res 2007;67(8):3853–61]

Introduction

Pancreatic cancer is now the fourth leading cause of deaths in the United States with an overall 5-year survival rate of <5%. In 2006, it was estimated that 32,300 men and women would die of pancreatic cancer in the United States (1). The poor prognosis of pancreatic

cancer is attributable to its tendency for late presentation, aggressive local invasion, early metastases, and poor response to chemotherapy (2). Currently, gemcitabine remains the best chemotherapeutic agent available for the treatment of advanced pancreatic cancer. However, gemcitabine treatment results in an objective tumor response rate of <10% and only a marginal survival advantage (3) and is associated with multiple adverse events and drug resistance. Thus, there is a need for novel strategies involving less toxic agents that can sensitize pancreatic cancer cells to chemotherapy.

The transcription factor nuclear factor- κ B (NF- κ B) has been linked with cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis, and chemoresistance in multiple tumors (4, 5). In addition, numerous lines of evidence suggest that NF- κ B plays a major role in the growth and chemoresistance of pancreatic cancer. First, NF- κ B is constitutively active in pancreatic cancer cells (6) but not in immortalized, nontumorigenic pancreatic ductal epithelial cells (7). Second, NF- κ B activation has been reported in animal models of pancreatic cancer (8) and in human pancreatic cancer tissue (6). Third, NF- κ B promotes pancreatic cancer growth via inhibition of apoptosis (6, 9) and mediates induction of mitogenic gene products, such as c-myc and cyclin D1 (10). Fourth, cyclin D1 is overexpressed in human pancreatic cancer tissue and inversely correlated with patient survival (11). Fifth, NF- κ B enhances the angiogenic potential of pancreatic cancer cells via increased expression of proangiogenic factors, including vascular endothelial growth factor (VEGF; ref. 12). Sixth, NF- κ B–regulated gene products promote migration and invasion of pancreatic cancer cells (13). Finally, NF- κ B plays a pivotal role in promoting gemcitabine resistance in pancreatic cancer (5). Together, this evidence implicates the role of NF- κ B in pancreatic cancer and suggests that agents that block NF- κ B activation could reduce chemoresistance to gemcitabine and perhaps be used in combination with gemcitabine as a novel therapeutic regimen for pancreatic cancer.

Curcumin (diferuloylmethane), a derivative of the spice turmeric (*Curcuma longa*), is one such agent (Fig. 1A) that is nontoxic to humans (14). Curcumin has been shown to suppress NF- κ B activation (15) and down-regulate the expression of NF- κ B–regulated gene products with roles in antiapoptosis [Bcl-2, Bcl-xL, X-linked inhibitor of apoptosis protein, and cellular inhibitor of apoptosis protein-1 (cIAP-1)], proliferation [cyclooxygenase-2 (COX-2), cyclin D1, and c-myc], angiogenesis (VEGF and interleukin-8), invasion [matrix metalloproteinase-9 (MMP-9)], and metastasis [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1, and endothelial leukocyte adhesion molecule-1; refs. 16–18]. Furthermore, this phytochemical has been shown to induce the

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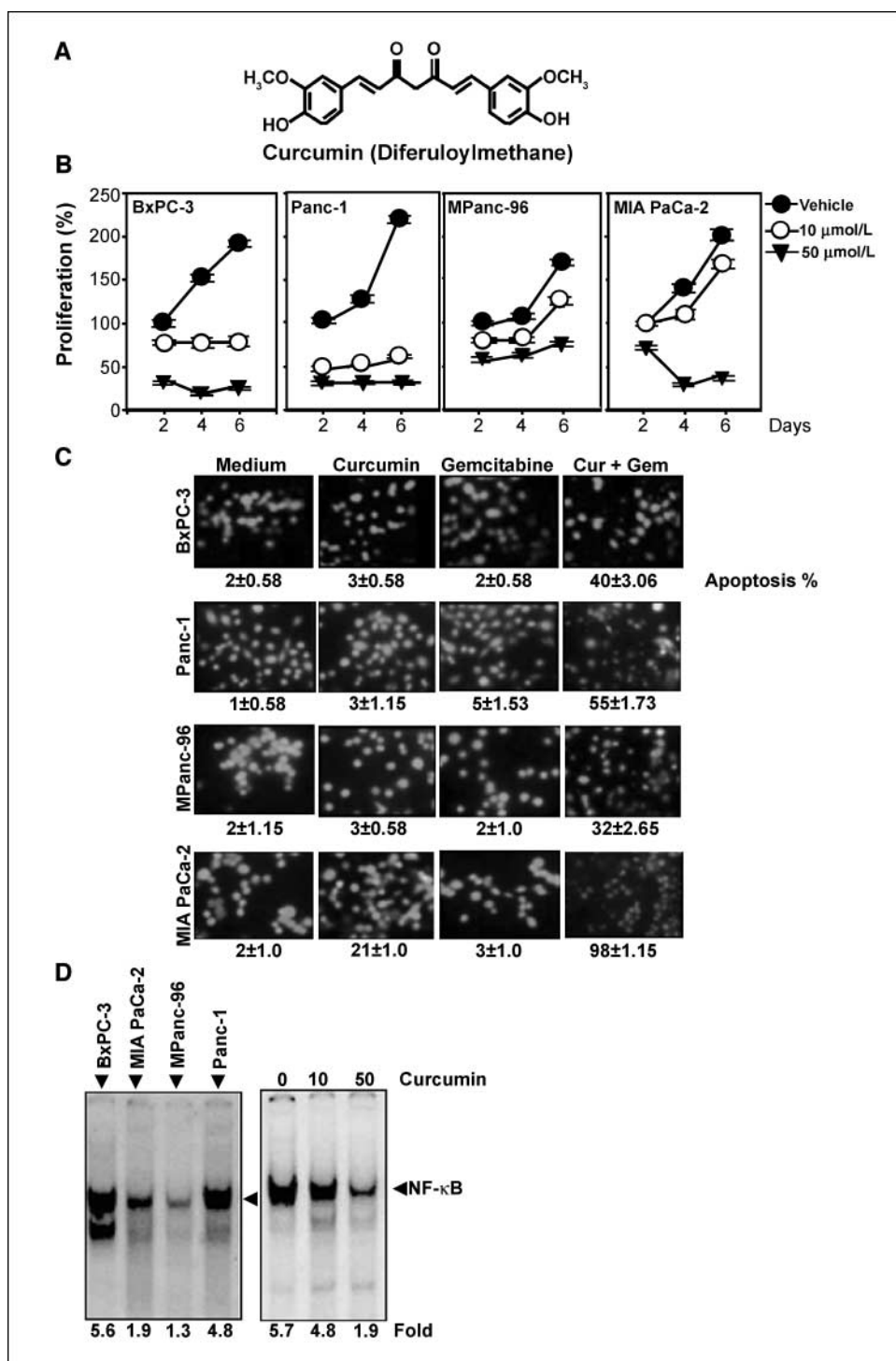


Figure 1. Curcumin inhibits proliferation, potentiates the apoptotic effects of gemcitabine, and inhibits constitutive NF- κ B activation in pancreatic cancer cells *in vitro*. **A**, structure of curcumin. **B**, MTT assay results showed dose-dependent suppression of cell proliferation in all four pancreatic cancer cell lines tested. Points, mean of triplicate; bars, SE. Data are a representative of two independent experiments (the significance value for control versus curcumin is <0.05). **C**, Live/Dead assay results indicated that curcumin (*Cur*) potentiates gemcitabine (*Gem*)-induced cytotoxicity. Percentages, proportions of apoptotic pancreatic cancer cells. Values are mean \pm SE of triplicate. Data are a representative of two independent experiments. **D**, left, EMSA results showing constitutive activation of NF- κ B in pancreatic cancer cells; right, EMSA results showing that curcumin suppresses the constitutive activation of NF- κ B in MIA PaCa-2 cells in a dose-dependent manner. The MIA PaCa-2 (1×10^6) cells were treated with curcumin (10 and 50 μ mol/L) for 4 h, and nuclear extracts were prepared and assayed for NF- κ B activation by EMSA. Numbers, fold activation in relation to human myeloid KBM-5 cells as one. Data are a representative of two independent experiments.

apoptosis of tumor cells through activation of caspases, cleavage of the proapoptotic protein BID, and release of cytochrome *c* (19). Curcumin has also been shown to suppress angiogenesis *in vivo* (20–22). Previously, we have shown that curcumin can suppress the growth of human pancreatic cancer cells *in vitro* (23) and *in vivo* (24). This has led to testing of curcumin in patients with advanced pancreatic cancer (25).

In this study, we investigated whether curcumin can potentiate the antitumor effects of gemcitabine against pancreatic cancer cells growing *in vitro* or *in vivo*. We found that curcumin

potentiated the apoptotic effects of gemcitabine in pancreatic cancer cells *in vitro* and significantly enhanced the antitumor effects of gemcitabine in orthotopic pancreatic tumors in nude mice by down-regulating NF- κ B-regulated gene products and suppression of angiogenesis.

Materials and Methods

Materials. Curcumin (77.5% curcumin; 4.21% bisdemethoxycurcumin, 18.27% demethoxycurcumin; also called C3 complex) was kindly supplied by

Sabinsa (Piscataway, NJ). The following polyclonal antibodies against p65 (recognizing the epitope within the NH₂-terminal domain of human NF- κ B p65), ICAM-1, cyclin D1, MMP-9, survivin, cIAP-1, procaspase-3, and procaspase-9 and monoclonal antibodies against VEGF, COX-2, c-myc, Bcl-2, and Bcl-xL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The liquid DAB+ Substrate Chromogen System-HRP used for immunocytochemistry was obtained from DakoCytomation (Carpinteria, CA). Penicillin, streptomycin, RPMI 1640, and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY). Tris, glycine, NaCl, SDS, and bovine serum albumin (BSA) were obtained from Sigma Chemical (St. Louis, MO). Gemcitabine (Gemzar; kindly supplied by Eli Lilly, Indianapolis, IN) was stored at 4°C and dissolved in sterile PBS on the day of use. D-luciferin potassium salt (Xenogen, Hopkinton, MA) was dissolved in sterile PBS at 40 mg/mL concentration.

Cell lines. The pancreatic cancer cell lines BxPC-3, MIA PaCa-2, and Panc-1 were obtained from the American Type Culture Collection (Manassas, VA). MPanc-96 was a kind gift from Dr. C. Logsdon [University of Texas M. D. Anderson Cancer Center (UTMDACC), Houston, TX]. All cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin.

Proliferation assay. The effect of curcumin on cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as described previously (26). The cells (2,000 per well) were incubated with curcumin in triplicate in a 96-well plate and then incubated for 2, 4, or 6 days at 37°C. A MTT solution was added to each well and incubated for 2 h at 37°C. An extraction buffer (20% SDS and 50% dimethylformamide) was added, and the cells were incubated overnight at 37°C. The absorbance of the cell suspension was measured at 570 nm using an MRX Revelation 96-well multiscanner (Dynex Technologies, Chantilly, VA). This experiment was repeated twice, and the statistical analysis (simple linear regression analysis initially and then unpaired Student's *t* test that revealed significant differences between two sample means) was done to obtain the final values.

Apoptosis assay. To determine whether curcumin can potentiate the apoptotic effects of gemcitabine in pancreatic cancer cells, we used a Live/Dead assay kit (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic, green fluorescent dye that is retained within live cells, and a red fluorescent ethidium bromide homodimer dye that can enter cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells (26). Briefly, cells (5,000 per well) were incubated in chamber slides, pretreated with curcumin for 4 h, and treated with gemcitabine for 24 h. Cells were then stained with the assay reagents for 30 min at room temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells. This experiment was repeated twice and the statistical analysis was done. The values were initially subjected to one-way ANOVA, which revealed significant differences between groups, and then later compared among groups using unpaired Student's *t* test, which revealed significant differences between two sample means.

Animals. Male athymic *nu/nu* mice (4 weeks old) were obtained from the breeding colony of the Department of Experimental Radiation Oncology at UTMDACC. The animals were housed four per cage in the standard mice plexiglass cages in a room maintained at constant temperature and humidity under 12-h light and darkness cycle and fed with regular autoclave chow diet with water *ad libitum*. None of the mice exhibited any lesions, and all were tested pathogen-free. Before initiating the experiment, we acclimatized all mice to a pulverized diet for 3 days. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at UTMDACC.

Orthotopic implantation of MIA PaCa-2 cells. MIA PaCa-2 cells were stably transfected with luciferase as previously described for Panc-1 cells (27). Luciferase-transfected MIA PaCa-2 cells were harvested from subconfluent cultures after a brief exposure to 0.25% trypsin and 0.2% EDTA. Trypsinization was stopped with medium containing 10% FBS. The cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of single cells, with >90% viability, were used

for the injections. Mice were anesthetized with ketamine-xylazine solution, a small left abdominal flank incision was made, and MIA PaCa-2 cells (1×10^6) in 100 μ L PBS were injected into the subcapsular region of the pancreas using a 27-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Co., Reno, NV). To prevent leakage, a cotton swab was held cautiously for 1 min over the site of injection. The abdominal wound was closed in one layer with wound clips (Bainbridge Scientific, Inc., Braintree, MA).

Experimental protocol. After 1 week of implantation, mice were randomized into the following treatment groups (*n* = 6) based on the bioluminescence measured after the first IVIS imaging: (a) untreated control (olive oil, 100 μ L daily); (b) curcumin alone (1 g/kg), once daily p.o.; (c) gemcitabine alone (25 mg/kg), twice weekly by i.p. injection; and (d) combination of curcumin (1 g/kg), once daily p.o., and gemcitabine (25 mg/kg), twice weekly by i.p. injection. Tumor volumes were monitored weekly by the bioluminescence IVIS Imaging System 200 using a cryogenically cooled imaging system coupled to a data acquisition computer running Living Image software (Xenogen Corp., Alameda, CA). Before imaging, animals were anesthetized in an acrylic chamber with 2.5% isoflurane/air mixture and injected i.p. with 40 mg/mL D-luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. After 10 min of incubation with luciferin, mice were placed in a right lateral decubitus position and a digital grayscale animal image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest per second. Mice were imaged on days 0, 7, 14, 21, 24, and 31 of treatment. Therapy was continued for 4 weeks and animals were sacrificed 1 week later. Primary tumors in the pancreas were excised and the final tumor volume was measured as $V = 2 / 3\pi r^3$, where *r* is the mean of the three dimensions (length, width, and depth). The final tumor volumes were initially subjected to one-way ANOVA and then later compared among groups using unpaired Student's *t* test. Half of the tumor tissue was formalin fixed and paraffin embedded for immunohistochemistry and routine H&E staining. The other half was snap frozen in liquid nitrogen and stored at -80°C. H&E staining confirmed the presence of tumor(s) in each pancreas.

Preparation of nuclear extract from tumor samples. Pancreatic tumor tissues (75–100 mg/mouse) from control and experimental mice were minced and incubated on ice for 30 min in 0.5 mL of ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 \times *g* at 4°C for 10 min. The resulting nuclear pellet was suspended in 0.2 mL of buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 0.5 mmol/L PMSF, 2 μ g/mL leupeptin] and incubated on ice for 2 h with intermittent mixing. The suspension was then centrifuged at 16,000 \times *g* at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at -70°C until use. Protein concentration was measured by the Bradford assay with BSA as the standard.

NF- κ B activation in pancreatic cancer cells and tumor samples. To assess NF- κ B activation, we isolated nuclei from pancreatic cancer cell lines and tumor samples and carried out electrophoretic mobility shift assays (EMSA) essentially as described previously (28). Briefly, nuclear extracts prepared from pancreatic cancer cells (1×10^6 /mL) and tumor samples were incubated with ³²P-end-labeled 45-mer double-stranded NF- κ B oligonucleotide (4 μ g of protein with 16 fmol of DNA) from the HIV long terminal repeat (5'-TTGTTACAAGGGACTTTCGCTGGGGACTTTCAGG-GGGAGGCGTGG-3'; italicized indicates NF- κ B-binding sites) for 15 min at 37°C. The resulting DNA-protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutant oligonucleotide (5'-TTGTTACAACACTTTCGCTGCTCACTTTC-CAGGGAGGCGTGG-3') was used to examine the specificity of binding of NF- κ B to the DNA. The dried gels were visualized, and radioactive bands were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Immunolocalization of NF- κ B p65, VEGF, and COX-2 in tumor samples. The nuclear localization of p65 and expression of COX-2, and VEGF were examined using an immunohistochemical method described previously (29). Briefly, pancreatic cancer tumor samples were embedded in paraffin and fixed with paraformaldehyde. After being washed in PBS, the slides were blocked with protein block solution (DakoCytomation) for 20 min and then incubated overnight with rabbit polyclonal anti-human p65, mouse monoclonal anti-human VEGF, and anti-COX-2 antibodies (1:100, 1:50, and 1:75, respectively). After the incubation, the slides were washed and then incubated with biotinylated link universal antiserum followed by horseradish peroxidase-streptavidin conjugate (LSAB+ kit). The slides were rinsed, and color was developed using 3,3'-diaminobenzidine hydrochloride as a chromogen. Finally, sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted with DPX mounting medium for evaluation. Pictures were captured with a Photometrics CoolSNAP CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

Ki-67 immunohistochemistry. Formalin-fixed, paraffin-embedded sections (5 μ m) were stained with anti-Ki-67 (rabbit monoclonal clone SP6; NeoMarkers, Fremont, CA) antibody as described previously (30). Results were expressed as percentage of Ki-67⁺ cells \pm SE per $\times 40$ magnification. A total of ten $\times 40$ fields was examined and counted from three tumors of each of the treatment groups. The values were initially subjected to one-way ANOVA and then later compared among groups using unpaired Student's *t* test.

Microvessel density. Ethanol-fixed, paraffin-embedded sections (5 μ m) were stained with rat anti-mouse CD31 monoclonal antibody (PharMingen, San Diego, CA) as described previously (30). Areas of greatest vessel density were then examined under higher magnification ($\times 100$) and counted. Any distinct area of positive staining for CD31 was counted as a single vessel. Results were expressed as the mean number of vessels \pm SE per high-power field ($\times 100$). A total of 20 high-power fields was examined and counted from three tumors of each of the treatment groups. The values were initially subjected to one-way ANOVA and then later compared among groups using unpaired Student's *t* test.

Western blot analysis. Pancreatic tumor tissues (75–100 mg/mouse) from control and experimental mice were minced and incubated on ice for 30 min in 0.5 mL of ice-cold whole-cell lysate buffer (10% NP40, 5 mol/L NaCl, 1 mol/L HEPES, 0.1 mol/L EGTA, 0.5 mol/L EDTA, 0.1 mol/L PMSF, 0.2 mol/L sodium orthovanadate, 1 mol/L NaF, 2 μ g/mL aprotinin, 2 μ g/mL leupeptin). The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 $\times g$ at 4°C for 10 min. The proteins were then fractionated by SDS-PAGE, electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

The goal of this study was, first, to determine whether curcumin, also called diferuloylmethane (Fig. 1A), potentiates the antitumor effects of gemcitabine *in vitro* and *in vivo* against human pancreatic cancer and, second, to delineate the mechanism of potentiation. Four different pancreatic cancer cell lines were selected for this investigation. To facilitate the imaging of the tumor in the animals, luciferase-transfected MIA PaCa-2 cells were used.

Curcumin inhibits proliferation, potentiates the apoptotic effects of gemcitabine, and inhibits constitutive NF- κ B activation in pancreatic cancer cells *in vitro*. To determine whether curcumin inhibits the proliferation of human pancreatic cancer cells, we used MIA PaCa-2, MPanc-96, and Panc-1, all of which exhibit *K-Ras* and *p53* mutations, and BxPC-3 with wild-type *K-Ras*. These cell lines were tested for viability using a MTT assay after incubation with 0, 10, or 50 μ mol/L of curcumin for 2, 4, or 6 days. Curcumin inhibited the proliferation of all four pancreatic cancer cell lines in a dose-dependent manner irrespective of the genetic background (Fig. 1B).

To determine whether curcumin enhances the induction of apoptosis by gemcitabine, we tested the four cell lines with a Live/Dead apoptosis assay. Our results showed that, at a dose at which curcumin (10 μ mol/L) and gemcitabine (50 nmol/L) alone were minimally effective, the two together were highly effective (Fig. 1C). Because this combined effect was the greatest in MIA PaCa-2 cells, this cell line was used in further experiments.

We next used a DNA-binding assay to examine whether pancreatic cancer cells express constitutive NF- κ B. All four cell lines expressed constitutive NF- κ B, although levels were higher in BxPC-3 and Panc-1 cells than in MIA PaCa-2 and MPanc-96 cells (Fig. 1D, *left*). Treatment of MIA PaCa-2 cells with curcumin down-regulated NF- κ B activation in a dose-dependent manner (Fig. 1D, *right*).

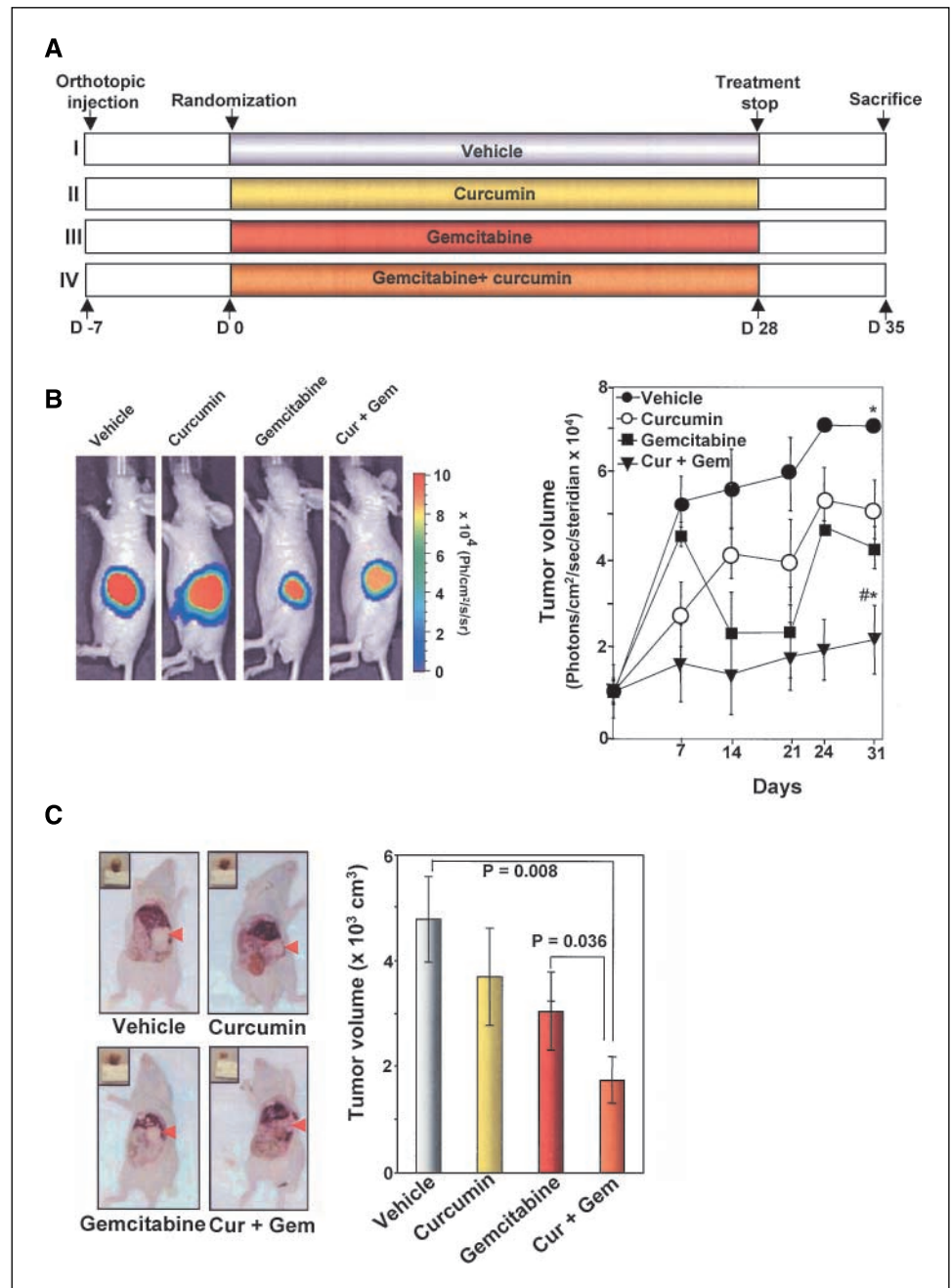
Curcumin potentiates the antitumor, antiproliferative, and antiangiogenic effects of gemcitabine in orthotopic pancreatic tumors in nude mice. We examined the effects of curcumin and gemcitabine, alone or in combination, on the growth of orthotopically implanted pancreatic tumors (Fig. 2A). MIA PaCa-2 cells were implanted in the pancreatic tails of nude mice. Based on the IVIS imaging, 1 week later, the mice were randomized into four groups. The treatment was started 1 week after the tumor cell implantation, continued as per experimental protocol for 4 weeks. The animals were sacrificed 6 weeks after tumor cell injection and 5 weeks from the date of treatment (Fig. 2A). We did the IVIS imaging on days 7, 14, 21, 24, and 31 after the start of treatment. The bioluminescence imaging results (Fig. 2B, *right and left*) indicated a gradual increase in tumor volume in the control group compared with the three treatment groups. The tumor volume in the combination of curcumin and gemcitabine group was significantly lower than the gemcitabine alone or control group by day 31 of treatment ($P < 0.05$ versus gemcitabine; $P < 0.001$ versus control). The final tumor volumes on day 35 after the start of treatment showed significant decrease in the curcumin + gemcitabine group compared with control ($P < 0.01$ versus control) or with gemcitabine alone ($P < 0.05$ versus gemcitabine; Fig. 2C, *right and left*). The tumor volumes in curcumin alone and gemcitabine alone groups were not statistically significant compared with control group ($P = 0.399$ and 0.147, respectively).

We next examined the expression of the cell proliferation marker Ki-67 and the microvessel density marker CD31 in tumor tissues from the four groups. The results in Fig. 3A and B showed that curcumin in combination with gemcitabine significantly down-regulated the expression of Ki-67 in tumor tissues compared with the control group ($P < 0.05$ versus control). The results also showed that curcumin alone significantly suppressed the expression of CD31 ($P < 0.05$ versus control), and the presence of gemcitabine further enhanced the down-regulation of CD31 ($P < 0.05$ versus control; Fig. 3C and D).

Curcumin inhibits NF- κ B activation in orthotopic pancreatic tumors. We investigated whether the effect of curcumin on tumor growth in mice is associated with the inhibition of NF- κ B activation. The EMSA results showed that curcumin alone completely suppressed NF- κ B activation in tumor samples (Fig. 4A). We found that curcumin also suppressed NF- κ B activation in gemcitabine-treated animals.

Curcumin potentiates the effect of gemcitabine in down-regulating the expression of NF- κ B-regulated gene products. NF- κ B is known to regulate the expression of COX-2 (involved in proliferation), MMP-9 (involved in invasion), and ICAM-1 (involved in metastasis; ref. 4). Accordingly, we observed increased expression of these molecules in orthotopic pancreatic tumors.

Figure 2. Curcumin potentiates the effect of gemcitabine in blocking the growth of pancreatic cancer in nude mice. **A**, schematic representation of experimental protocol described in Materials and Methods. Group I was given with olive oil (100 μ L, p.o., daily) and PBS (100 μ L, i.p., twice weekly), group II was given with curcumin (1 g/kg, p.o., daily), group III was given with gemcitabine (25 mg/kg, i.p., twice weekly), and group IV was given with curcumin (1 g/kg, p.o., daily) and gemcitabine (25 mg/kg, i.p., twice weekly; $n = 6$). **B**, left, bioluminescence IVIS images of orthotopically implanted pancreatic tumors in live, anesthetized mice; right, measurements of photons per second depicting tumor volume at various time points using live IVIS imaging at the indicated times ($n = 6$). Points, mean; bars, SE. **C**, left, necropsy photographs of mice bearing orthotopically implanted pancreatic tumors; right, tumor volumes in mice measured on the last day of the experiment at autopsy using Vernier calipers and calculated using the formula $V = 2/3\pi r^3$ ($n = 6$). Columns, mean; bars, SE. $P = 0.399$ (control versus curcumin) and 0.147 (control versus gemcitabine).



Immunohistochemical analysis (Fig. 4B) and Western blotting (Fig. 4C) revealed significant reductions in the expression of COX-2 in tumors from the curcumin-treated groups compared with those from the control group. Gemcitabine alone, in contrast, did not significantly reduce the expression of COX-2. Western blotting (Fig. 4C) showed that curcumin alone also significantly decreased the expression of MMP-9 and ICAM-1 compared with the control treatment.

We also assessed the expression of the NF- κ B-regulated genes *VEGF*, which plays an important role in angiogenesis; *cyclin D1* and *c-myc*, which are involved in tumor cell proliferation; and *survivin*, *Bcl-2*, *Bcl-xL*, and *IAP-1*, the overexpression of which has been linked to tumor survival, chemoresistance, and radioresistance (31–34). Immunohistochemical analysis (Fig. 4B) and Western blotting (Fig. 4C) revealed that curcumin, alone or in combination with

gemcitabine, significantly decreased the expression of all of these molecules compared with the control treatment in pancreatic tumor tissues. The Western blot results also showed that curcumin, alone or in combination with gemcitabine, decreased the expression of procaspase-3 and procaspase-9 (Fig. 4D).

Discussion

The aim of this study was to determine whether curcumin, a well-known dietary component, can sensitize pancreatic cancer to gemcitabine. In various pancreatic cancer cell lines, curcumin inhibited proliferation, potentiated the apoptosis induced by gemcitabine, and inhibited constitutive NF- κ B activation. In an orthotopic nude mice model, curcumin potentiated the antitumor

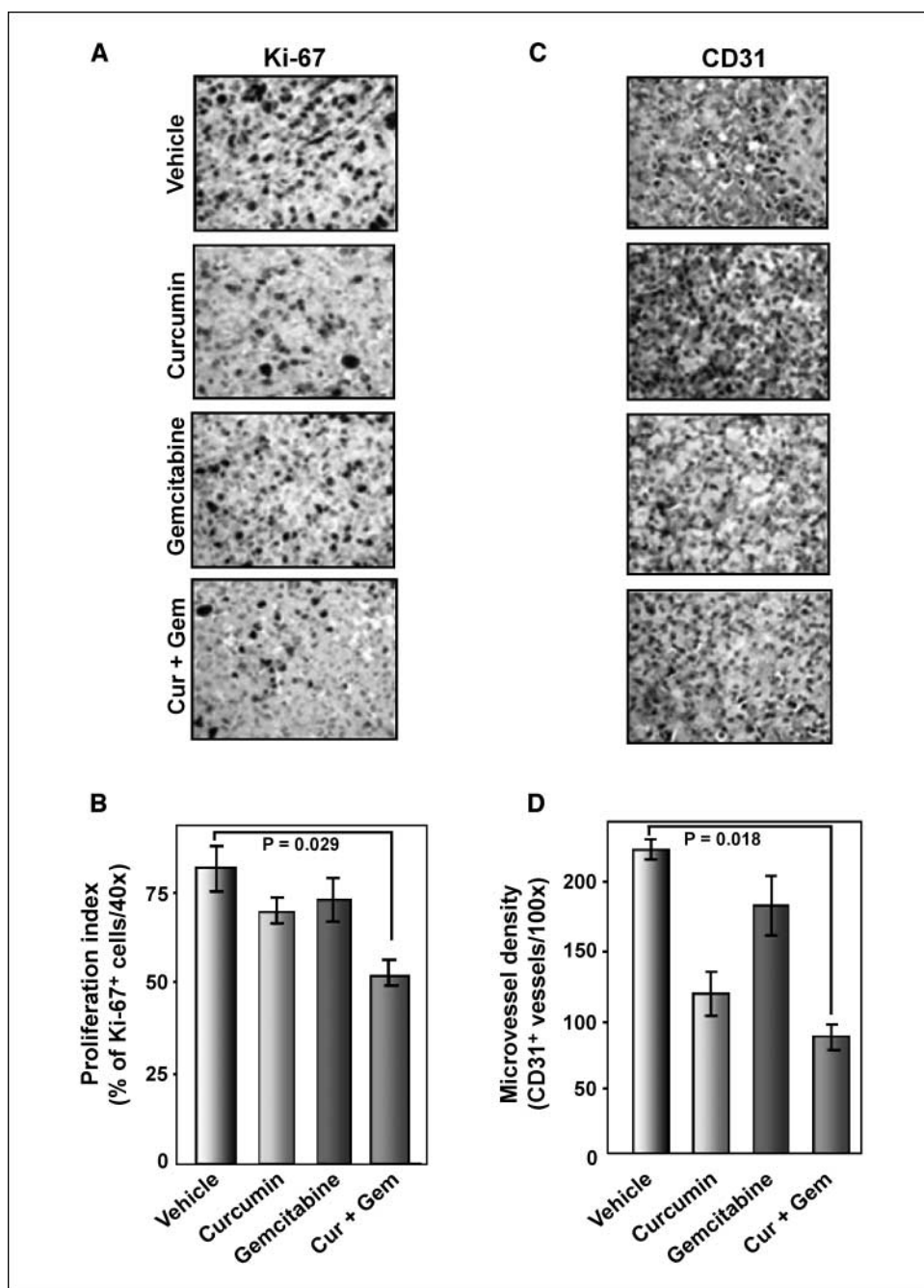


Figure 3. Curcumin enhances the effect of gemcitabine against tumor cell proliferation and angiogenesis in pancreatic cancer. *A*, immunohistochemical analysis of proliferation marker Ki-67 indicates the inhibition of pancreatic cancer cell proliferation in curcumin alone or in combination with gemcitabine-treated groups of animals. *B*, quantification of Ki-67⁺ cells as described in Materials and Methods. *Columns*, mean of triplicate; *bars*, SE. *C*, immunohistochemical analysis of CD31 for microvessel density in pancreatic cancer tumors indicates the inhibition of angiogenesis by curcumin alone and in combination with gemcitabine. *D*, quantification of CD31⁺ microvessel density as described in Materials and Methods. *Columns*, mean of triplicate; *bars*, SE.

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effects of gemcitabine; this effect was associated with suppression of Ki-67, down-regulation of NF- κ B activation and NF- κ B-regulated gene products, and inhibition of angiogenesis as indicated by a decrease in microvessel density.

This is the first report to show that curcumin enhances the apoptotic effects of gemcitabine in cultured pancreatic cancer cells. Gemcitabine alone had a minimal effect on apoptosis in BxPC-3, Panc-1, MIA PaCa-2 and MPanc-96 cell lines. Another report similarly showed that BxPC-3, Capan-1, and PancTu-1 cells are unaffected by gemcitabine (5). The mechanism by which curcumin potentiates the apoptotic effects of gemcitabine may involve suppression of NF- κ B. All four pancreatic cancer cell lines studied showed constitutive NF- κ B activation, which was down-regulated by curcumin. NF- κ B has been linked with chemo-

resistance (35), so it is very likely that the down-regulation of NF- κ B by curcumin sensitized the cells to gemcitabine. These results agree with a previous report in which MG132, sulfasalazine, and the κ B α superrepressor sensitized pancreatic cancer cells to gemcitabine (5). Similarly, genistein has been shown to sensitize prostate cancer cells to gemcitabine through the down-regulation of NF- κ B (36).

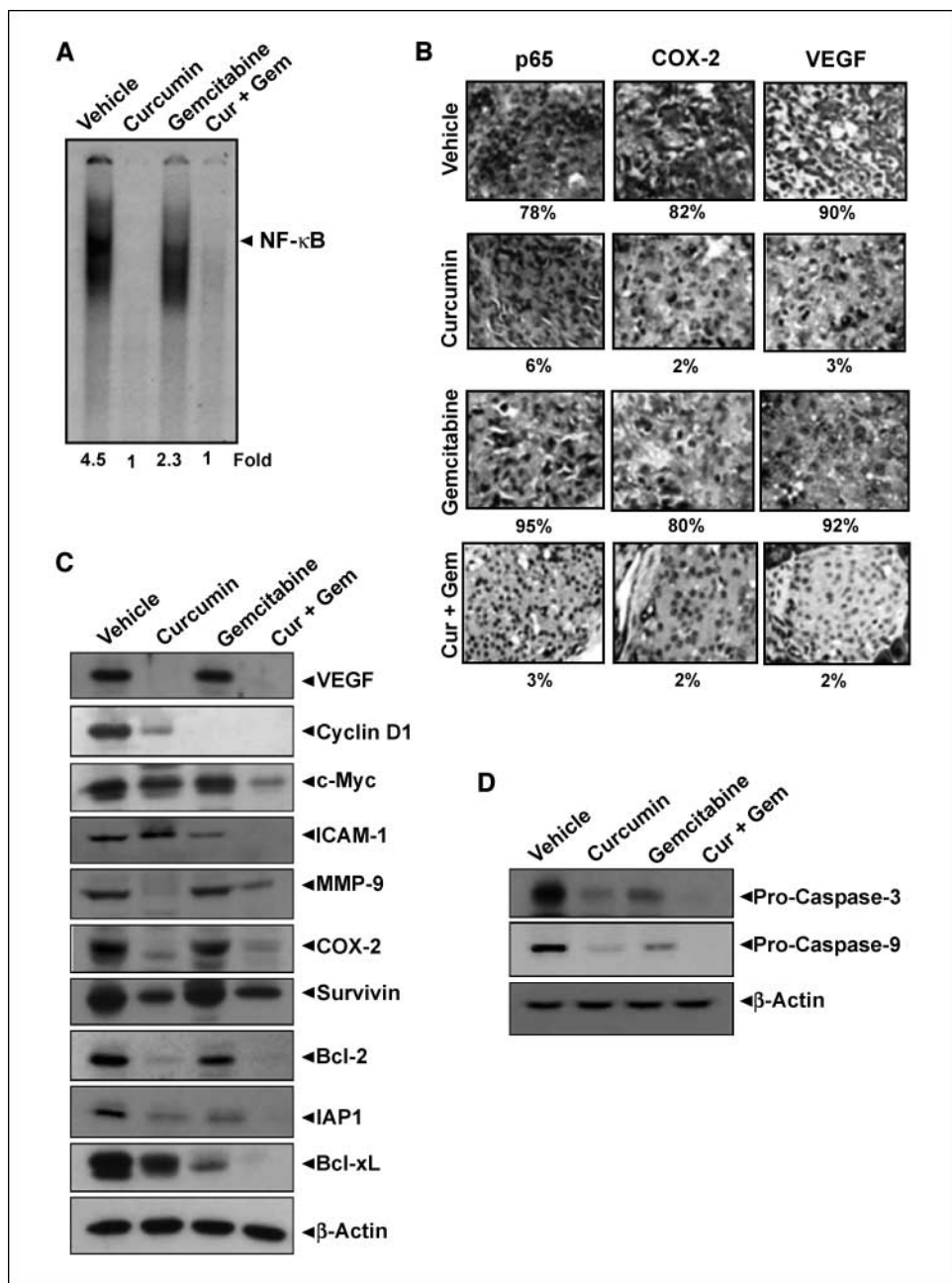
In addition to these *in vitro* results, we found that curcumin potentiates the antitumor effects of gemcitabine in an orthotopic model of pancreatic cancer as indicated by a decrease in tumor volume measured by two independent methods. Similarly, genistein in combination with gemcitabine has been shown to have a much more potent antitumor effect than either agent alone in an orthotopic tumor model (36).

How curcumin potentiates the effects of gemcitabine *in vivo* was investigated in several ways. First, analysis of microvessel density (CD31) and proliferation index (Ki-67) revealed that these two markers were decreased by curcumin. Second, NF- κ B analysis of the tumor tissues showed that curcumin alone completely suppressed constitutive NF- κ B activation in pancreatic cancer tissues, a finding consistent with the immunohistochemical analysis of the NF- κ B subunit p65. Third, immunohistochemical analysis also showed that curcumin down-regulated the expression of VEGF, which is closely linked with angiogenesis. Fourth, the expression of COX-2, which is linked with growth and metastasis, was significantly suppressed in tumor tissues from curcumin-treated mice. Finally, Western blot analysis of Bcl-2, survivin, Bcl-xL, and cIAP-1 showed that curcumin alone could inhibit the expression of these antiapoptotic proteins in pancreatic tumor samples.

Our finding that curcumin down-regulated COX-2 expression in pancreatic cancer tissues is notable, as COX-2 is overexpressed in chronic pancreatitis (37) and in human pancreatic cancer tissue. COX-2 expression has been associated with a lower apoptotic index (38), increased cell proliferation (39), increased risk of metastasis (40, 41), and enhanced VEGF production, leading to angiogenesis in pancreatic tumors (42, 43). This is consistent with a recent report that indicates a synergistic action of curcumin with celecoxib, a specific COX-2 inhibitor, against pancreatic adenocarcinoma cells (44).

Our results also showed that curcumin inhibited the expression of several important NF- κ B-regulated proteins *in vivo*. For instance, cyclin D1, known to be overexpressed in pancreatic tumor tissues, was down-regulated by curcumin. We found that curcumin also suppressed the ICAM-1 expression in tumor tissues. Overexpression of ICAM-1 has been well documented in malignant cells, including

Figure 4. Curcumin enhances the effect of gemcitabine against expression of NF- κ B and NF- κ B-regulated gene products in pancreatic cancer samples. **A**, detection of NF- κ B by DNA binding in orthotopic tumor tissue samples showed the inhibition of NF- κ B by curcumin. *Numbers*, fold activation in relation to human myeloid KBM-5 cells as one. **B**, immunohistochemical analysis of nuclear p65, COX-2, and VEGF showed the inhibition of NF- κ B, COX-2, and VEGF by curcumin alone or in combination with gemcitabine. *Percentage*, positive staining for the given biomarker. **C**, Western blot showing that curcumin and gemcitabine together inhibit the expression of NF- κ B-dependent gene products VEGF, cyclin D1, c-myc, ICAM-1, MMP-9, COX-2, survivin, Bcl-2, Bcl-xL, and IAP-1 in pancreatic tumor tissues. **D**, Western blot showing that curcumin and gemcitabine together inhibit the expression of procaspase-3 and procaspase-9 in pancreatic tumor tissues. Samples from three animals in each group were analyzed and representative data are shown.



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pancreatic cancer cells (45). Similarly, we also found that curcumin inhibited MMP-9 expression *in vivo*. Multiple studies used newly developed synthetic MMP inhibitors in experimental models of pancreatic cancer and showed that the agents reduced tumor growth and increased survival of mice (46). The myc proteins, which are known to play a central role in cellular proliferation, differentiation, apoptosis, and tumorigenesis, are overexpressed in pancreatic cancer (47). We found that curcumin inhibited c-myc expression *in vivo*. Finally, our results also showed that curcumin inhibited several NF- κ B-regulated antiapoptotic proteins, including survivin, Bcl-2, Bcl-xL, and cIAP-1. The results suggested that curcumin enhanced the apoptotic effect of gemcitabine in pancreatic cancer tumor tissues by inhibiting these antiapoptotic proteins.

Overall, our results showed that curcumin potentiates the antitumor effects of gemcitabine by inhibiting NF- κ B and its downstream targets, leading to the inhibition of proliferation, angiogenesis, and invasion. The dose of gemcitabine (25 mg/kg)

and curcumin (1 g/kg) used in the current animal studies is quite relevant to that in human subjects. Because curcumin is very well tolerated in human subjects, even at very high doses (21), the combination of curcumin with gemcitabine has significant potential as an effective therapy for pancreatic cancer that can enhance the effect of gemcitabine and overcome chemoresistance. Further clinical studies are necessary to confirm our findings in patients with pancreatic cancer.

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