

# Antitumor Activity of Gemcitabine and Oxaliplatin Is Augmented by Thymoquinone in Pancreatic Cancer

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## Abstract

Previous studies have shown biological activity of thymoquinone, an active compound extracted from *Nigella sativa*, in pancreatic cancer cells; however, preclinical animal studies are lacking. Here, we report, for the first time, the chemosensitizing effect of thymoquinone to conventional chemotherapeutic agents both *in vitro* and *in vivo* using an orthotopic model of pancreatic cancer. *In vitro* studies revealed that preexposure of cells with thymoquinone (25  $\mu\text{mol/L}$ ) for 48 h followed by gemcitabine or oxaliplatin resulted in 60% to 80% growth inhibition compared with 15% to 25% when gemcitabine or oxaliplatin was used alone. Moreover, we found that thymoquinone could potentiate the killing of pancreatic cancer cells induced by chemotherapeutic agents by down-regulation of nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ), Bcl-2 family, and NF- $\kappa\text{B}$ -dependent antiapoptotic genes (X-linked inhibitors of apoptosis, survivin, and cyclooxygenase-2). As shown previously by our laboratory, NF- $\kappa\text{B}$  gets activated on exposure of pancreatic cancer cells to conventional chemotherapeutic agents; interestingly, thymoquinone was able to down-regulate NF- $\kappa\text{B}$  *in vitro*, resulting in chemosensitization. In addition to *in vitro* results, here we show for the first time, that thymoquinone in combination with gemcitabine and/or oxaliplatin is much more effective as an antitumor agent compared with either agent alone. Most importantly, our data also showed that a specific target, such as NF- $\kappa\text{B}$ , was inactivated in animal tumors pretreated with thymoquinone followed by gemcitabine and/or oxaliplatin. These results provide strong *in vivo* molecular evidence in support of our hypothesis that thymoquinone could abrogate gemcitabine- or oxaliplatin-induced activation of NF- $\kappa\text{B}$ , resulting in the chemosensitization of pancreatic tumors to conventional therapeutics. [Cancer Res 2009;69(13):5575–83]

## Introduction

Pancreatic cancer is the fourth major cause of cancer-related deaths worldwide, with a 5-year survival rate of <3%. According to NIH-National Cancer Institute statistics, of the 38,000 new cases of pancreatic cancer diagnosed in 2008, 35,000 are expected to succumb to this disease. Despite the availability of myriad of treatment modalities, including preferred cytotoxic chemotherapy, dose-limiting toxicity to normal tissues and acquisition of acquired

resistance fails to transcend into optimal clinical benefit in terms of cure rate in an overwhelming majority of patients (1). Emerging evidence suggest DNA-binding ability of the transcription factor nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) as a major mechanism contributing to chemoresistant phenotype of pancreatic cancer (2–4) and that several conventional cancer chemotherapeutic agents activate NF- $\kappa\text{B}$  leading to unfavorable clinical outcome (4–8). In recent years, naturally occurring compounds are being investigated because of their alleged therapeutic effects attributable to inactivation of NF- $\kappa\text{B}$  and other survival signaling cascade within tumors and fewer associated toxicities (5, 9, 10). Here, we report, for the first time, the therapeutic benefit and mechanism of action of a naturally occurring compound thymoquinone in sensitizing pancreatic cancer cells to conventional cytotoxic drugs.

Thymoquinone is the bioactive compound derived from black seed (*Nigella sativa*) oil. In folklore medicine, the seed is reportedly associated with diverse therapeutic benefits as related to bronchial asthma, dysentery, headache, gastrointestinal problems, eczema, hypertension, and obesity. We recently reported in a comprehensive review the therapeutic and chemopreventive potential of black cummin seeds (11). In the context of cancer, thymoquinone has been reported to exhibit antiproliferative effects on cell lines derived from breast, colon, ovarian, larynx, lung, myeloblastic leukemia, and osteosarcoma (12–18). Additionally, thymoquinone inhibited hormone-refractory prostate cancer by targeting androgen receptor and transcription factor E2F (19). Mechanistically, thymoquinone reportedly induced apoptosis in tumor cells by suppressing NF- $\kappa\text{B}$ , Akt activation, and extracellular signal-regulated kinase signaling pathways and also inhibits tumor angiogenesis (20, 21). These limited studies suggest that thymoquinone could be useful as an adjunct to conventional chemotherapeutics; however, *in vivo* studies in support of this statement are lacking and our current findings filled that gap.

Gemcitabine is the first-line conventional chemotherapy for pancreatic cancer, but oxaliplatin is used as an alternative option (22, 23). Conventional dosing with either cytotoxic drug given at maximum tolerated dose caused side effects and fails to respond adequately due to acquisition of chemoresistance phenomenon (23, 24). In the present study, first we sought to understand the molecular mechanism of action of thymoquinone in pancreatic cancer cells in inducing apoptosis and then examined the chemosensitization potential of thymoquinone in combination with oxaliplatin and gemcitabine to reduce viable cells and potentiate apoptosis using low concentrations of chemotherapeutic drugs. Importantly, we also tested our hypothesis *in vivo* using an orthotopic model of pancreatic cancer in severe combined immunodeficient (SCID) animals. Using *in vivo* imaging data, we show, for the first time, that thymoquinone pretreatment in combination with either gemcitabine or oxaliplatin caused greater antitumor activity than each drug given alone. These results correlated with

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down-regulation of NF- $\kappa$ B activity and its downstream proteins such as survivin, Bcl-xL, and X-linked inhibitor of apoptosis (XIAP) in tumor extracts.

## Materials and Methods

**Cell culture.** The human pancreatic cancer cell lines BxPC-3 and HPAC were obtained from the American Type Culture Collection. Human pancreatic ductal epithelial cells and COLO-357 were obtained from M. D. Anderson Cancer Center. The cell lines were maintained in continuous exponential growth in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 10 mg/mL streptomycin in a humidified incubator containing 5% CO<sub>2</sub> in air at 37°C.

Antibodies were obtained from the following commercial sources: caspase-3, caspase-9, cytochrome *c*, cytochrome *c* oxidase, and p65 (Cell Signaling); anti-mouse Bcl-2, Bcl-xL, Bax, Mcl-1, and anti-retinoblastoma antibody (Santa Cruz Biotechnology); anti-poly(ADP-ribose) polymerase (PARP) antibody (Biomol Research); and anti- $\beta$ -actin (Sigma). Thymoquinone (Sigma) was dissolved in DMSO to make 20 mmol/L stock solution. Oxaliplatin and gemcitabine were obtained from the Barbara Ann Karmanos Cancer Institute pharmacy.

**Cell viability inhibition by thymoquinone.** Cells ( $3 \times 10^3$  per well) in 96-well culture plates were seeded and replaced next day with fresh medium containing thymoquinone (0-50  $\mu$ mol/L) diluted from stock solution. After 72 h, MTT solution was added and incubated further for 2 h. MTT formazan formed by metabolically viable cells was dissolved in isopropanol, and absorbance was measured at 595 nm on a plate reader (TECAN).

**Cell viability inhibition by cytotoxic agents.** Cells were plated and incubated with medium containing thymoquinone (25  $\mu$ mol/L) for 48 h and exposed to 500 nmol/L gemcitabine or 62  $\mu$ mol/L oxaliplatin for an additional 24 h. The effect of thymoquinone pretreatment on cell viability was examined by MTT as described above. To investigate the synergistic effect between thymoquinone, gemcitabine, and oxaliplatin, cells were exposed to drugs in fixed ratio as above and combination index (CI) was calculated using CalcuSyn software (Biosoft).

**Quantification of apoptosis.** The Cell Apoptosis ELISA Detection Kit (Roche) was used to detect apoptosis in pancreatic cancer according to the manufacturer's protocol.

**Protein extraction and Western blot analysis.** HPAC cells were plated and allowed to attach for 36 h. Thymoquinone was directly added to cell cultures at the indicated concentrations and incubated for 72 h. Total protein (40  $\mu$ g) was separated on SDS-PAGE, electrotransferred, and probed with specific antibodies.

**Analysis of cytochrome *c* release.** Mitochondria and cytosolic fractions were obtained as described previously (6) to detect cytochrome *c* release by Western immunoblotting.

**Caspase activity assays.** Caspase-9 and caspase-3 activities were measured in HPAC cells and tissue lysates by a colorimetric assay according to the manufacturer's protocol (R&D Systems).

**Determination of prostaglandin E<sub>2</sub>.** HPAC cells were seeded in 50 mm dishes and treated with thymoquinone (0-50  $\mu$ mol/L) for 72 h in serum-free medium. Conditioned medium was collected and analyzed for prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) using PGE<sub>2</sub> high-sensitivity immunoassay kit (R&D Systems).

**Cell cycle analysis.** HPAC cells were treated with thymoquinone for 48 h followed by either gemcitabine (500 nmol/L) or oxaliplatin (62  $\mu$ mol/L) for 24 h. The fixed cells were stained with propidium iodide for 30 min and analyzed by flow cytometry.

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared from treated samples and electrophoretic mobility shift assay was done by incubating 10  $\mu$ g nuclear extract with IRDye-700-labeled NF- $\kappa$ B oligonucleotide as described earlier (6).

**Experimental animals and orthotopic implantation of tumor cells.** Female ICR/SCID mice were purchased from Taconic Farms. HPAC cells were harvested from subconfluent cultures, washed in serum-free medium, and resuspended in PBS. Cells ( $1 \times 10^6$ ) in 15  $\mu$ L PBS were injected into the parenchyma of pancreas with a 27-gauge hypodermic needle.

**Experimental protocol.** Mice were randomized into the following treatment groups ( $n = 7$ ): (a) untreated control; (b) thymoquinone 3 mg/mouse given daily by intragastric intubation for 25 days; (c) oxaliplatin 5 mg/kg body weight, intraperitoneally, given twice per week; (d) gemcitabine 50 mg/kg body weight, intraperitoneally, given thrice per week; and (e) and (f) thymoquinone and gemcitabine or oxaliplatin following schedule as for individual treatments. All mice were sacrificed on day 35 following last dose of thymoquinone, and their body weight was recorded. On autopsy, the pancreas was excised neatly and weighed. One part of the tissue was fixed in formalin and another part was frozen in liquid nitrogen.

**Mice imaging.** EGF-IRDye-800CW (epidermal growth factor receptor antibody) was delivered via tail vein (1 nmol/L per mice) 96 h before euthanizing the animals. Near IR fluorescence imaging of live animals was done using Odyssey Infrared Imaging System.

**Tumor tissue nuclear protein extraction and electrophoretic mobility shift assay.** Nuclear protein extracts were prepared from tumor tissues and electrophoretic mobility shift assay was done by incubating 12  $\mu$ g nuclear extract with IRDye-700-labeled NF- $\kappa$ B oligonucleotide as described earlier (5).

**Histopathology and immunohistochemistry.** H&E and immunohistochemistry for Ki-67 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) for apoptosis were done on paraffin-embedded tissue sections.

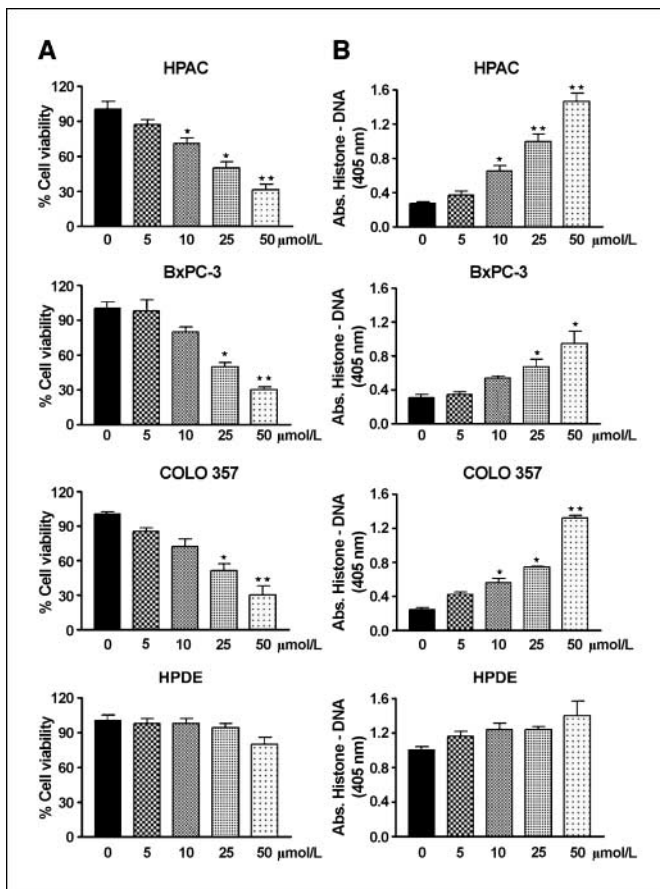
**Statistical analysis.** Data are represented as mean  $\pm$  SD for the absolute values or percent of controls as indicated in the vertical axis legend of Figs. 1 to 6. Statistical significance was determined by Student's *t* test.  $P < 0.05$  were considered statistically significant.

## Results

**Effect of thymoquinone on cell viability and apoptosis induction.** In Fig. 1A, thymoquinone inhibited cell viability in a dose-dependent manner. In almost all pancreatic cancer cell lines, cell viability was reduced up to 70% with 50  $\mu$ mol/L thymoquinone treatment for 72 h. In contrast, treatment of human pancreatic ductal epithelial cells resulted in minimal loss of viable cell when exposed to identical concentrations of thymoquinone for a similar period. BxPC-3 cells harboring wild-type *K-ras* oncogene and mutated p53 was sensitive to the effect of thymoquinone in as much as pancreatic cancer cells with mutant *k-ras* and wild-type p53 (HPAC and COLO-357). Further, to assess whether the loss of cell viability could in part be due to apoptosis, we evaluated apoptosis using histone-DNA ELISA. Figure 1B shows a significant increase in apoptotic cells, which closely parallels the loss of viable cells following thymoquinone treatment. In contrast, human pancreatic ductal epithelial cells were minimally affected to apoptosis by equivalent concentrations of thymoquinone.

**Thymoquinone inhibits antiapoptotic molecules in HPAC cells.** To understand the molecular basis of thymoquinone-induced apoptosis, we performed Western immunoblotting using HPAC cells as representative. As shown in Fig. 2A, the proapoptotic protein Bax was markedly induced, whereas Bcl-2 and Bcl-xL were significantly inhibited in a concentration-dependent manner, indicating that the apoptotic effects of thymoquinone are partly caused by up-regulating the Bax/Bcl-2 or Bax/Bcl-xL protein ratio, which is a critical determinant of apoptosis. Additionally, we assessed other antiapoptotic molecules Mcl-1, survivin, and XIAP. Relative to control, Mcl-1, survivin, and XIAP expression was down-regulated in cells exposed to thymoquinone. These results provide additional molecular evidence of apoptosis induction by thymoquinone treatment.

Western immunoblotting also revealed thymoquinone treatment resulted in appearance of cleaved active component of caspase-3, caspase-9, and PARP in HPAC cells (Fig. 2A). In parallel, caspase-3



**Figure 1.** Evaluation of cell viability by MTT (A) and histone DNA ELISA for apoptosis (B) by thymoquinone treatment for 72 h. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . HPDE, human pancreatic ductal epithelial cells.

and caspase-9 activities were also significantly elevated (Fig. 2B). An upstream event in activation of the caspase cascade is the release of cytochrome *c* from mitochondria. Thymoquinone induced release of cytochrome *c* in dose-responsive manner (Fig. 2A), suggesting that thymoquinone-induced apoptosis in HPAC cells is mediated at least in part by the mitochondrial pathway.

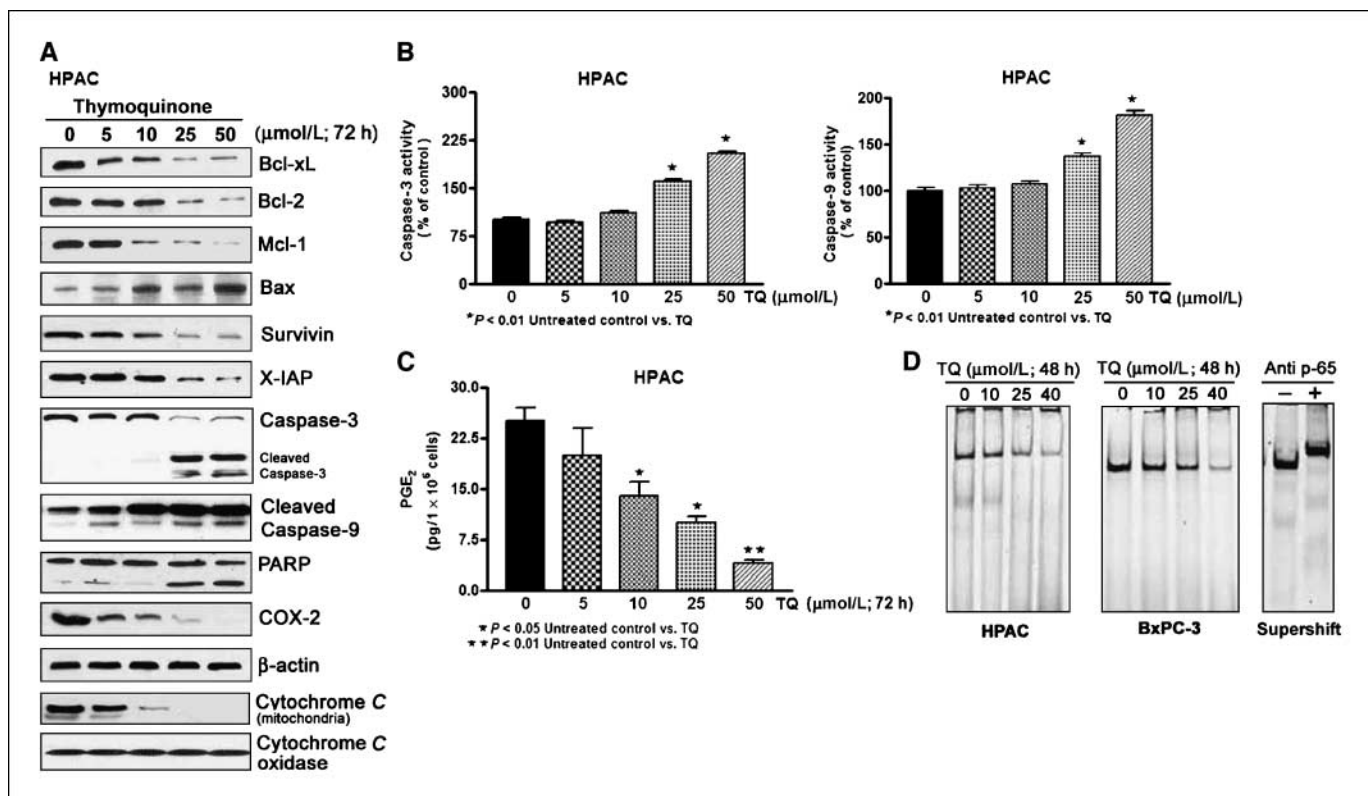
**Inhibition of cyclooxygenase-2 expression and PGE<sub>2</sub> accumulation by thymoquinone.** The cyclooxygenase-2 (COX-2) enzyme is overexpressed in pancreatic cancer and is involved in the inhibition of apoptosis, potentiation of cell growth, and angiogenesis and thus considered as a putative target for drug development (25). To determine whether thymoquinone reduces the expression of COX-2 and consequently the production of PGE<sub>2</sub>, COX-2-expressing HPAC cells were treated with thymoquinone. Western immunoblotting showed a significant decrease in COX-2 protein expression in a dose-dependent manner (Fig. 2A). Further, to confirm whether the catalytic activity of COX-2 and thus the production of PGE<sub>2</sub> were inhibited by thymoquinone or not, culture supernatant was collected from exponentially growing HPAC cells in the presence of thymoquinone, and the PGE<sub>2</sub> levels were measured. As shown in Fig. 2C, the synthesis of PGE<sub>2</sub> was significantly decreased in a dose-dependent manner after thymoquinone treatment, which well correlated with the down-regulation of COX-2 expression. These results are consistent with the ability of thymoquinone in inhibiting cell growth and induction of apoptosis.

**Thymoquinone inhibits activation of NF- $\kappa$ B.** To investigate whether thymoquinone could abrogate constitutively active NF- $\kappa$ B in pancreatic cancer cells, cells were treated with increasing doses of thymoquinone (0–40  $\mu$ mol/L) for 48 h and subjected to gel shift assay (electrophoretic mobility shift assay). Thymoquinone resulted in a concentration-dependent decrease in NF- $\kappa$ B DNA-binding activity in HPAC and BxPC-3 cells (Fig. 2D), which was further confirmed by supershift experiment. These results are consistent with down-regulation of COX-2, survivin, and Bcl family proteins because these proteins are transcriptionally regulated by NF- $\kappa$ B and contribute to the survival of cells by inhibiting apoptosis.

**Thymoquinone sensitizes pancreatic cancer cells to chemotherapeutic agents.** The effect of gemcitabine or oxaliplatin on the viability of pancreatic cancer cells was determined by MTT assay (data not shown). Subsequent studies were undertaken to examine whether cells pretreated with thymoquinone could be more sensitive to gemcitabine and oxaliplatin. Cells were either treated with thymoquinone (25  $\mu$ mol/L alone for 48 h) followed by 24 h incubation with suboptimal dose of either drugs, gemcitabine and oxaliplatin, and cell viability was determined (Fig. 3A). We noticed treatment of cells with either thymoquinone for 48 h or gemcitabine and oxaliplatin alone for 24 h caused 15% to 50% loss of viable pancreatic cancer cells depending on cell type. However, pretreatment with thymoquinone followed by cytotoxic chemotherapeutic drugs resulted in 65% to 80% ( $P < 0.001$ ) loss of viable cells in all pancreatic cancer cell evaluated, which we believe may be associated with less toxic side effects on normal cells. Interestingly, pretreatment with thymoquinone proved more efficacious in sensitizing the cells to cytotoxic agents compared with cotreatment or washing away thymoquinone before adding drugs (data not shown). Further, to confirm synergism, we determined CI values for two combination treatment groups. CI is a quantitative measure of the degree of drug interaction; CI  $< 1$  indicates synergism, CI  $> 1$  indicates antagonism, and CI = 1 indicates additive effect. Our results show that cells pretreated with thymoquinone showed synergistic loss of the cell viability when combined with gemcitabine or oxaliplatin (CI = 0.735 and 0.784 for thymoquinone and gemcitabine and 0.545 and 0.685 for thymoquinone and oxaliplatin).

**Thymoquinone sensitizes HPAC cells to apoptosis by gemcitabine and oxaliplatin.** We next confirmed whether enhanced cytotoxicity by thymoquinone pretreatment was mediated by apoptosis. Accordingly, relative to single agent, thymoquinone pretreatment (25  $\mu$ mol/L) followed by either gemcitabine or oxaliplatin elicited significantly ( $\leq 30$ –50%;  $P < 0.01$ ) higher apoptosis in all investigated pancreatic cancer cell lines, suggesting that the loss of viable cells by thymoquinone and gemcitabine/or oxaliplatin results due to the induction of cell death pathway (Fig. 3D).

**Thymoquinone pretreatment enhances apoptosis by oxaliplatin and gemcitabine in HPAC cells.** To understand sensitization mechanism and apoptosis by thymoquinone pretreatment, we assessed by Western immunoblotting the status on caspase-3, caspase-9, and PARP cleavage and modulation of other pro-survival molecules (Fig. 4A). Cells were exposed to thymoquinone (25  $\mu$ mol/L) for 48 h before addition of gemcitabine or oxaliplatin for 24 h. Our data showed that whereas treatment of HPAC cells with single agents showed appearance of cleaved caspase-3, 9, and PARP, thymoquinone pretreatment significantly augmented and showed comparatively strong band for cleaved caspase-3, caspase-9, and PARP (Fig. 4A). Additionally, caspase-3 activity was also observed significantly elevated in the combination treatment group



**Figure 2.** A, Western blot depicting alterations in apoptosis-related proteins and COX-2 in lysates prepared from HPAC cells after treatment for 72 h. B, percent changes in caspase-3 and caspase-9 activities relative to untreated control. C, PGE<sub>2</sub> quantitation by ELISA in conditioned medium from HPAC cells. D, gel shift assay showing down-regulation of NF-κB in pancreatic cancer cells. TQ, thymoquinone.

(Fig. 4C). Based on our results showing apoptosis, we evaluated other prosurvival molecules such as Bcl-xL, survivin, and XIAP. Our results showed significant down-regulation of these proteins consistent with results obtained for PARP and caspases, indicating that thymoquinone indeed synergize the cytotoxic effect of gemcitabine and oxaliplatin (Fig. 4A).

**Thymoquinone down-regulates NF-κB activation stimulated by gemcitabine and oxaliplatin.** HPAC cells were exposed to 25 μmol/L thymoquinone for 48 h followed by 3 h of either gemcitabine (500 nmol/L) or oxaliplatin (62 μmol/L), and their nuclear extracts were subjected to NF-κB DNA-binding activity assay by electrophoretic mobility shift assay. Consistent with previously published data (5, 6), we found that gemcitabine and oxaliplatin treatment alone for 3 h induced NF-κB DNA-binding activity (Fig. 4B). Interestingly, pretreatment with 25 μmol/L thymoquinone abrogated gemcitabine- or oxaliplatin-induced activation of NF-κB (Fig. 4C). These results show that thymoquinone not only down-regulates preexisting basal levels of NF-κB DNA-binding activity in unstimulated pancreatic cancer cells but also could inhibit gemcitabine- or oxaliplatin-induced NF-κB activation, and we strongly believe the chemosensitizing effect of thymoquinone is in part due to inactivation of NF-κB and its downstream genes.

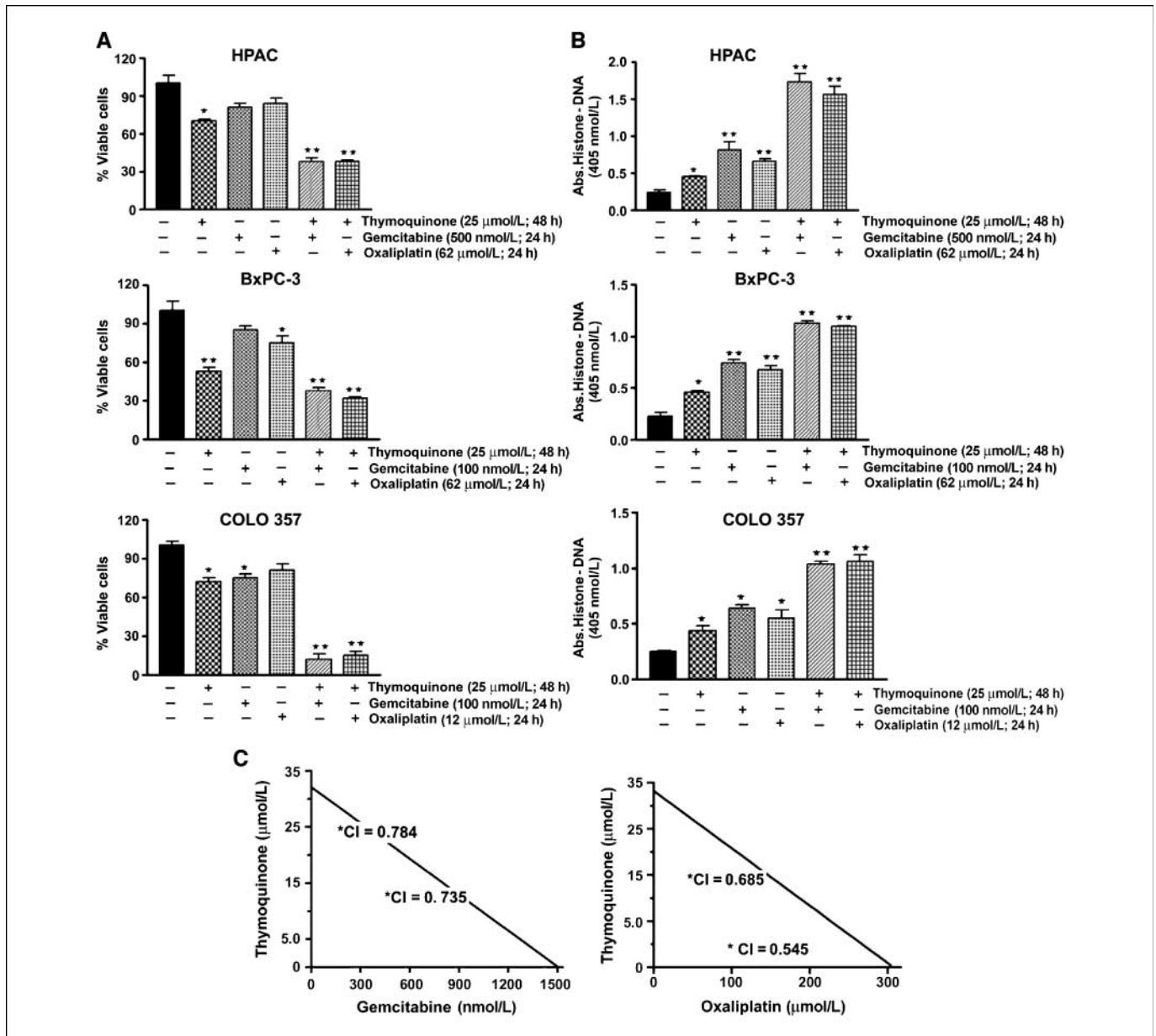
**Effect of thymoquinone exposure on cell cycle distribution.** Untreated HPAC cells showed a relatively normal pattern, with most cells in the S phase (49.37%), a lower G<sub>0</sub>-G<sub>1</sub> phase (32.96%), and G<sub>2</sub>-M (17.66%) peak of the cell cycle. The change in the cell cycle distribution of HPAC cells treated for 48 h with 25 μmol/L thymoquinone followed by 24 h treatment with either

gemcitabine (500 nmol/L) or oxaliplatin (62 μmol/L) is shown in Fig. 4D. Gemcitabine (500 nmol/L) alone caused G<sub>0</sub>-G<sub>1</sub> cell cycle arrest (46.40% versus 32.96% in control), whereas oxaliplatin (62 μmol/L) alone resulted in S-phase arrest (62.96% versus 32.96% in control) after 24 h treatment. Interestingly, thymoquinone pretreatment led to increased cell population at the G<sub>0</sub>-G<sub>1</sub> phase from 46.40% to 56.61% in thymoquinone-gemcitabine group. Thymoquinone-oxaliplatin augmented S-phase arrest from 62.96% to 73.28% in pancreatic cancer cells, whereas the proportion of G<sub>2</sub>-M-phase cells decreased. This indicates that thymoquinone pretreatment potentiates arrest of cells in progression of the cell cycle.

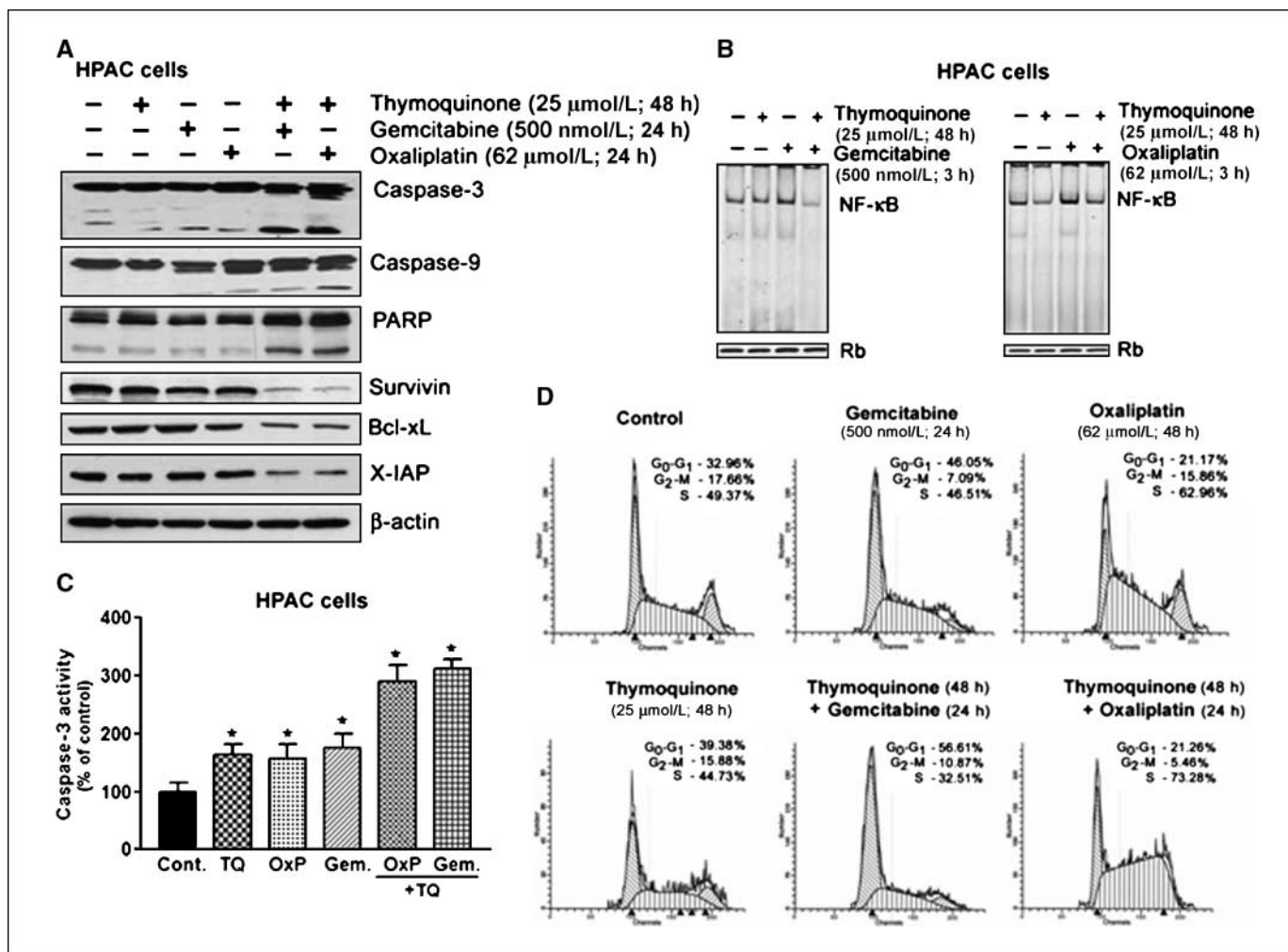
**Thymoquinone enhances *in vivo* therapeutic effect of gemcitabine and oxaliplatin on HPAC orthotopic tumor.** Building on aforementioned results, which strongly support better killing of pancreatic cancer cells when preexposed to thymoquinone, we evaluated therapeutic advantage of thymoquinone and gemcitabine or oxaliplatin in SCID mice bearing orthotopically implanted HPAC cells. Such studies have never been documented *in vivo* to the best of our knowledge. A dose of 3.0 mg/d thymoquinone/mouse was selected for intragastric administration, whereas gemcitabine (50 mg/kg body weight) and oxaliplatin (5 mg/kg body weight) dosages were based on previously published reports and administered as depicted in Fig. 5A. The efficacy of treatment was determined by considering mean pancreatic tumor weight immediately following euthanization. Administration of thymoquinone caused 38% reduction in tumor weight. Gemcitabine or oxaliplatin alone caused 66% and 58% reduction in tumor

weight, respectively (Fig. 5B). However, the combination of thymoquinone-gemcitabine or thymoquinone-oxaliplatin showed significant decrease ( $P < 0.01$ ) in tumor weight relative to untreated control, thymoquinone alone, gemcitabine alone, or only oxaliplatin. Thymoquinone also suppressed local invasion and nodal metastasis. On autopsy, 86% of mice from the control group showed evidence of nodal metastasis. In contrast, a progressive decline in the percentage of mice harboring nodal metastasis, as well as metastatic tumor size, was noticed in the combination groups as shown in Fig. 5C. Other than nodal metastasis, no macroscopic evidence of spreading to other visceral organs was evident in any experimental groups.

Because pancreatic cancer cells, especially HPAC cells, over-express epidermal growth factor receptor, we validated our findings using a noninvasive IR fluorescent-labeled probe for evaluating tumor size *in vivo* using Li-COR Odyssey Infrared Imaging Detection System (26). Scanning pancreatic tumor images of mice randomly selected from each group revealed significantly reduced fluorescence intensity ( $P < 0.01$ ) in the combination group suggestive of a superior effect relative to monotherapy, consistent with inhibition of tumor weights assessed at autopsy. Our treatment conditions did not cause any severe toxicity as assessed by the weight loss criteria or aversion to food intake or diarrhea, suggesting that thymoquinone did not induce any deleterious effects.



**Figure 3.** A, chemosensitization by thymoquinone preexposure in pancreatic cancer cells by MTT as described in Materials and Methods. B, increased apoptosis in pancreatic cancer cells by histone-DNA ELISA relative to untreated control or monotherapy treatment. C, isobologram showing synergistic interaction between thymoquinone and drug combinations. \*,  $P < 0.05$ , control versus monotherapy (only thymoquinone, gemcitabine, or oxaliplatin) for HPAC and COLO-357 cells. \*\*,  $P < 0.05$ , control versus monotherapy (only thymoquinone or oxaliplatin) for BxPC-3 cells; \*\*,  $P < 0.001$ , monotherapy versus combination (thymoquinone + gemcitabine, thymoquinone + oxaliplatin) for HPAC, BxPC-3, and COLO-357 cells.



**Figure 4.** A, Western blot showing down-regulation of antiapoptotic and prosurvival molecules in HPAC cells under conditions of preexposure to thymoquinone followed by either cytotoxic chemotherapeutic drug. B, NF- $\kappa$ B DNA binding in HPAC cells in presence and/or absence of thymoquinone and gemcitabine or oxaliplatin as detailed in Materials and Methods. Thymoquinone pretreatment down-regulated gemcitabine- and oxaliplatin-induced NF- $\kappa$ B in HPAC cells. Western immunoblotting for retinoblastoma protein in the nuclear extract was used as loading control. C, caspase-3 activity relative to untreated control in lysates from HPAC cells treated as described above, \*,  $P < 0.01$ , treatment versus control. D, effect of thymoquinone on % cells in different phases of cell cycle by flow cytometry in HPAC cells.

These results, for the first time, confirm the chemosensitization efficacy of thymoquinone in an *in vivo* orthotopic model.

**NF- $\kappa$ B DNA-binding activity and antiapoptotic protein expression *in vivo*.** Our results clearly show that NF- $\kappa$ B in tumor samples was moderately down-regulated by thymoquinone alone, but unlike *in vitro* situation, neither gemcitabine- nor oxaliplatin-treated animals revealed any overtly induction of NF- $\kappa$ B DNA-binding activity relative to control specimens. However, similar to our *in vitro* studies, constitutively active NF- $\kappa$ B was abrogated in tumor samples from mice treated with thymoquinone in combination with either gemcitabine or oxaliplatin (Fig. 6B). These results are in part similar to our *in vitro* findings and complement our hypothesis that the inactivation of NF- $\kappa$ B is, at least, one of the molecular mechanisms by which thymoquinone potentiates gemcitabine- or oxaliplatin-induced antitumor activity *in vivo*. Tumors also revealed down-regulation of a few important NF- $\kappa$ B-regulated molecules such as Bcl-xL, survivin, and XIAP proteins and also caspase-3 activity, providing evidence of apoptosis within tumors (Fig. 6B and C).

**Tumor histology and immunohistochemistry.** H&E evaluation of the tumors from all groups showed high-grade carcinoma

associated with tumor apoptosis and necrosis (Fig. 6D, left column). In the control and thymoquinone group, the tumor was largely viable with high mitosis and minimal intratumoral stroma. In contrast, in the group receiving combined treatment, large areas of cell debris due to marked tumor destruction in the tissue sections were seen. The peripheral tumor is composed of smaller nests or clusters of neoplastic cells. Similar but milder changes were also seen in the tumors from the group treated with gemcitabine or oxaliplatin alone. Immunohistochemistry revealed significant reduction in Ki-67-positive cells in tumors derived from mice treated with thymoquinone and gemcitabine ( $P < 0.05$ ) or thymoquinone and oxaliplatin ( $P < 0.05$ ) compared with single-agent-treated mice. Significant differences in percentage of TUNEL-positive cells were also noted in tumors derived from the combination group relative to single-agent-treated animals ( $P < 0.05$ ).

## Discussion

Loss of viability and induction of apoptotic cell death are two major mechanisms by which chemotherapeutic agents kill cancer

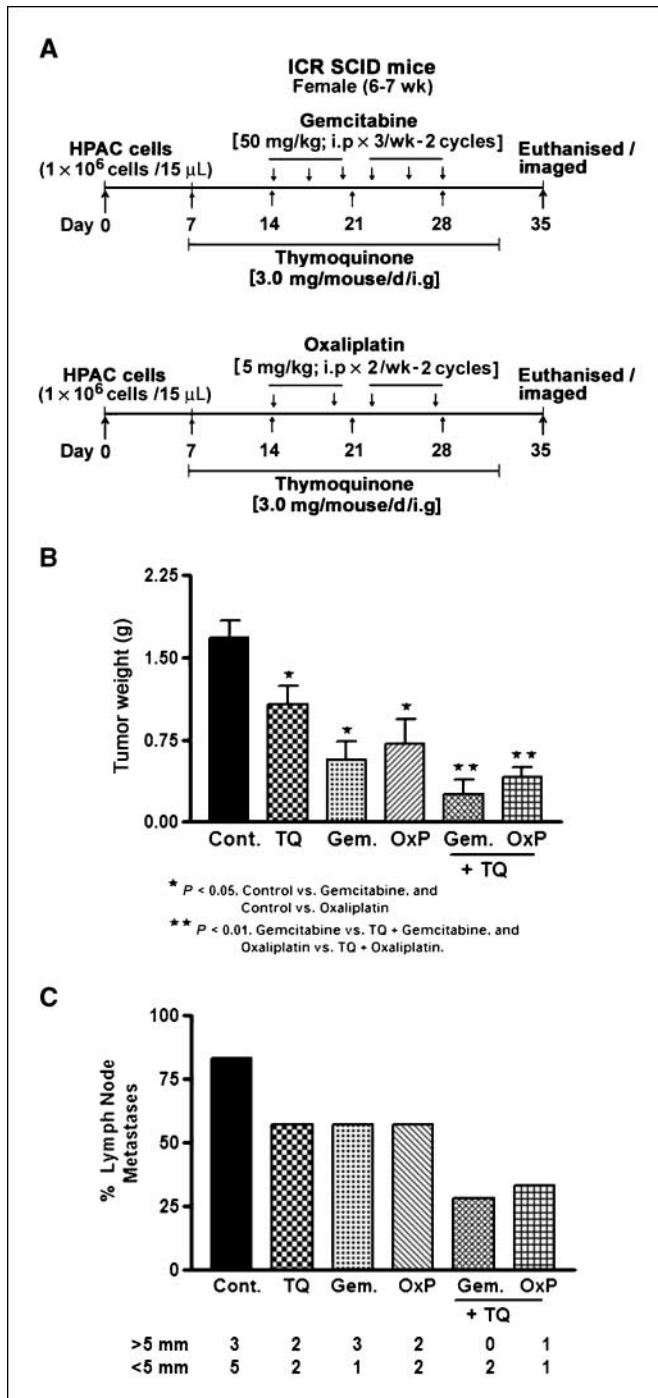
cells. Unfortunately, in pancreatic tumors, acquisition of drug resistance during chemotherapy constitutes a major impediment and challenge in curing patients. In clinical setting, suboptimal therapeutic benefit was achieved when prognostic combinations of different classes of chemotherapeutic agents were attempted owing to dose-limiting toxicities (27). Extensive studies from our laboratory and elsewhere acknowledge the underlying resistance to therapeutic

response and aggressiveness of pancreatic cancer is partly due to constitutive activation of the transcription factor, NF- $\kappa$ B (5, 28, 29). We therefore conceptualized that both *de novo* and acquired resistance to therapy could be attenuated using combination therapy based on sound rationale and hypothesized that thymoquinone might be a novel chemosensitizing agent, which mechanistically at molecular level could be due to inactivation of the DNA-binding activity of NF- $\kappa$ B resulting in inactivation of multiple downstream survival factors. Additionally, Bcl-2 and Bcl-xL proteins predominantly overexpressed in pancreatic cancer cells also reportedly contribute to pancreatic cancer chemoresistance, which can be suppressed by NF- $\kappa$ B inhibition (30). Emerging evidence also indicates that overexpression of XIAP and survivin, members of IAP proteins in human pancreatic cancer, is associated with poor prognosis and increased tumor recurrence (31, 32). Survivin has been validated as a therapeutic target because of its dual function in inhibiting apoptosis as well as its role in regulation of mitosis in concert with different cell cycle regulators (33, 34). Small interfering RNA directed against survivin leading to enhanced pancreatic cancer chemosensitivity to gemcitabine has been reported (35). Additionally, expression of these proteins has also been shown to be associated with resistance to chemotherapy and poor prognosis in carcinomas of the lung, breast, ovary, and esophagus (36–39).

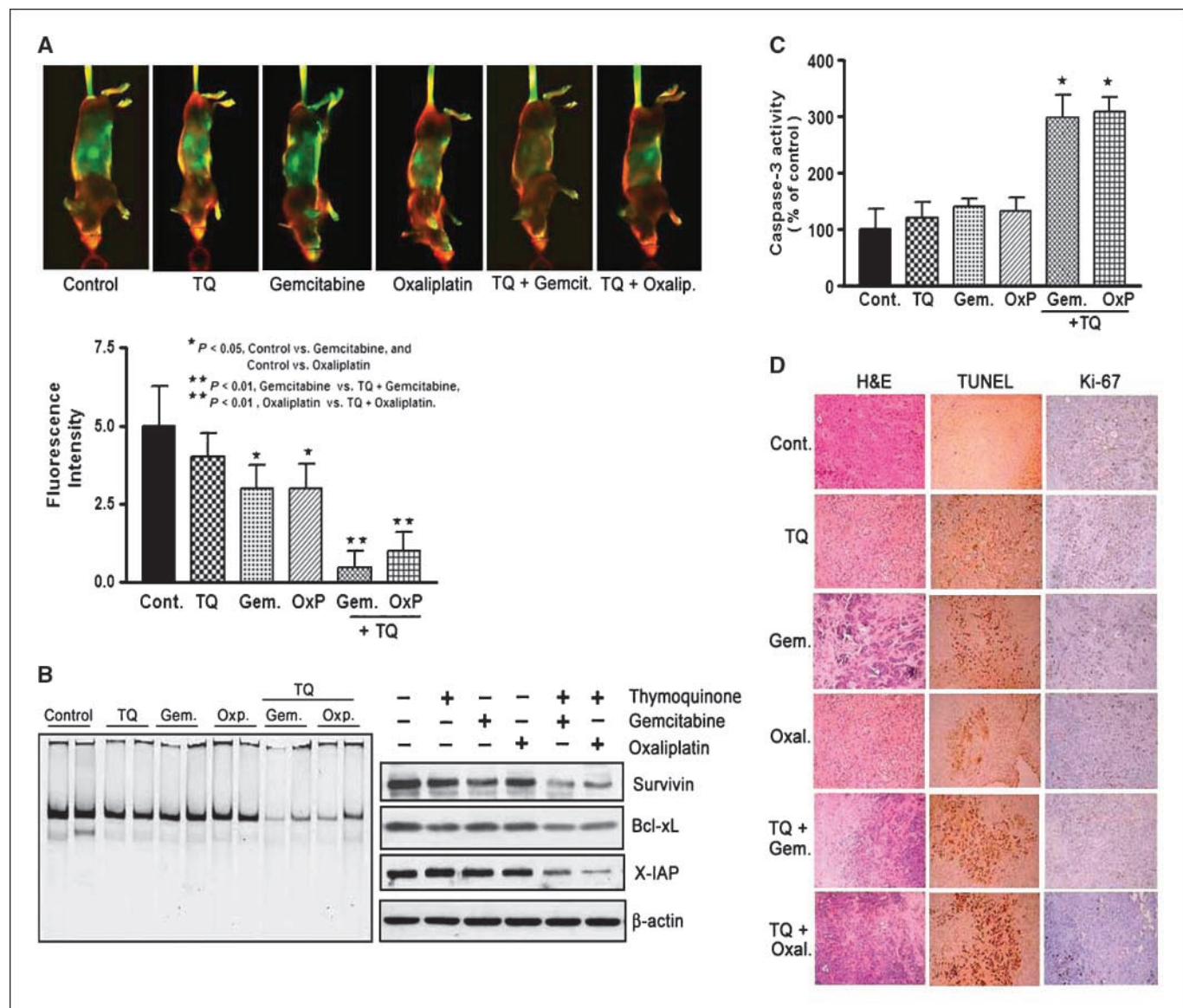
To understand how thymoquinone inhibits cell viability and induce apoptosis, we noticed that thymoquinone per se was effective as a general inducer of apoptosis in pancreatic cancer by down-regulating several antiapoptotic proteins using a dose that had no effect on normal human pancreatic ductal epithelial cells. Corollary to our hypothesis, we present evidence documenting significant reduction of tumors *in vivo* by combination of thymoquinone and gemcitabine or oxaliplatin that were found associated with the inhibition of antiapoptotic Bcl-xL. Thymoquinone per se was effective in down-regulating IAP proteins, XIAP and survivin, not only in pancreatic cancer cells *in vitro* but also in preclinical *in vivo* conditions along with gemcitabine and oxaliplatin. Furthermore, our observations with cell cycle progression revealed that preexposure to thymoquinone augmented gemcitabine-induced G<sub>0</sub>-G<sub>1</sub>-phase cell cycle arrest, whereas thymoquinone and oxaliplatin resulted in increased S-phase cell cycle arrest. It is conceivable that thymoquinone prevented the progression of cell cycle through the M phase resulting from inhibition of survivin, leading them to undergo apoptosis.

COX-2 and its synthesized product, PGE<sub>2</sub>, is also overexpressed in pancreatic cancer and therefore considered as promising chemotherapeutic target for treatment and reversal of chemoresistance phenotype (25, 40–42). The effect of thymoquinone on COX-2 expression and PGE<sub>2</sub> production in a mouse model of allergic airway inflammation has been reported (43). In line with this, significant down-regulation of COX-2 protein expression and the level of PGE<sub>2</sub> were observed in HPAC cells treated with thymoquinone. Because the promoter sequence of COX-2 contain binding sites for NF- $\kappa$ B, it is conceivable that thymoquinone complemented down-regulation of COX-2 by inhibition of NF- $\kappa$ B. These observations highlight therapeutic benefit of thymoquinone as natural inhibitor of COX-2 and PGE<sub>2</sub>, which open avenues for inclusion of thymoquinone as natural adjuvant in pancreatic cancer treatment.

Interestingly, our *in vitro* results were recapitulated *in vivo* in an orthotopic pancreatic cancer model, wherein thymoquinone significantly augmented antitumor efficacy of chemotherapeutics. Although none of the mice from combination treatment group were



**Figure 5.** A, flow chart for *in vivo* experimental design and treatment schedule. B, isolated pancreatic tumor weight between different groups of mice. C, metastasis frequency and tumor size distribution between different treatment groups of mice.



**Figure 6.** A, IR fluorescence imaging of pancreatic tumors in SCID mice showing therapeutic benefit of thymoquinone pretreatment and chemotherapy. Reduced fluorescence of IRDye-800CW EGF-targeting agent in the combination group parallel with reduced tumor size. *Bottom*, quantification of imaging data using Odyssey software. B, gel shift assay for NF- $\kappa$ B on two randomly selected primary pancreatic tumors from each group and Western immunoblotting in tumor lysates (*right*). C, percent change in caspase-3 activity relative to control in primary tumor extracts. \*,  $P < 0.01$ , monotherapy versus combination. D, H&E and immunohistochemistry for apoptosis by TUNEL and Ki-67 in tumor tissue sections.

found tumor-free, the therapeutic effect was significant compared with single-drug treatment. These findings draw a parallel with increased TUNEL staining and reduced Ki-67 immunoreactivity indicative of apoptosis and reduced cellular proliferation within tumors. These features are of significant value in predicting improved therapeutic outcome and warrant further studies.

In conclusion, we have presented evidence showing that cells with *de novo* and acquired resistance to chemotherapeutic drugs (such as gemcitabine and oxaliplatin) could be reversed by thymoquinone pretreatment. Thymoquinone down-regulated antiapoptotic and prosurvival proteins that are transcriptionally regulated by NF- $\kappa$ B, resulting in loss of pancreatic cancer cells to survive and proliferate. Our *in vitro* findings along with *in vivo* results provide confidence in support of further

development of thymoquinone as an adjunct to conventional chemotherapeutics by targeted inactivation of NF- $\kappa$ B for treatment of human pancreatic tumors in the future and initiation of clinical trial.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Antitumor Activity of Gemcitabine and Oxaliplatin Is Augmented by Thymoquinone in Pancreatic Cancer

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