

Molecular Evidence for Increased Antitumor Activity of Gemcitabine by Genistein *In vitro* and *In vivo* Using an Orthotopic Model of Pancreatic Cancer

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Abstract

Soy isoflavone genistein exhibits growth inhibitory activity against human pancreatic cancer cell lines. We previously reported the potential of genistein to augment chemotherapeutic response of pancreatic cancer cells *in vitro*. In the present study, we investigated whether genistein pretreatment could be used as a novel strategy for gemcitabine-induced killing *in vitro* and enhanced antitumor activity *in vivo* using an orthotopic tumor model. We conducted our studies using paired isogenic human pancreatic cancer cell line with differences in metastatic behavior (COLO 357 and L3.6pl). *In vitro* studies were done to measure growth inhibition and degree of apoptotic cell death induced by either genistein alone, gemcitabine alone, or genistein followed by gemcitabine. Our results show that pretreatment of cells with genistein for 24 hours followed by gemcitabine resulted in 60% to 80% growth inhibition compared with 25% to 30% when gemcitabine was used alone. The overall growth inhibition was directly correlated with apoptotic cell death irrespective of the metastatic potential of cells. Several genes that are known to inhibit apoptosis and contribute to chemoresistance such as nuclear factor- κ B (NF- κ B) and Akt were assessed to investigate the basis for the observed chemosensitizing effects of genistein. Genistein potentiated the gemcitabine-induced killing by down-regulation of NF- κ B and Akt. In contrast, Akt and NF- κ B were found to be up-regulated when pancreatic cancer cells were exposed to gemcitabine alone, suggesting the potential mechanism of acquired chemoresistance. In addition to *in vitro* results, we show here for the first time, that genistein in combination with gemcitabine is much more effective as an antitumor agent compared with either agent alone in our orthotopic tumor model. But most importantly, our data also showed that a specific target, such as NF- κ B, was inactivated in genistein-treated animal tumors and that gemcitabine-induced activation of NF- κ B was completely inhibited in animal tumors treated with genistein and gemcitabine. These results provide strong molecular *in vivo* evidence in support of our hypothesis that inactivation of NF- κ B signaling pathway by genistein could also abrogate gemcitabine-induced activa-

tion of NF- κ B resulting in the chemosensitization of pancreatic tumors to gemcitabine, which is likely to be an important and novel strategy for the treatment of pancreatic cancer. (Cancer Res 2005; 65(19): 9064-72)

Introduction

Pancreatic ductal adenocarcinoma is a highly aggressive malignant disease, which is currently treated with limited success and dismal outcomes using conventional therapeutics including chemotherapy and irradiation. Currently, it is ranked as the fourth most common cause of cancer related mortality in the United States and other industrialized countries (1). Despite therapeutic advances, the prognosis of patients with pancreatic ductal adenocarcinoma is extremely poor, with a median survival of 6 months (2). In recent years, novel strategies for sensitizing pancreatic tumor cells with naturally occurring dietary chemopreventive compounds have gained considerable attention because of their beneficial effects in overcoming intrinsic tumor cell resistance to apoptosis (3). We previously reported that genistein augments therapeutic efficacy of cytotoxic chemotherapeutic agents (4). As a corollary to our previous study, we report herein the potential of genistein to augment gemcitabine-based chemotherapy for pancreatic cancer. Furthermore, to rule out the possibility of pancreatic adenocarcinoma cell specificity, we extended our studies using paired isogenic pancreatic cancer cell lines (COLO 357 and L3.6pl) differing in their metastatic behavior.

Genistein (4',5,7-trihydroxyisoflavone) is an isoflavone having a heterocyclic diphenolic structure similar to estrogen and is present in dietary items such as soy (5). Genistein has been reported to exert potent antitumor activity in a variety of human cancer cell lines *in vitro* and in several xenograft models with minimal or no toxicity to nonmalignant human cells (6, 7). Intracellularly, genistein has been shown to inhibit protein tyrosine kinase (8), topoisomerase I and II (9), protein histidine kinase, and 5 α -reductase (10). Additionally, previous reports from our laboratory have shown that genistein regulates genes related to the control of cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, angiogenesis, and cancer cell invasion and metastasis (11–15).

Although gemcitabine monotherapy (2',2'-difluorodeoxycytidine), a deoxycytidine analogue, or its combination with other agents has become standard chemotherapy for the treatment of advanced pancreatic cancer (16–19), the outcome is very disappointing. A recently published study involving combination of gemcitabine and monoclonal antibody targeting epidermal growth factor receptor revealed marginal effects on survival and objective tumor response against advanced pancreatic cancer in a multicenter phase II trial (20).

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Therefore, further improvements in chemotherapeutic response in pancreatic cancer are urgently needed.

Because most human pancreatic tumors show high levels of activated Akt, a serine/threonine protein kinase that mediates survival signaling and also confers resistance to conventional therapeutics (21, 22), targeting Akt, could potentially be an effective therapeutic approach. Consistent with this notion, *in vitro* studies showed that Akt activation inhibits gemcitabine-induced apoptosis and that the addition of Akt inhibitors could enhance apoptosis (23, 24). The mechanism by which Akt stimulates cell survival is not fully understood; however, recent studies have shown that activation of Akt leads to the activation of a series of survival factors, including the activation of nuclear factor- κ B (NF- κ B), arming cancer cells to resist induction of apoptosis (25, 26). It has been shown that many conventional cancer chemotherapeutic agents such as vinblastine, vincristine, daunomycin, doxorubicin, camptothecin, cisplatin, and etoposide, activate NF- κ B (27, 28), thereby resisting apoptosis which results in poor clinical outcome for patients with pancreatic cancer. Preclinical studies *in vitro* from our laboratory have shown that genistein acts as a double-edged sword, inactivating NF- κ B and Akt activity, resulting in the induction of apoptosis and, at the same time, abrogating *de novo* or acquired chemoresistance (25, 29). Based on our results and others, we hypothesized that dietary genistein may block multiple intracellular signaling pathways that are known to confer a high degree of chemoresistance by pancreatic cancer cells, thereby abrogating either *de novo* or acquired chemoresistance. Although search for the development of alternative gemcitabine schedules and chemotherapy combinations continues, here we report our preclinical observations in support of our hypothesis that better cell killing is feasible by presensitizing pancreatic cancer cells with genistein during gemcitabine-induced killing. These results are primarily due to inactivation of NF- κ B signaling *in vitro*, and most importantly, *in vivo* using our orthotopic model of pancreatic tumors.

Materials and Methods

Cell culture. Human pancreatic carcinoma cell lines, COLO-357 and L3.6pl, were obtained from M.D. Anderson Cancer Center (Houston, TX). The human pancreatic L3.6pl cells were established from COLO 357 cells by injecting them into the pancreas of nude mice. Hepatic metastasis were harvested and reinjected into the pancreas. This process was repeated six times, resulting in the isolation of the L3.6pl cell line, which produces significantly higher incidence and number of lymph nodes and liver metastases than parental cells (30). The cell lines were maintained in continuous exponential growth by twice-a-week passage in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator containing 5% CO₂ in air at 37°C. Each cell line was split regularly before attaining 70% to 80% confluency. BxPC-3 cells were procured from American Type Culture Collection (Rockville, MD) and were maintained and grown in RPMI containing supplements as above.

Antibodies and reagents. Antibodies were obtained from the following commercial sources: caspase-3, cytochrome *c*, and Ser⁴⁷³ (phosphorylated Akt, p-Akt) were purchased from Cell Signaling (Beverly, MA); anti-poly(ADP-ribose) polymerase (PARP) antibody was from Biomol Research (Plymouth, PA); anti-Bcl-2, anti-Bcl-xL, and anti-retinoblastoma antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti β -actin antibody was from Sigma Chemical Co. (St. Louis, MO). Genistein was obtained from Toronto Research Chemicals (North York, Ontario, Canada) and was dissolved in 0.1 mol/L Na₂CO₃ to make 10 mmol/L stock solution. Gemcitabine (Eli Lilly, Indianapolis, IN) was dissolved in sterile PBS to make 100 μ mol/L stock solution.

Cell growth inhibition by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. COLO 357 and L3.6pl cells were seeded at a density of 3×10^3 cells per well in 96-well microtiter culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing different concentrations of genistein (0-100 μ mol/L) diluted from a 10 mmol/L stock. On completion of 72 hours of incubation, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) were added to each well and incubated further for 2 hours. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100 μ L of isopropanol. The plates were mixed for 30 minutes on a gyratory shaker, and absorbance was measured at 595 nm using a plate reader (TECAN, Durham, NC).

Cell growth inhibition by cytotoxic agents. Cells were plated as described above and allowed to attach overnight. The cells were replaced with fresh medium containing 25 μ mol/L of genistein for 24 hours and then exposed to 25 nmol/L of the chemotherapeutic agent, gemcitabine, for an additional 72 hours. Thus, for a single-agent treatment, cells were exposed to genistein for 96 hours and gemcitabine for 72 hours. The effect of genistein pretreatment on cell viability was examined by the MTT assay method as stated above.

Quantification of apoptosis by ELISA. The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis in COLO 357 and L3.6pl cells with different treatments according to the manufacturer's protocol. Briefly, COLO 357 and L3.6pl cells were treated with 25 μ mol/L genistein for 24 hours and then exposed to a chemotherapeutic agent, 25 nmol/L of gemcitabine, for an additional 72 hours. For single-agent treatment, COLO 357 and L3.6pl cells were treated with genistein for 96 hours and gemcitabine for 72 hours. After treatment, the cytoplasmic histone DNA fragments from COLO 357 and L3.6pl cells with different treatments were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined using ULTRA Multifunctional Microplate Reader (TECAN) at 405 nm.

DNA ladder analysis for detecting apoptosis. COLO 357 and L3.6pl cells were treated with 25 μ mol/L genistein for 24 hours and then exposed to 25 nmol/L gemcitabine for additional 72 hours. After treatment, cellular cytoplasmic DNA from COLO 357 and L3.6pl cells with different treatments was extracted using 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 15 minutes at $13,000 \times g$ to separate the fragment DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase, followed by SDS-Proteinase K digestion, phenol chloroform extraction, and isopropanol precipitation. DNA was separated through a 1.5% agarose gel. After electrophoresis, gels were stained with ethidium bromide and the DNA was visualized under UV light and photographed.

Protein extraction and Western blot analysis. The pancreatic cancer cells COLO 357 and L3.6pl were plated and allowed to attach for 36 hours. Genistein was directly added to cell cultures at 30 μ mol/L concentration and incubated for 48 hours followed by the addition of gemcitabine. Control cells were incubated in the medium containing an equivalent concentration of Na₂CO₃. After 24 hours of incubation with gemcitabine, the cells were harvested in PBS and whole cell lysate was prepared by suspending the cells in 200 μ L of lysis buffer [1 mol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EGTA, 0.1% Triton X-100; 0.1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 μ g/mL leupeptin, 2 μ g/mL aprotinin]. The cells were disrupted by sonication and the total proteins were extracted by centrifuging the tubes at 4°C for 30 minutes at maximal microfuge speed to remove debris. For immunoblotting, each extract was prepared as above and an equivalent to 50 μ g total proteins was separated on SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with specific antibodies. Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit according to manufacturer's instructions. The same membrane was reprobed with the anti- β actin antibody, which was used as an internal control for protein loading.

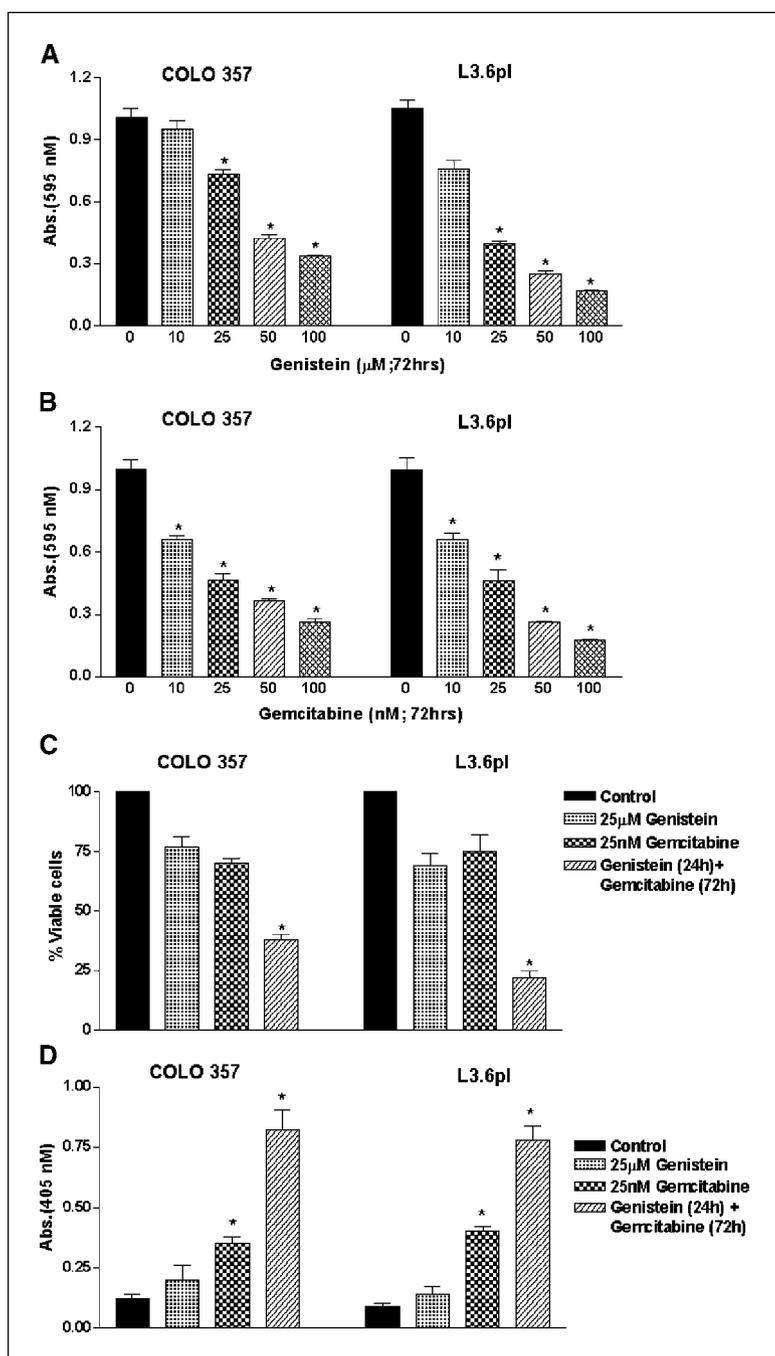


Figure 1. Evaluation of cell viability and apoptosis by genistein pretreatment using MTT and ELISA technique in pancreatic cancer cells, COLO 357 and L3.6pl. Cells were either untreated or treated with either increasing concentration of genistein (A) or gemcitabine, for 72 hours (B) and analyzed for viable cells by MTT assay as described in Materials and Methods. C, COLO 357 and L3.6pl cells pretreated with 25 μmol/L genistein for 24 hours followed by coincubation with gemcitabine (25 nmol/L) for 72 hours. On termination of incubation, viable cells were evaluated relative to untreated control and interpreted as % viable cells. Points, averages of four to five independent experiments. D, sensitization of pancreatic tumor cells, COLO 357 and L3.6pl, to genistein- and/or gemcitabine-induced apoptotic cell death by ELISA after 24 hours of pretreatment with genistein (25 μmol/L), gemcitabine (25 nmol/L), or combination of genistein and gemcitabine for 72 hours. Increased apoptotic response was evident in the combination treatment group of the cells relative to untreated control or individual treatment groups; *, $P < 0.05$, statistical significance.

Analysis of cytochrome *c* release. COLO 357 cells were plated at a density of 5×10^6 cells in 100-mm dish and allowed to attach overnight. Genistein and gemcitabine were added in the sequence as described above. Following termination of incubation period, cells were collected and washed once with ice-cold PBS, and gently lysed using Dounce homogenizer in 400 μL of ice-cold lysis buffer [250 mmol/L sucrose, 1 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 7.2), 1 mmol/L DTT, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and protease inhibitors, Complete Cocktail from Roche Molecular Biochemicals, Indianapolis, IN]. Lysate was centrifuged at $500 \times g$ for 5 minutes at 4°C. The supernatant containing mitochondria was collected and centrifuged again at $10,000 \times g$ for 10 minutes to obtain cytosolic extract and the pellet (fraction containing mitochondria). The pellet was resuspended in 50 μL of homogenizing buffer and used for detecting mitochondrial cytochrome *c*.

Electrophoretic mobility shift assay. Nuclear extracts were prepared according to the method described by Chaturvedi et al. (31). Briefly, respective treatment cells were washed with cold PBS and suspended in 0.15 mL of lysis buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine]. The cells were allowed to swell on ice for 20 minutes and then 4.8 μL of 10% NP40 were added. The tubes were then vigorously mixed on a vortex mixer for a few seconds and centrifuged in a microfuge. The nuclear pellet was resuspended in 30 μL of ice-cold nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine] and incubated on ice with intermittent mixing. The tubes were then centrifuged for 5 minutes in a microfuge at 4°C, and

the supernatant (nuclear extract) was collected in cold Eppendorf tubes and stored at -70°C for later use. The protein content was measured by bicinchoninic acid method.

Electrophoretic mobility shift assay (EMSA) was done by incubating 5 μg of nuclear proteins with IRDye-700 labeled NF- κB oligonucleotide. The incubation mixture included 2 μg of poly(deoxyinosinic-deoxycytidylic acid) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 8.0% native polyacrylamide gel using buffer containing 50 mmol/L Tris, 200 mmol/L glycine (pH 8.5), and 1 mmol/L EDTA and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1. Equal protein loading was ensured by immunoblotting 10 μg of nuclear protein with anti-retinoblastoma antibody.

Experimental animals. Female nude mice (ICR-SCID) were purchased from Taconic Farms (Germantown, NY). The mice were housed and maintained under sterile conditions in facilities accredited by the American Association for the 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 the NIH. The mice were used in accordance with Animal Care and User Guidelines of Wayne State University under a protocol approved by the Institutional Animal Care and Use Committee. The mice received Lab Diet 5021 (Purina Mills, Inc., Richmond, IN).

Orthotopic implantation of tumor cells. COLO 357 and L3.6pl 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% fetal bovine serum. The cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of a single cell with >90% viability were used for the injections. The pancreas of anesthetized mice was exposed through a midline laparotomy incision and by retraction of the spleen. Cells (2×10^5) in 20 μL PBS were injected into the parenchyma of the pancreas with a 27-gauge hypodermic needle and a Hamilton syringe. The abdominal wound was sutured using a 5.0 chromic gut suture in a running fashion. Based on our previous experience with this model, we found a tumor take rate approaching >90%.

Experimental protocol. Mice were randomized into the following treatment groups ($n = 7$): (a) untreated control; (b) only gemcitabine (80 mg/kg body weight), once every other day (i.v. injection); (c) genistein, everyday orally for 10 days; and (d) genistein and gemcitabine, following schedule as for individual treatments. All mice were killed on day 3 following last dose of treatment, and their body weight was determined. The pancreas were excised and weighed. For routine H&E staining, one part of the tissue was fixed in formalin and embedded in paraffin. Another part was rapidly frozen in liquid nitrogen and stored at -70°C . H&E staining confirmed the presence of tumor(s) in each pancreas.

Tumor tissue nuclear protein extraction and electrophoretic mobility shift assay. Tissues were minced and incubated on ice for 30 minutes in 0.5 mL of ice-cold buffer A, composed of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl_2 , 0.5 mmol/L DTT, 0.1% IGEPAL CA-630, and 0.5 mmol/L PMSF. The minced tissue was homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) followed by centrifuging at $5,000 \times g$ at 4°C for 10 minutes. The crude nuclear pellet was suspended in 200 μL of buffer B [20 mmol/L HEPES (pH 7.9); 25% glycerol, 1.5 mmol/L MgCl_2 ; 420 mmol/L NaCl, 0.5 mmol/L DTT; 0.2 mmol/L EDTA; 0.5 mmol/L PMSF; and 4 $\mu\text{mol/L}$ leupeptidin] and incubated on ice for 30 minutes. The suspension was centrifuged at $16,000 \times g$ at 4°C for 30 minutes. The supernatant (nuclear proteins) was collected and kept at -70°C until use. The protein concentration was determined using the bicinchoninic acid assay kit with bovine serum albumin as the standard (Pierce Chemical Co., Rockford, IL). Anti-retinoblastoma immunoblotting with nuclear protein was done as loading control.

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 and 6. The statistical significance of differential findings between experimental groups and control was determined by Student's t test as implemented by Excel 2000 (Microsoft Corp., Redmond, WA). P s < 0.05 were considered statistically significant.

Results

Effect of genistein and gemcitabine on cell proliferation. To test the effect of genistein on cell growth, pancreatic cancer cells were treated with increasing concentrations of genistein (0–100 $\mu\text{mol/L}$) for 72 hours. As shown in Fig. 1A, cell growth was inhibited by genistein treatment in a dose- and time-dependent manner. In COLO 357 cells, treatment with 10, 25, 50, and 100 $\mu\text{mol/L}$ of genistein for 72 hours resulted in 95%, 71%, 42%, and 33% of cell growth relative to control, respectively. Similarly, treatment of L3.6pl cells resulted in 71%, 37%, 23%, and 15% of cell growth, respectively, relative to control when exposed to identical concentrations of genistein for similar period of time. These results indicate that genistein was overall an effective inhibitor of pancreatic cancer cell growth as a single agent, and L3.6pl cells were more sensitive to genistein compared with parental COLO 357 cells. We subsequently evaluated the effect of gemcitabine on cell growth *in vitro* and found that gemcitabine was effective in inhibiting cell growth with equal potency in both cell lines tested (Fig. 1B). Subsequent studies were undertaken to examine whether or not cells pretreated with genistein were more sensitive to the cytotoxic effect of gemcitabine.

Genistein potentiates growth inhibition induced by gemcitabine in COLO 357 and L3.6pl cells. We assessed the effect of pretreatment and cotreatment of a combination of genistein and gemcitabine on cell viability by MTT assay. For these studies, cells were either pretreated with genistein (25 $\mu\text{mol/L}$) alone or in combination with a single dose of gemcitabine (25 nmol/L), and viable cells were evaluated at 96 hours posttreatment by MTT assay. The dose used here was chosen based upon a preliminary dose escalation study done by us. We found that treatment with either genistein or gemcitabine alone for 96 hours resulted in only 25% to 30% loss of viability of COLO 357 and L3.6pl cells. However, pretreatment with genistein for 24 hours followed by treatment with gemcitabine resulted in the loss of 60% to 80% of viable cells in both the cell types investigated (Fig. 1C). Similarly, treatment with genistein plus gemcitabine simultaneously was also effective (data not shown),

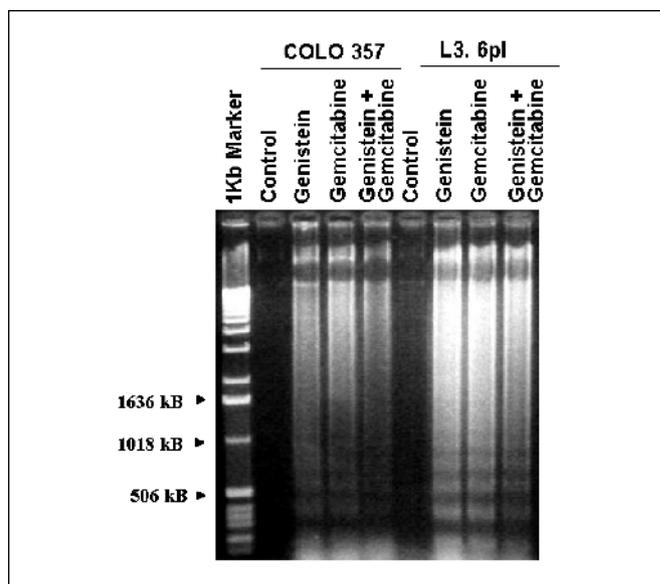


Figure 2. DNA ladder indicative of apoptosis in pancreatic cancer cells either untreated or treated with individual agents or under conditions of pretreatment with 25 $\mu\text{mol/L}$ genistein for 24 hours followed by 25 nmol/L gemcitabine for 72 hours.

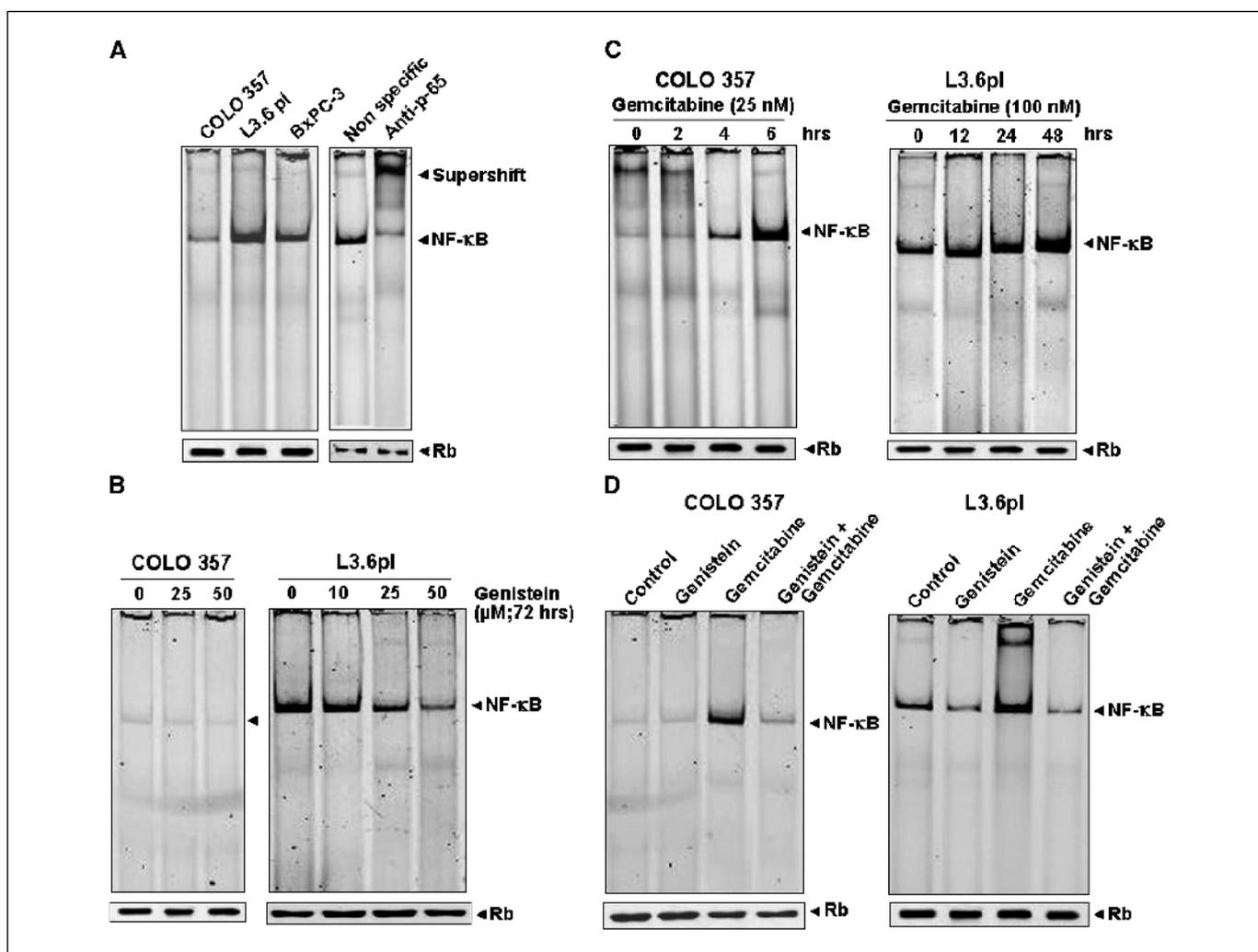


Figure 3. Abrogation of constitutive and induced NF- κ B by genistein in the investigated cell lines, COLO 357 and L3.6pl. *A*, NF- κ B status in investigated pancreatic cancer cell types and confirmation of the band by supershift. Nuclear extracts prepared from BxPC-3 cells were included as positive control. Equal loading of nuclear protein was ensured by immunoblotting 10 μ g of nuclear extract with anti-retinoblastoma antibody by Western blot technique. *B*, dose response by genistein in down-regulating NF- κ B in cells expressing constitutive NF- κ B. Nuclear proteins were prepared from cells after incubation with specified concentrations of genistein for up to 72 hours as described in Materials and Methods. Five micrograms of nuclear protein extract were separated on an 8% Tris-glycine gel. *C*, time course representation of NF- κ B induction by gemcitabine in pancreatic cancer cells, COLO 357 and L3.6pl. The cells were incubated with the indicated concentration of gemcitabine and then the extracted nuclear protein subjected to Gel Shift Assay for evaluation of NF- κ B induction. Nonstimulated COLO 357 cells do not express basal NF- κ B but upon gemcitabine treatment induction of NF- κ B as early as 4 hours was evident. In contrast, a high-dose and prolonged incubation with gemcitabine was prerequisite for inducing NF- κ B in L3.6pl cells harboring constitutive basal NF- κ B. *D*, NF- κ B DNA binding activity in the nuclear extract of the investigated pancreatic cancer cells in the presence and/or absence of genistein as detailed in Materials and Methods. *Lane 1*, untreated control; *lane 2*, treated with genistein (30 μ mol/L, 48 hours); *lane 3*, treated with gemcitabine (6 hours in COLO 357 and 24 hours for L3.6pl); *lane 4*, genistein pretreatment followed by gemcitabine for either 6 or 24 hours according to the cell type being investigated. Genistein pretreatment down-regulated gemcitabine-induced NF- κ B in both cell types. Western immunoblotting for retinoblastoma protein in the nuclear extract was performed as loading control representative.

but pretreatment of cells with genistein was more effective in gemcitabine-induced killing. These results suggest that the combination of genistein with low therapeutic doses of gemcitabine elicits significantly greater inhibition of cancer cell growth compared with either agent, suggesting that lower toxic side effects are likely to occur in normal cells. Inhibition of cell growth and viability as assessed by MTT could also be due to the induction of apoptotic cell death induced by genistein and gemcitabine. We therefore investigated whether gemcitabine in combination with genistein could induce more apoptosis compared with either agent alone.

Genistein sensitizes COLO 357 and L3.6pl cells to apoptosis induced by gemcitabine. We observed induction of apoptosis in pancreatic cancer cells treated with either genistein (25 μ mol/L) or gemcitabine (25 nmol/L) alone. Relative to single agents, genistein

pretreatment followed by gemcitabine treatment induced much more apoptosis in both the cell lines as shown by both histone DNA ELISA (Fig. 1D) as well as DNA ladder analysis (Fig. 2). These results are consistent with cell growth inhibition studies by MTT, suggesting that the loss of viable cells by genistein and gemcitabine is partly due to the induction of an apoptotic cell death mechanism. Collectively, the above results clearly suggest that the enhanced cell growth inhibition and induction of apoptosis by gemcitabine in genistein-pretreated cells is not cell type specific, because we observed similar effects in both the cell lines tested, and also supports our previous observation in BxPC-3 cells (4).

Genistein inhibits nuclear factor- κ B DNA-binding activity. Consistent with earlier reports, constitutively active NF- κ B DNA-binding activity was found in nuclear extracts from BxPC-3 as well

as L3.6pl cells, whereas very weak basal NF- κ B DNA-binding activity was detected in COLO 357 cells (Fig. 3A). The specificity of the band was confirmed by supershift using p65 antibody. Furthermore, as a corollary to previous reports from our laboratory indicating the potential of genistein to abrogate constitutive and inducible NF- κ B in prostate cancer cells, we analyzed whether genistein can abrogate the basal constitutively active NF- κ B in these cells. To evaluate the effect of genistein in COLO 357 and L3.6pl cells, semiconfluent cells were treated with varying concentrations (0, 10, 25, and 50 μ mol/L) of genistein for 72 hours. As shown in Fig. 3B, incubation with 50 μ mol/L genistein for 72 hours resulted in a decrease in NF- κ B DNA-binding activity in L3.6pl cells. These results are consistent with our findings using BxPC-3 cells as reported earlier (4), which clearly suggest that genistein is effective in down-regulating NF- κ B DNA-binding activity. We found no alterations in the nuclear protein content of retinoblastoma, which was used as protein loading control.

Genistein abrogates nuclear factor- κ B activation induced by gemcitabine. Next, we analyzed whether gemcitabine could induce NF- κ B DNA-binding activity and whether inactivation of NF- κ B by genistein could abrogate chemoresistant phenotype of COLO 357 and L3.6pl cells, resulting in more pronounced gemcitabine-induced apoptosis. First, we analyzed dose and time response to gemcitabine in the induction of NF- κ B in COLO 357 cells. For this, nuclear extracts were prepared from COLO 357 cells treated with incremental doses of gemcitabine for up to 6 hours and analyzed for NF- κ B DNA-binding activity by EMSA. Our results showed that relative to untreated control, gemcitabine (25 nmol/L) treatment induced NF- κ B DNA-binding activity as early as 4 hours in COLO 357 cells (Fig. 3C). Furthermore, pretreatment of cells with

30 μ mol/L genistein for 48 hours abrogated gemcitabine-induced activation of NF- κ B DNA-binding activity (Fig. 3D, left). Under similar assay conditions, we also assessed the NF- κ B activation in L3.6pl pancreatic cancer cells expressing constitutively active NF- κ B DNA-binding activity in response to gemcitabine. A dose escalation study with gemcitabine revealed modest up-regulation of constitutive NF- κ B DNA-binding activity at 100 nmol/L concentration of the drug after 48 hours of treatment. Thus, a low concentration of gemcitabine may not be sufficient to effectively up regulate NF- κ B from its basal level in this cell line. Moreover, we tested whether pretreatment of L3.6pl cells with 30 μ mol/L genistein for 48 hours could abrogate gemcitabine-induced NF- κ B DNA-binding activity. Our results showed that gemcitabine-induced NF- κ B activity could be inhibited by genistein pretreatment in L3.6pl cells (Fig. 3D, right). These results show that genistein not only down-regulates NF- κ B DNA-binding activity in unstimulated conditions but also inhibits gemcitabine-induced NF- κ B activity, which could be the molecular mechanism of gemcitabine-induced cell death in genistein-pretreated cells. We found similar results in BxPC-3 cells (Fig. 4A and B). These observations provide strong evidence that gemcitabine induced NF- κ B, and its down-regulation by genistein could be a common phenomenon in pancreatic cancer cells.

Genistein augments apoptosis signaling by gemcitabine in COLO 357 and L3.6pl cells. In an attempt to explore the mechanism of enhanced apoptotic process induced by treatment of cells with genistein and gemcitabine, we assessed the levels of caspase-3, PARP, antiapoptotic Bcl-2, Bcl-xL, and changes in the status of Akt phosphorylation in COLO 357 and L3.6pl cells (Fig. 5). Initial experiments were done to determine the optimal treatment schedule and dose of individual agents. Cells were pretreated with genistein (30 μ mol/L) for 48 hours before addition of 25 nmol/L (for COLO 357) or 100 nmol/L (for L3.6pl) gemcitabine for 24 hours. Our results showed that whereas treatment of these cells with gemcitabine alone had no change in procaspase-3 level, their combination with genistein pretreatment substantially reduced the expression level of procaspase-3 as determined by Western immunoblotting.

PARP is a substrate for caspase activity and a reliable marker of apoptosis. Although each of the agents by themselves showed no PARP cleavage in COLO 357 and L3.6pl cells to a detectable level, combination of gemcitabine with genistein resulted in PARP cleavage. Furthermore, based on our results showing strong caspase activity and PARP cleavage by genistein and gemcitabine combination, we assessed cytochrome *c* release into cytosol by immunoblotting mitochondrial extract and mitochondria-free cytosolic extract. A significant decrease in the mitochondrial cytochrome *c* level in the combination treatment was observed compared with each agent alone (Fig. 5B). Results for antiapoptotic Bcl-2 and Bcl-xL proteins also showed down-regulation in the combination group relative to single-agent treatment and untreated control. Analysis for p-Akt (Ser⁴⁷³) revealed an up-regulation by gemcitabine treatment that was down-regulated by genistein pretreatment and evident in both the cell types. These data provide insight into mechanistic basis of chemosensitization by genistein pretreatment.

Genistein augments *in vivo* therapeutic effect of gemcitabine on primary tumor. Two sets of independent experiments were done to investigate the therapeutic utility of genistein and gemcitabine combination in SCID mice bearing orthotopically implanted pancreatic tumor cells, COLO 357 and L3.6pl. A dose of 1 mg genistein/d/mouse was selected for oral administration,

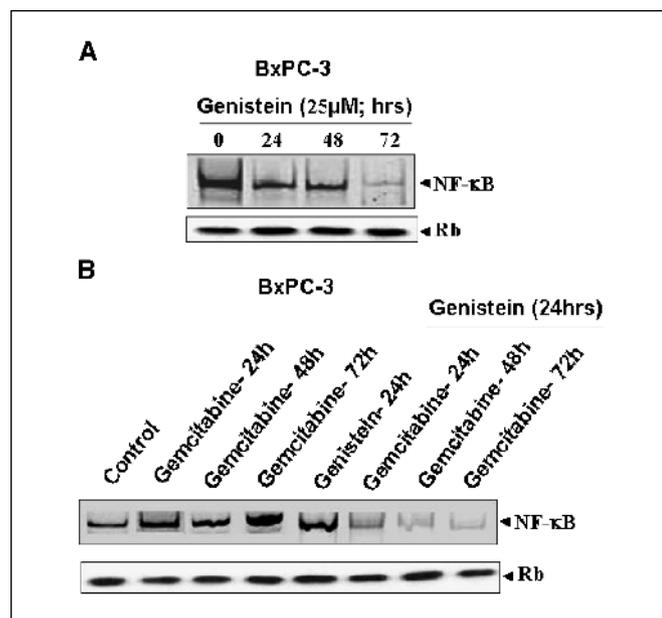


Figure 4. A, genistein inhibits constitutively active NF- κ B in BxPC-3 cells. BxPC-3 cells were treated with 25 μ mol/L genistein for indicated time and nuclear extracts were probed for NF- κ B binding to DNA consensus sequence. Results of NF- κ B DNA binding activity by EMSA. B, genistein abrogates gemcitabine induced NF- κ B in BxPC-3 cells exposed to 25 μ mol/L genistein for 24 hours followed by incubation with 100 nmol/L gemcitabine. Nuclear extracts were prepared from cells harvested at different time points and analyzed by EMSA. Genistein pretreatment down-regulated gemcitabine-induced NF- κ B as early as 24 hours posttreatment with 100 nmol/L gemcitabine. Retinoblastoma protein in the nuclear extract was used as a loading control.

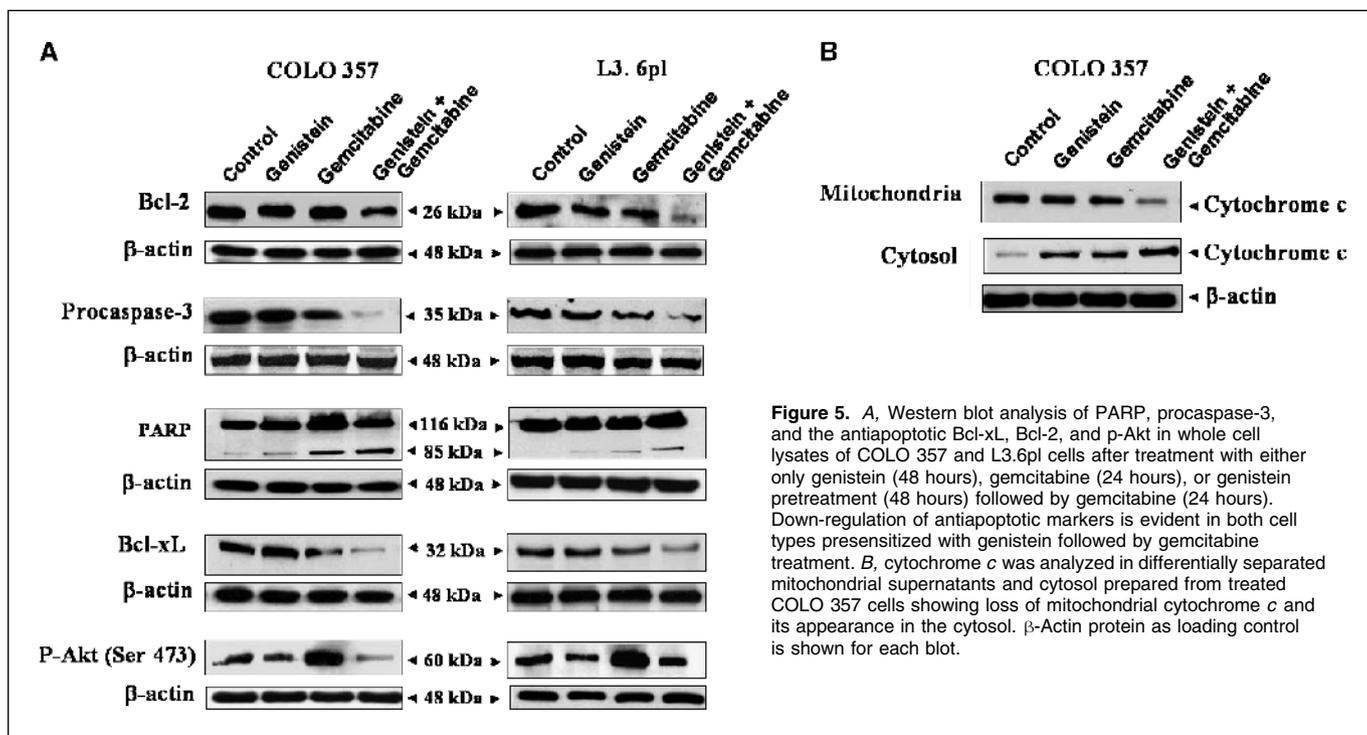


Figure 5. A, Western blot analysis of PARP, procaspase-3, and the antiapoptotic Bcl-xL, Bcl-2, and p-Akt in whole cell lysates of COLO 357 and L3.6pl cells after treatment with either only genistein (48 hours), gemcitabine (24 hours), or genistein pretreatment (48 hours) followed by gemcitabine (24 hours). Down-regulation of antiapoptotic markers is evident in both cell types presensitized with genistein followed by gemcitabine treatment. B, cytochrome c was analyzed in differentially separated mitochondrial supernatants and cytosol prepared from treated COLO 357 cells showing loss of mitochondrial cytochrome c and its appearance in the cytosol. β -Actin protein as loading control is shown for each blot.

whereas gemcitabine dose was based on previously published reports (80 mg/kg body weight, i.v., and every other day for a total of three injections). For *in vivo* experiment, 28 mice were divided into four groups as described in Materials and Methods. In an effort to establish the efficacy of a single-agent treatment compared with combinations, we determined the mean tumor weight in all treated groups. In both set of experiments, administration of genistein by gavage treatment resulted in nonsignificant reduction in the mean tumor weight compared with placebo treatment after 13 days as described in Materials and Methods. Genistein treatment did not cause any weight loss of the animals, suggesting that genistein does not induce any deleterious effect under the present experimental conditions. Analysis of the average pancreas weight in the different treatment groups showed that single modality treatment with either genistein or gemcitabine alone in mice harboring COLO 357 cells caused 13% and 27% reduction in tumor weight, respectively, compared with control tumors (Fig. 6B). Under identical experimental conditions, combination of genistein and gemcitabine treatment showed significant decrease (75%, $P < 0.01$) in tumor weight compared with untreated control, genistein alone-, or gemcitabine alone-treated group. To further investigate the therapeutic potential of genistein and gemcitabine combination in metastatic pancreatic cancer cell line L3.6pl, a second set of experiments was done including the same treatment groups as used in the first set of experiments. The profile of pancreas weight harvested at termination of the experiment revealed identical trends as the former set of experiments, showing that combination of genistein and gemcitabine was significantly better in lowering total pancreas weight than either no treatment or only genistein or gemcitabine treatment (Fig. 6B). Under the current experimental conditions, we did not observe any metastasis in the liver when examined at autopsy, suggesting that the number of cells implanted and the time course was not sufficient to allow metastatic tumor growth.

Nevertheless, our study documents, for the first time, that combination of genistein and gemcitabine can be given safely to inhibit pancreatic tumor growth.

Nuclear factor- κ B DNA-binding activity *in vivo*. We subsequently asked the most important question whether treatment of animals with genistein, gemcitabine, or their combination could effectively target a specific signaling molecule such as NF- κ B in tumor tissues. Our results clearly show that NF- κ B was down-regulated by genistein, but most importantly, that the gemcitabine-induced activation of NF- κ B DNA-binding activity was abrogated when gemcitabine was given together with genistein (Fig. 6C). These *in vivo* results were similar to our *in vitro* findings, suggesting that the inactivation of NF- κ B is at least one of the molecular mechanisms by which genistein potentiates gemcitabine-induced antitumor activity in our experimental animal model.

Discussion

Despite rapid advances in diagnostic and operative techniques, pancreatic cancer remains one of the most difficult human malignancies to treat, which is partly due to the advanced stage of the disease and *de novo* chemoresistant behavior to cytotoxic chemotherapeutic agents and/or radiotherapy. In recent years, this problem has been addressed by combinatorial approach. Several randomized studies have shown significant increase in patient response rate by the use of combinations of different class of chemotherapeutic agents, but the major problem is due to treatment associated high toxicity with no added benefit in significant overall survival (32–34). However, these limitations could be overcome by the use of rational chemotherapeutic combinations, in which toxic agents are used in lower doses, and the efficacy of treatment is complemented by using a nontoxic agent that has a different mechanism of action. Based on this rationale, in the present study, we used genistein, a nontoxic

flavonoid compound, in combination with a commonly used chemotherapeutic agent, gemcitabine, to test its efficacy against two isogenic pancreatic cancer cell lines differing in their metastatic potential. This preclinical study documents that sensitization of cancer cells could be achieved by genistein during gemcitabine-induced killing, as shown by more pronounced cell death compared with single-agent treatment, and this effect was independent of metastatic behavior of pancreatic cancer cells.

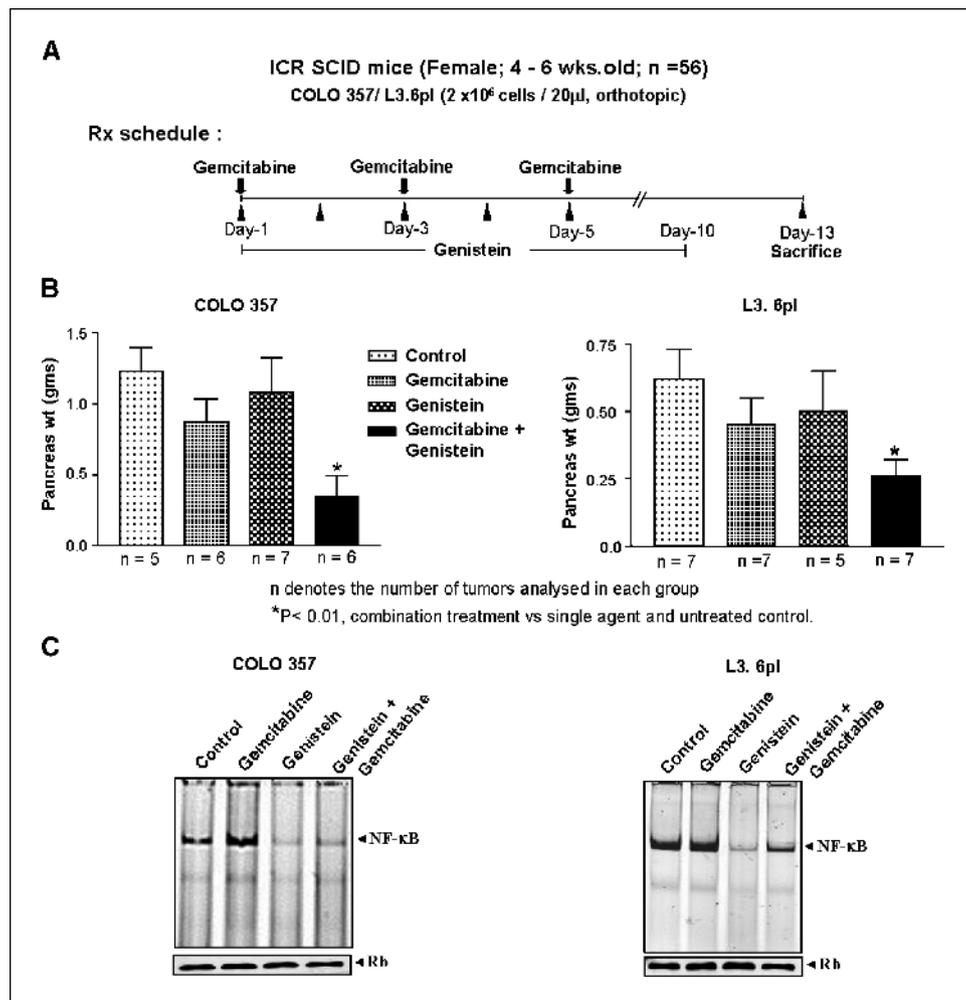
Genistein has been shown to synergize with cisplatin and docetaxel, documented by enhanced antiproliferative effect on BxPC-3 pancreatic cancer cells (4). We have recently shown that genistein has a strong modulatory effect on a number of crucial mitogenic pathways in human prostate cancer cells and is effective in inhibiting tumor xenograft in nude mice (6, 11). In this study, we found that genistein pretreatment enhanced significant tumor cell killing compared with either agents alone. This observation is of high significance because 60% to 80% growth inhibition could be achieved using the same doses of gemcitabine that produce only 25% to 30% growth inhibition when used alone. Inhibition of cell growth was also correlated with apoptotic cell death.

Gemcitabine-induced apoptosis in cells pretreated with genistein was mediated by caspase pathway as evidenced by DNA fragmentation and PARP cleavage, the latter designated as sensitive marker of caspase-induced apoptosis. In fact, many cancer-preventive and chemotherapeutic agents have been shown

to activate the apoptosome pathway that involves the release of cytochrome *c* from mitochondria, which then oligomerizes with procaspase-9 leading to formation of active caspase-9 that activates downstream executioner caspases such as caspases-3 and caspases-7 (35). In accordance with these reports, our results are consistent showing decreased levels of procaspase-3 and cleavage of the PARP concomitant with a decrease in mitochondrial cytochrome *c*. Furthermore, pretreatment of cells with caspase inhibitor totally abolished PARP cleavage, further supporting the role of caspase activation during gemcitabine-induced apoptosis of pancreatic cancer cells. Moreover, antiapoptotic molecules, such as Bcl-2, Bcl-xL, and p-Akt, were substantially down-regulated by pretreatment with genistein followed by gemcitabine treatment, and the inactivation of these molecules could be due to inactivation of NF- κ B during sensitization of pancreatic cancer cells to gemcitabine-induced killing. Together, these observations suggest that genistein strongly sensitizes pancreatic cancer cells to gemcitabine-induced apoptosis.

Our results also showed that gemcitabine alone could activate NF- κ B, resulting in reduced apoptosis supporting the notion that NF- κ B activation inhibits apoptosis. We also found that genistein inhibits NF- κ B in COLO cells as well as in other cancer cells (25, 29). In addition, our *in vitro* results showed that genistein alone or genistein pretreatment followed by gemcitabine treatment abrogates NF- κ B activation and increases apoptotic index, suggesting

Figure 6. A, flow chart representation of *in vivo* experimental design and treatment schedule. B, changes in the isolated pancreatic tumor weight revealing *in vivo* therapeutic efficacy of genistein and gemcitabine treatment as deduced from pancreas weight. Student's *t* test was used to explore the statistical significance between the data sets. C, gel shift assay for NF- κ B done on randomly selected tumor tissues obtained from each treatment groups of animals. Bottom, loading control by Western immunoblotting of retinoblastoma protein in the nuclear extract.



that inhibition of NF- κ B by genistein is mechanistically associated with sensitization of pancreatic cancer cells to apoptotic cell death. Interestingly, these *in vitro* results (such as antitumor activity and inactivation of NF- κ B) were recapitulated *in vivo* using the orthotopic mouse model which provides a scientific rationale for therapeutic exploitation of our strategy for the treatment of patients with pancreatic cancer. Collectively, these results provide strong molecular evidence in support of our hypothesis that genistein pretreatment could be useful to sensitize pancreatic cancer cells to gemcitabine-induced killing. These results are also relevant in the context of clinical course of pancreatic adenocarcinoma, which is characterized by an early propensity to metastasize and a high risk for disease recurrence following resection. The remarkable ability of genistein and gemcitabine within a therapeutic range to induce apoptosis opens new and novel avenues by which our strategy could provide therapeutic benefit for patients diagnosed with unresectable pancreatic adenocarcinoma as well as for those patients who have undergone resection but are at a high risk for disease recurrence. It is of interest to note that a report by Busby et al. (36) showed that up to

27 μ mol/L of genistein in human plasma could be achievable after genistein supplementation at a dose of 16 mg/kg, suggesting the bioavailability of genistein from supplements.

In conclusion, our current findings are consistent with the hypothesis that it is possible to enhance chemosensitivity of pancreatic cancer cells by pretreatment with genistein, which is mediated by inactivation of NF- κ B DNA-binding activity leading to apoptotic cell death. However, further mechanistic studies could be useful to fully support our strategy for the treatment of patients with pancreatic tumors.

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Molecular Evidence for Increased Antitumor Activity of Gemcitabine by Genistein *In vitro* and *In vivo* Using an Orthotopic Model of Pancreatic Cancer

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