

Inactivation of Nuclear Factor κ B by Soy Isoflavone Genistein Contributes to Increased Apoptosis Induced by Chemotherapeutic Agents in Human Cancer Cells

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Abstract

Cancer chemotherapeutic strategies commonly require multiple agents. However, use of multiple agents contributes to added toxicity resulting in poor treatment outcome. Thus, combination chemotherapy must be optimized to increase tumor response and at the same time lower its toxicity. Chemotherapeutic agents are known to induce nuclear factor κ B (NF- κ B) activity in tumor cells, resulting in lower cell killing and drug resistance. In contrast, genistein has been shown to inhibit the activity of NF- κ B and the growth of various cancer cells without causing systemic toxicity. We therefore investigated whether the inactivation of NF- κ B by genistein before treatment of various cancer cells with chemotherapeutic agents could lead to better tumor cell killing as tested by *in vitro* studies using gene transfections and also by animal studies. PC-3 (prostate), MDA-MB-231 (breast), H460 (lung), and BxPC-3 (pancreas) cancer cells were pretreated with 15 to 30 μ mol/L genistein for 24 hours and then exposed to low doses of chemotherapeutic agents for an additional 48 to 72 hours. We found that 15 to 30 μ mol/L genistein combined with 100 to 500 nmol/L cisplatin, 0.5 to 2 nmol/L docetaxel, or 50 ng/mL doxorubicin resulted in significantly greater inhibition of cell growth and induction of apoptosis compared with either agent alone. Moreover, we found that the NF- κ B activity was significantly increased within 2 hours of cisplatin and docetaxel treatment and that the NF- κ B inducing activity of these agents was completely abrogated in cells pretreated with genistein. These results were also supported, for the first time, by animal experiments, p65 cDNA transfection and p65 small interfering RNA studies, which clearly showed that a specific target (NF- κ B) was affected *in vivo*. Collectively, our results clearly suggest that genistein pretreatment inactivates NF- κ B and may contribute to increased growth inhibition and apoptosis induced by cisplatin, docetaxel, and doxorubicin in prostate, breast, lung, and pancreatic cancer cells. These results warrant carefully designed clinical studies investigating the combination of soy isoflavones and commonly used chemotherapeutic agents for the treatment of human cancers. (Cancer Res 2005; 65(15): 6934-42)

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Introduction

Cancer chemotherapeutic strategies commonly require multiple agents. However, use of multiple agents contributes to added toxicity requiring dose reduction, which may result in poor treatment outcome. *De novo* and acquired resistance to chemotherapeutic agents and the toxicity to normal cells are the major causes of treatment failure in most solid tumors (1). To overcome such problems, strategies must be devised for better cancer cell killing and lower systemic toxicity for combination chemotherapy. Therefore, there is a tremendous need for the development of optimal mechanism-based and targeted therapeutic strategies for human cancers to alleviate treatment failure.

Among chemotherapeutic agents, cisplatin (Platinol), docetaxel (Taxotere), and doxorubicin (Adriamycin) have been frequently used for treatment of cancers including prostate, breast, lung, and pancreatic cancers, alone or in combination with other agents (2–8). Several clinical trials have reported that these agents, used in combination with other drugs, show improved outcomes in objective response rates and survival (2, 8–10). However, results have also been reported showing considerable toxicity or no advantage for the combination of these agents in terms of response rate, clinical benefit, and survival (11, 12). The drug resistance acquired by cancer cells has been contributed to treatment failure. It has been reported that some chemotherapeutic agents including cisplatin, docetaxel, and doxorubicin induce the activation of nuclear factor κ B (NF- κ B) in cancer cells and this has been believed to be responsible in part for drug resistance in cancer cells (13–15). NF- κ B plays important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiologic processes. NF- κ B mediates survival signals that inhibit apoptosis and promote cancer cell growth. It has been found that inhibition of NF- κ B activation in tumor cells may increase the efficacy of topoisomerase II inhibitors as a chemotherapeutic agent (16). Therefore, targeted inactivation of NF- κ B without systemic toxicity in combination with chemotherapeutic agents may lead to better tumor cell killing in human cancers.

Genistein, a predominant isoflavone found in soybeans, has been shown to inhibit the growth of various cancer cells *in vitro* and *in vivo* without toxicity to normal cells (17–19). We have previously shown that genistein regulates genes that are involved in the control of cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, angiogenesis, and cancer cell invasion and metastasis (20). Moreover, we have also shown that genistein inhibits activities of NF- κ B and Akt, resulting in the inhibition of cancer cell growth and the induction of apoptosis (21, 22). Because chemotherapeutic

agents are known to induce NF- κ B activation that leads to chemoresistance, we hypothesized that the inactivation of NF- κ B by genistein before treatment of cells with chemotherapeutic agents would sensitize cancer cells to apoptosis, resulting in better cancer cell killing. In this study, we tested our hypothesis by evaluating the effects of genistein and three chemotherapeutic agents on cell growth, apoptosis, and NF- κ B activation in cells from four different types of human cancers, which are among the most common and leading causes of cancer death in the United States.

Materials and Methods

Cell culture and reagents. PC-3 human prostate cancer cells [American Type Culture Collection (ATCC), Manassas, VA], H460 human lung cancer cells (ATCC), and BxPC-3 human pancreatic cancer cells (ATCC) were

cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a 5% CO₂ atmosphere at 37°C. MDA-MB-231 human breast cancer cells (ATCC) were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. Genistein (Toronto Research Chemicals, North York, Ontario, Canada) was dissolved in 0.1 mol/L Na₂CO₃ to make a 10 mmol/L stock solution. Docetaxel (Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in DMSO to make a 4 μ mol/L stock solution. Cisplatin (Sigma, St. Louis, MO) was dissolved in PBS to make a 100 μ mol/L stock solution. Doxorubicin (Sigma) was dissolved in PBS to make a 100 μ g/mL stock solution.

Cell growth inhibition by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Four cancer cell lines were seeded in 96-well culture dishes. After 24 hours of incubation, PC-3, MDA-MB-231, and BxPC-3 cells were treated with 15 or 30 μ mol/L genistein for 24 hours and then exposed to chemotherapeutic agents (1 nmol/L docetaxel, 100 nmol/L

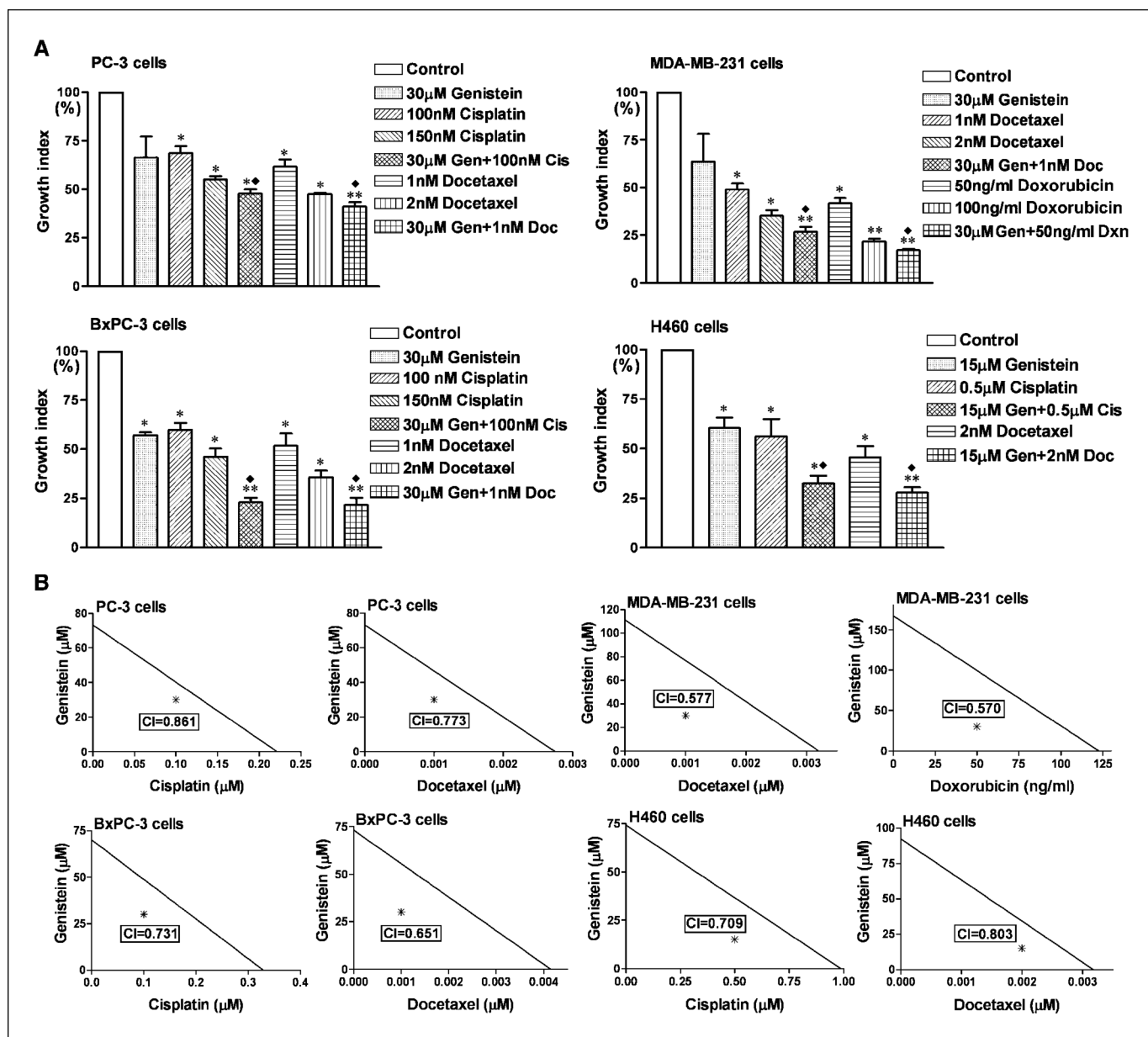


Figure 1. A, inhibition of cancer cell growth tested by MTT assay (*, $P < 0.05$, compared with control; **, $P < 0.01$, compared with control; \blacklozenge , $P < 0.05$, compared with monotherapy; $n = 3$). B, isobologram plots for combination treatments with genistein and chemotherapeutic agents in the doses shown in (A). CI, combination index.

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cisplatin, or 50 ng/mL doxorubicin) for additional 48 hours. H460 cells were treated with 15 or 30 $\mu\text{mol/L}$ genistein for 24 hours and then exposed to 2 nmol/L docetaxel or 500 nmol/L cisplatin for additional 72 hours. For single-agent treatment, PC-3, MDA-MB-231, BxPC-3, and H460 cells were treated with genistein (15, 30, or 50 $\mu\text{mol/L}$), docetaxel (0.5, 1 or 2 nmol/L), cisplatin (50, 100, 150, or 500 nmol/L), or doxorubicin (25, 50, or 100 ng/mL) alone for 72 to 96 hours. It is important to note that different concentrations of various reagents were used in different cell lines because of their relative sensitivity or resistance to the reagents tested. After treatment, cancer cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL, Sigma) at 37°C for 2 hours and then with isopropyl alcohol at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined by ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 595 nm. Combination index and isobologram for combination treatment were calculated and plotted using CalcuSyn software (Biosoft, Ferguson, MO).

Histone/DNA ELISA for detecting apoptosis. Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis according to the protocol of the manufacturer. Briefly, PC-3, MDA-MB-231, BxPC-3, and H460 cells were treated with genistein and/or chemotherapeutic agents (docetaxel, cisplatin, or doxorubicin) as described above. After treatment, the cytoplasmic histone/DNA fragments from cancer 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 by ULTRA Multifunctional Microplate Reader (TECAN) at 405 nm.

DNA ladder analysis for detecting apoptosis. PC-3 and BxPC-3 cells were treated with 30 $\mu\text{mol/L}$ genistein for 24 hours and then exposed to chemotherapeutic agents (1 nmol/L docetaxel or 100 nmol/L cisplatin) for additional 48 hours. For single-agent treatment, PC-3 and BxPC-3 cells were treated with 50 $\mu\text{mol/L}$ genistein, 2 nmol/L docetaxel, or 150 nmol/L cisplatin alone for 72 hours. After treatment, cellular cytoplasmic DNA from PC-3 and BxPC-3 cells with different treatments was extracted and the DNA ladder was visualized as described previously (22).

Nuclear factor κB DNA-binding activity measurement. PC-3, MDA-MB-231, BxPC-3, and H460 cells were plated at a density of 1×10^5 cells in 100 mm dishes and incubated. After 24 hours, the cells were treated with combination of genistein and chemotherapeutic agents as described above. For single-agent treatment, cancer cells were treated with genistein (30 or 50 $\mu\text{mol/L}$), 2 nmol/L docetaxel, or 200 nmol/L cisplatin alone for 2 hours. For positive control, the cells were treated with tumor necrosis factor- α (TNF- α ; 30 ng/mL) for 30 minutes. Following treatments, nuclear protein in

the cells was extracted as described previously (22). For electrophoretic mobility shift assay (EMSA), 10 μg of nuclear protein were assembled with $5 \times$ Gel Shift Binding buffer (20% glycerol, 5 mmol/L MgCl_2 , 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl), 0.25 mg/mL poly(dI)-poly(dC), and IRDye 700-labeled NF- κB oligonucleotide (LI-COR, Lincoln, NE). After incubation at room temperature for 30 minutes, the samples were loaded on a pre-run 8% polyacrylamide gel and electrophoresis was continued at 30 mA for 90 minutes. The signal was then detected and quantified with Odyssey infrared imaging system (LI-COR). Supershift assay using NF- κB p65 antibody and competition assay using unlabeled specific competitor (NF- κB oligo) were also conducted to confirm the specificity of NF- κB DNA-binding activity. For loading control, 10 μg of nuclear proteins from each sample were subjected to Western blot analysis for retinoblastoma protein, which showed no alternation after genistein or docetaxel treatment.

p65 cDNA transfection. PC-3 cells were seeded in a six-well plate (1.5×10^5 cells per well) and incubated at 37°C for 24 hours. Then, the cells were transfected with NF- κB p65 cDNA or control empty vector by ExGen 500 (Fermentas, Hanover, MD). After 5 hours, the p65 cDNA-transfected cells were treated with 30 to 50 $\mu\text{mol/L}$ genistein or left untreated. After 48 hours of incubation, the cells were treated with 2 nmol/L docetaxel for 2 to 24 hours. Then, the cytoplasmic proteins and nuclear proteins were extracted. p65 protein expression was detected by Western blot analysis and NF- κB DNA-binding activity was measured by EMSA. Also, the apoptotic cells in p65 cDNA-transfected cells with different treatments were detected using Cell Apoptosis ELISA Detection Kit (Roche).

p65 small interfering RNA assay. NF- κB p65 small interfering RNA (siRNA)/siAb Assay Kit (Upstate, Lake Placid, NY) was used to detect the effects of docetaxel, cisplatin, or genistein on NF- κB p65. PC-3 cells were seeded in a six-well plate (1.2×10^5 cells per well) and incubated at 37°C for 24 hours. Then, the cells were transfected with p65 siRNA or control RNA duplex by Lipofectamine 2000 (Invitrogen). After 8 hours, the siRNA-transfected cells were treated with 30 to 50 $\mu\text{mol/L}$ genistein or left untreated. After 48 hours of incubation, the cells were treated with 2 nmol/L docetaxel or 150 nmol/L cisplatin for 2 to 24 hours. Then, the total cellular proteins and nuclear proteins were extracted. p65 protein expression was detected by Western blot analysis and NF- κB DNA-binding activity was measured by EMSA. Also, the apoptotic cells in siRNA-transfected cells with different treatments were detected with Cell Apoptosis ELISA Detection Kit (Roche).

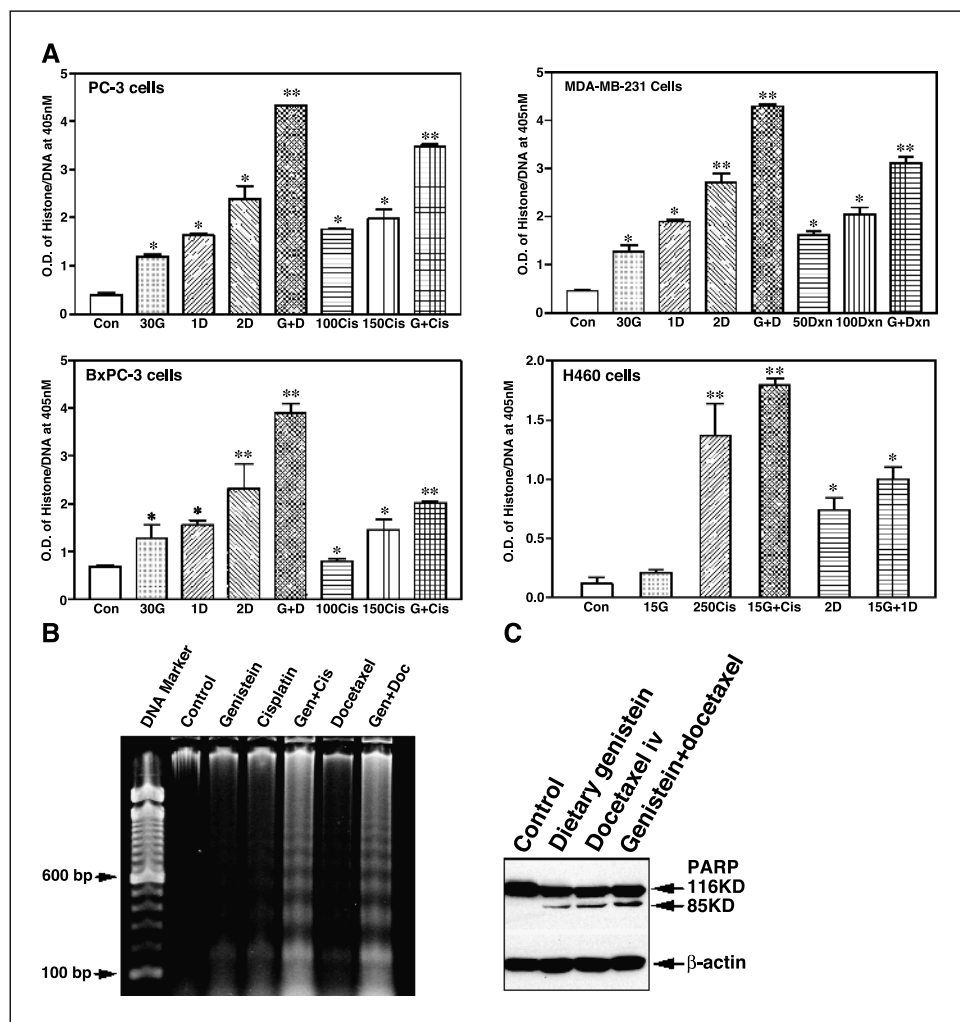
Animal studies. Male homozygous CB-17 scid/scid mice, ages 4 weeks, were purchased from Taconic Farms (Germantown, NY). The mice were maintained according to the NIH standards established in the Guidelines for the Care and Use of Experimental Animals. All experimental protocols were approved by the Animal Investigation Committee of Wayne State University (Detroit, MI). PC-3 s.c. tumors were created in SCID mice by injecting 5×10^6 PC-3 cells. The mice were given the genistein-containing diet (1 g genistein/

Table 1. Combination index values for combined treatments of genistein and chemotherapeutic agents in different cell lines

Cell line	Treatment	Combination Index Values at			
		Experimental doses	ED ₅₀	ED ₇₅	ED ₉₀
PC-3	Genistein and cisplatin (300:1)	0.861 (30 mmol/L genistein and 0.1 mmol/L cisplatin)	0.80343	0.57230	0.41106
	Genistein and docetaxel (30,000:1)	0.773 (30 mmol/L genistein and 1 nmol/L docetaxel)	0.78153	0.39857	0.21104
MDA-MB-231	Genistein and docetaxel (30,000:1)	0.577 (30 mmol/L genistein and 1 nmol/L docetaxel)	0.82201	0.45118	0.26980
	Genistein and doxorubicin (300:1)	0.57 (30 mmol/L genistein and 50 ng/mL doxorubicin)	0.79273	0.54229	0.37104
BxPC-3	Genistein and cisplatin (300:1)	0.731 (30 mmol/L genistein and 0.1 mmol/L cisplatin)	0.76083	0.60507	0.48547
	Genistein and docetaxel (30,000:1)	0.651 (30 mmol/L genistein and 1 nmol/L docetaxel)	0.86912	0.58180	0.41599
H460	Genistein and cisplatin (30:1)	0.709 (15 mmol/L genistein and 0.5 mmol/L cisplatin)	0.62426	0.61332	0.55015
	Genistein and docetaxel (7,500:1)	0.788 (15 mmol/L genistein and 2 nmol/L docetaxel)	0.66000	0.63858	0.51507

NOTE: Combination index (CI): a quantitative measure of the degree of drug interaction. CI < 1 indicates synergism; CI > 1 indicates antagonism; CI = 1 indicates additive effect.

Figure 2. A, induction of apoptosis in cancer cells tested by ELISA. (Con, control; 30G, 30 μ mol/L genistein; 1D, 1 nmol/L docetaxel; 2D, 2 nmol/L docetaxel; G + D, 30 μ mol/L genistein and 1 nmol/L docetaxel combination; 100Cis, 100 nmol/L cisplatin; 150Cis, 150 nmol/L cisplatin; G + Cis, 30 μ mol/L genistein and 100 nmol/L cisplatin combination; 50Dxn, 50 ng/mL doxorubicin; 100Dxn, 100 ng/mL doxorubicin; G + Dxn, 30 μ mol/L genistein and 50 ng/mL doxorubicin combination; 15G, 15 μ mol/L genistein; 250Cis, 250 nmol/L cisplatin; 15G + Cis, 15 μ mol/L genistein and 250 nmol/L cisplatin combination; 15G + 1D, 15 μ mol/L genistein and 1 nmol/L docetaxel combination.) *, $P < 0.05$; **, $P < 0.01$ ($n = 3$). B, induction of apoptosis in PC-3 cells tested by DNA ladder analysis. (Genistein, 50 μ mol/L genistein; Cisplatin, 150 nmol/L cisplatin; Gen + Cis, 30 μ mol/L genistein and 100 nmol/L cisplatin combination; Docetaxel, 2 nmol/L docetaxel; Gen + Doc, 30 μ mol/L genistein and 1 nmol/L docetaxel combination.) C, PARP cleavage assay showed that combination treatment with dietary genistein and docetaxel administrated i.v. induced more apoptosis in tumor tissue *in vivo*.



kg diet) or the exact same diet (AIN76A, Purina Test Mills, Richmond, IN) but without genistein after the s.c. tumors became measurable for a total of 10 days. In addition, the mice also received three doses of docetaxel (5 mg/kg, i.v.) every 48 hours after 4 days of dietary genistein intervention. The dose of genistein selected for this experiment was based on our previous studies showing antitumor activity (23). Then, the tumors were removed. The activity of NF- κ B of tumor cells was measured by EMSA and the poly(ADP-ribose) polymerase (PARP) cleavage in tumor cells was assessed by Western blot analysis.

Western blot analysis. Twenty-five micrograms of cell extracts from p65 cDNA transfection and p65 siRNA assay or 50 μ g of tumor lysate from animal experiments were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Membranes were incubated with polyclonal anti-NF- κ B p65 (1:1,000, Upstate), anti-survivin (1:200, Santa Cruz, Santa Cruz, CA), anti-Bcl-2 (1:200, Santa Cruz), anti-Bcl- x_L (1:200, Santa Cruz), anti-p21^{WAF1} (1:500, Upstate), anti-PARP (1:5,000, Biomol, Plymouth Meeting, PA), or anti- β -actin (1:5,000, Sigma) antibodies, washed with Tween 20-TBS, and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce, Rockford, IL).

Results

Genistein potentiated cancer cell growth inhibition caused by chemotherapeutic agents. Four types of cancer cells from prostate, breast, pancreas, or lung were treated with genistein,

docetaxel, cisplatin, doxorubicin, or genistein in combination with lower doses of docetaxel, cisplatin, or doxorubicin. The cell viability was determined by MTT assay, and the effect of genistein, docetaxel, cisplatin, or doxorubicin on the growth of different cancer cells is shown in Fig. 1A. We found that treatment of cells with genistein, docetaxel, cisplatin, or doxorubicin alone for 72 hours commonly caused ~50% growth inhibition in cancer cells using the doses tested. However, genistein in combination with lower doses of docetaxel, cisplatin, or doxorubicin resulted in ~75% growth inhibition in these cancer cells. Isobologram analysis showed that the combination index for every combination treatment we did was <1 (Fig. 1B; Table 1), suggesting the synergistic effect of each combination treatment. These results showed that combination of genistein with lower doses of docetaxel, cisplatin, or doxorubicin elicited significantly greater inhibition of cancer cell growth compared with either agent alone and this phenomenon was not cancer cell type specific.

Genistein sensitized cancer cells to apoptosis induced by chemotherapeutic agents. By apoptotic cell death ELISA and DNA ladder analysis, we observed similar results showing that genistein, docetaxel, cisplatin, or doxorubicin induced apoptosis in all cancer cells tested (Fig. 2A and B). Moreover, we found that 30 μ mol/L genistein combined with lower doses of docetaxel,

cisplatin, or doxorubicin induced more apoptosis in the cancer cells compared with single-agent treatment (Fig. 2A and B). Once again, this phenomenon was not cancer cell type specific. We also observed that genistein and docetaxel combination treatment *in vivo* produced more PARP cleavages compared with monotreatment (Fig. 2C), suggesting more apoptosis induced by the combination treatment *in vivo*. By Western blot analysis, we found that genistein alone or in combination with chemotherapeutic agents up-regulated the expression of p21^{WAF1} and down-regulated the expression of survivin, Bcl-2, and Bcl-x_L in p65 cDNA-transfected or parental PC-3 cells (Fig. 3). These results are consistent with cell growth inhibition observed by MTT assay, suggesting that greater cell growth inhibition by combination treatment may be mediated through the induction of more apoptosis in cancer cells.

Docetaxel or cisplatin treatment induced p65 expression and nuclear factor κ B DNA-binding activity. Nuclear proteins from cultured cancer cells treated with docetaxel or cisplatin were subjected to analysis for NF- κ B DNA-binding activity as measured by EMSA. The results showed that 2 nmol/L docetaxel or 200 nmol/L cisplatin treatment for 2 hours significantly induced NF- κ B DNA-binding activity in cancer cells compared with the untreated cells (Fig. 4A and B). Importantly, NF- κ B p65 cDNA transfection enhanced the NF- κ B DNA-binding activity induced by docetaxel whereas NF- κ B p65 siRNA transfection abrogated the expression and activation of p65 stimulated by docetaxel or cisplatin (Figs. 4B, C and 5). These results clearly suggest that docetaxel or cisplatin induces NF- κ B DNA-binding activity through increase in the expression and activation of p65.

Genistein abrogated activation of nuclear factor κ B activity stimulated by docetaxel or cisplatin *in vitro* and *in vivo*. By EMSA, we found that genistein *in vitro* significantly inhibited NF- κ B DNA-binding activity and also abrogated docetaxel-, cisplatin-, or p65 cDNA transfection-induced NF- κ B DNA-binding activity (Fig. 4B and C). More importantly, our animal studies showed that dietary genistein also inhibited NF- κ B DNA-binding activity induced by docetaxel treatment in SCID s.c. tumors (Fig. 4D). These results show that genistein *in vitro* and *in vivo* not only reduces NF- κ B DNA-binding activity in unstimulated conditions but also inhibits NF- κ B activation induced by docetaxel or cisplatin, resulting in better cancer cell killing.

Apoptosis-enhancing effect of genistein is mediated through the nuclear factor κ B pathway. We transfected NF- κ B p65 cDNA or siRNA into PC-3 cells, treated the transfected cells with genistein, docetaxel, or cisplatin, and detected NF- κ B DNA-binding activity and apoptosis. We found that p65 cDNA transfection enhanced the NF- κ B DNA-binding activity, up-regulated the expression of survivin, Bcl-2, and Bcl-x_L, and inhibited apoptosis in genistein-treated and untreated PC-3 cells (Figs. 3, 4C, and 6B). In contrast, p65 siRNA transfection inhibited the activation of NF- κ B and enhanced apoptosis induced by docetaxel or cisplatin (Figs. 4B and 6A). Moreover, we found that genistein treatment combined with p65 siRNA transfection exerted greater inhibitory effect on the activation of NF- κ B and a more pronounced effect on the induction of apoptosis (Figs. 4B and 6A). Genistein combined with docetaxel also showed greater inhibitory effect on the expression of survivin and Bcl-2 and a more pronounced effect on the induction of apoptosis in p65 cDNA-transfected PC-3 cells compared with

docetaxel monotreatment (Figs. 3 and 6B). These results provide mechanistic support in favor of our claim that the apoptosis-inducing effect of docetaxel or cisplatin is enhanced by genistein and it is partly mediated through the NF- κ B pathway.

Discussion

Cancer is the second leading cause of death with expected 1,368,030 new cases and 563,770 deaths in the United States in 2004, exceeded only by heart disease (24). Surgery and radiotherapy usually treat about 40% of all cancer patients whereas 60% of patients are usually under chemotherapy (25). However, chemotherapy has been known to induce drug resistance in cancer cells, resulting in treatment failure (26). Two of the major culprits involved in the development of drug resistance are *MDR* (multidrug resistance gene) and NF- κ B (15, 26, 27). It has been reported that increase in the resistance of human cervical carcinoma cells to cisplatin is partly mediated via enhancement of cisplatin-induced NF- κ B activation (27). Cancer

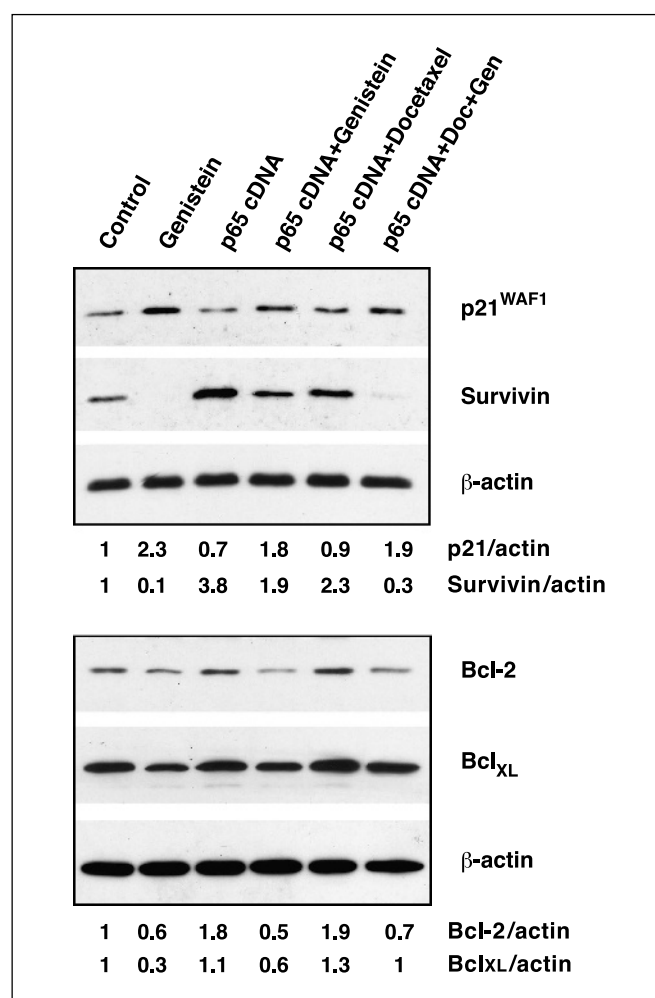
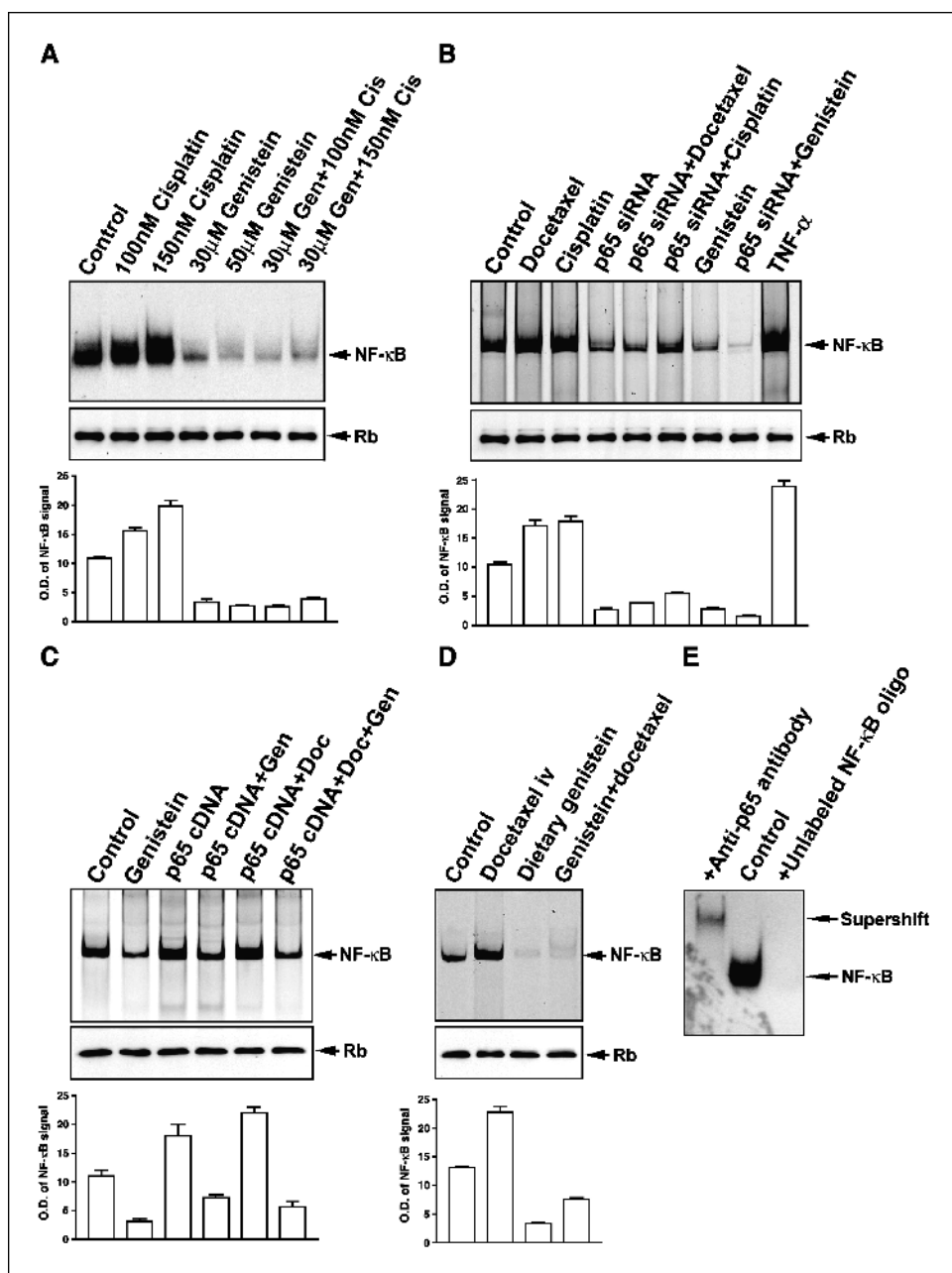


Figure 3. Western blot analysis for p21^{WAF1}, survivin, Bcl-2, and Bcl-x_L in NF- κ B p65 cDNA transfected and parental PC-3 cells. (Genistein, 50 μ mol/L genistein; p65 cDNA, p65 cDNA transfection; p65 cDNA + Genistein, p65 cDNA transfection and 50 μ mol/L genistein; p65 cDNA + Docetaxel, p65 cDNA transfection and 2 nmol/L docetaxel; Doc, 2 nmol/L docetaxel; Gen, 50 μ mol/L genistein.) The signal of each protein expression was quantified with Gel Doc 1000 image system (Bio-Rad, Hercules, CA). The ratios of p21^{WAF1}, survivin, Bcl-2, or Bcl-x_L against β -actin were calculated and shown.

Figure 4. NF- κ B DNA-binding activity in BxPC-3 cells (A), PC-3 cells (B and C), and SCID PC-3 s.c. tumors (D) tested by EMSA. (Docetaxel, 2 nmol/L docetaxel; Cisplatin, 150 nmol/L cisplatin; p65 siRNA, 100 nmol/L p65 siRNA; Genistein, 50 μ mol/L genistein; p65 cDNA transfection; Gen, 50 μ mol/L genistein; Doc, 2 nmol/L docetaxel; Doc + Gen: 2 nmol/L docetaxel and 50 μ mol/L genistein; TNF- α , 30 ng/mL TNF- α .) E, supershift and competitive assays. Supershift assay showed that NF- κ B band was shifted because of the formation of bigger complex after addition of anti-NF- κ B p65 antibody. Competitive assay showed no NF- κ B band because of competition with excess unlabeled NF- κ B specific oligonucleotide. Both assays confirmed the specificity of NF- κ B binding to the DNA consensus sequence.



cells without NF- κ B activity exhibit greater sensitivity to TNF- α and chemotherapeutic agents (28, 29). Chawla-Sarkar et al. (28) found that inhibition of NF- κ B potentiated the anticancer effect of chemotherapeutic agents. In the present study, we found that docetaxel or cisplatin treatment, like NF- κ B cDNA transfection, increased the expression of NF- κ B p65 and significantly induced NF- κ B DNA-binding activity in cancer cells, suggesting that the activation of NF- κ B by these chemotherapeutic agents may contribute to the resistance of cancer cells to these agents. More importantly, we found that genistein and p65 siRNA exerted inhibitory effects on p65 expression and NF- κ B DNA-binding activity to a similar degree. Both of them also abrogated the activation of NF- κ B stimulated by chemotherapeutic agents, suggesting that genistein may reduce cancer cell resistance to chemotherapeutic agents through inactivation of p65 expression

and NF- κ B DNA-binding activity. These results are consistent with our hypothesis and provide additional molecular explanation as previously postulated in our earlier publication (30).

Inhibition of cell proliferation and induction of apoptotic cell death are two major mechanisms by which chemotherapeutic agents kill cancer cells. To determine whether the down-regulation of NF- κ B by genistein could lead to sensitization of cancer cells to chemotherapeutic agents, we conducted cell growth inhibition and apoptosis assays. We found that docetaxel, cisplatin, or doxorubicin as a single agent inhibited the growth of cancer cells and induced apoptosis. However, pretreatment of cancer cells with genistein followed by docetaxel, cisplatin, or doxorubicin treatment resulted in significantly greater inhibition of cancer cell growth and induction of apoptosis, suggesting that the inactivation of NF- κ B by genistein before chemotherapy enhances the antiproliferative

and proapoptotic effects of chemotherapeutic agents. Docetaxel, cisplatin, and doxorubicin all have been shown to exert significant cell killing activity in a variety of cancer cells through induction of apoptosis (31–33). It has been well known that Bcl-2, Bcl-x_L, and survivin protect cells from apoptosis whereas p21^{WAF1} inhibits cell growth and induces apoptosis (34–36). We and others have reported that genistein down-regulates NF-κB, Bcl-2, Bcl-x_L, and survivin and up-regulates p21^{WAF1} (19, 37, 38) and that NF-κB binding sites were found in the promoter of Bcl-2, Bcl-x_L, and survivin (39–41), suggesting that genistein might inhibit the expression of Bcl-2, Bcl-x_L, and survivin through down-regulation of NF-κB. In the present study, our results showed that genistein pretreatment inhibited NF-κB activity, regulated the expression of apoptosis related genes (p21^{WAF1}, survivin, Bcl-2, and Bcl-x_L), and sensitized cancer cells to apoptosis induced by docetaxel, cisplatin, or doxorubicin. These results were consistent with more significant inhibition of cell growth as observed by MTT assay, suggesting that down-regulation

of NF-κB by genistein pretreatment is partly responsible for sensitization of cancer cells to chemotherapeutic agents. However, it is conceivable that other mechanisms may also contribute to this phenomenon of chemosensitization induced by genistein.

To investigate whether the enhanced cell growth inhibition and apoptosis by genistein was mediated through the NF-κB pathway, we conducted NF-κB cDNA transfection and NF-κB p65 siRNA assay. We found that docetaxel and cisplatin, functioning similarly as p65 cDNA transfection, induced the expression and activity of NF-κB. However, NF-κB p65 siRNA was functioning similarly as genistein, which inhibited NF-κB expression and NF-κB DNA-binding activity. Moreover, genistein treatment combined with p65 siRNA transfection exerted greater inhibitory effect on the activation of NF-κB and more enhanced effect on the induction of apoptosis, suggesting that genistein-enhanced cell growth inhibition and apoptosis is mediated through the NF-κB pathway. Importantly, we also found that genistein could abrogate the activation of NF-κB

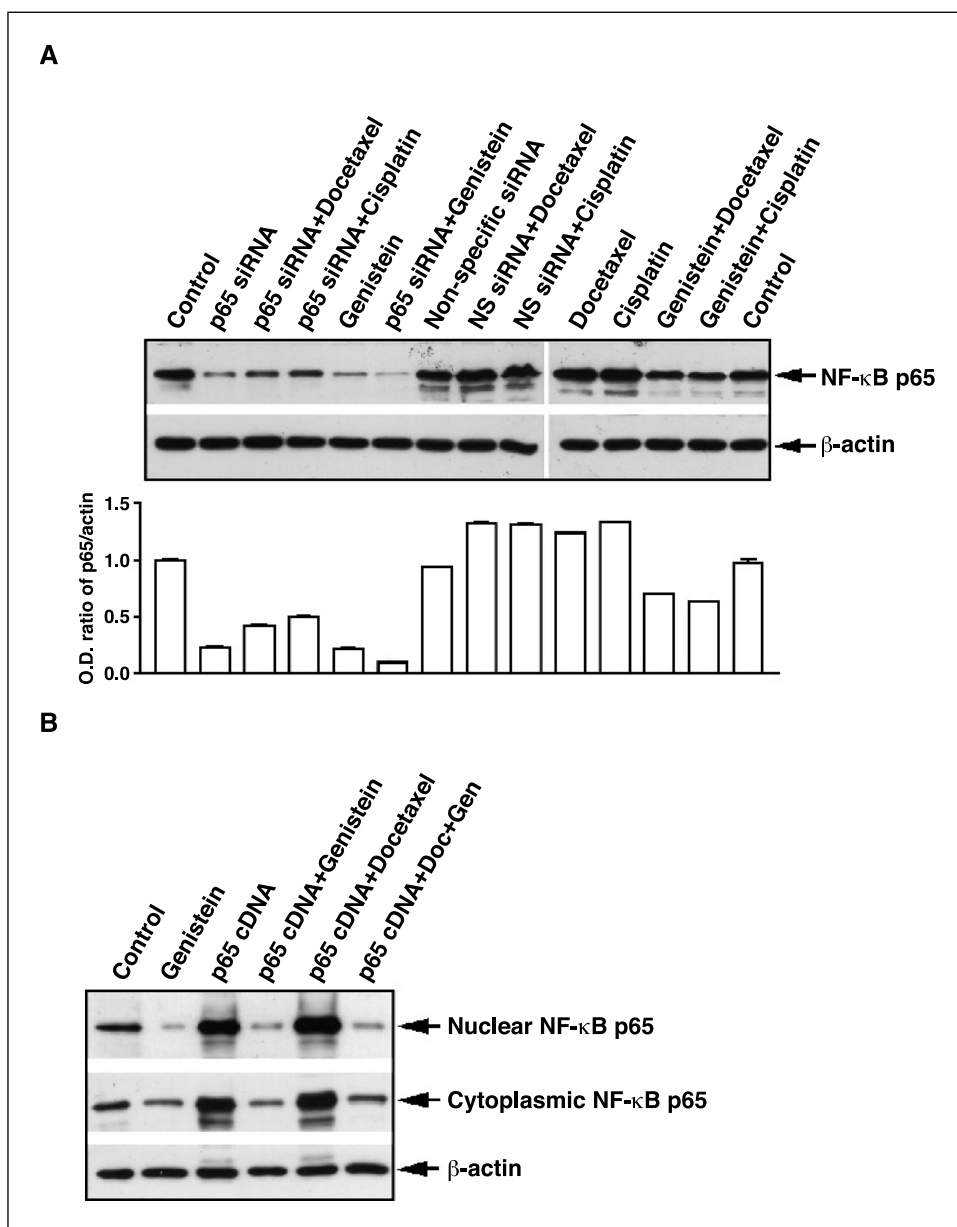


Figure 5. A, Western blot analysis for total cellular NF-κB p65 in PC-3 cells. (p65 siRNA, 100 nmol/L p65 siRNA; Docetaxel, 2 nmol/L docetaxel; Cisplatin, 150 nmol/L cisplatin; Genistein, 50 μmol/L genistein; NS siRNA, 100 nmol/L nonspecific siRNA.) B, Western blot analysis for nuclear and cytoplasmic NF-κB p65 in p65 cDNA-transfected PC-3 cells (Genistein, 50 μmol/L genistein; p65 cDNA, p65 cDNA transfection; Docetaxel, 2 nmol/L docetaxel; Doc, docetaxel; Gen, genistein).

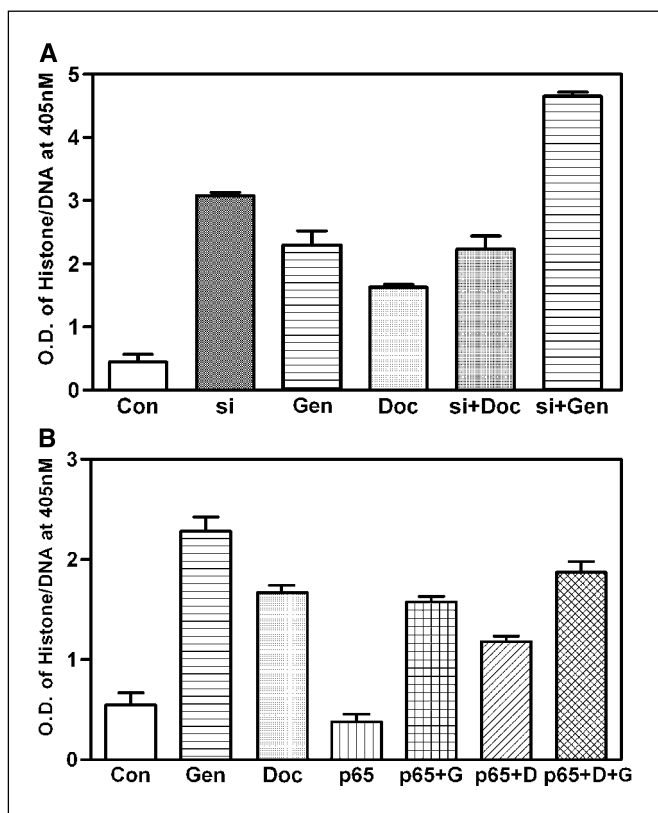


Figure 6. A, induction of apoptosis by p65 siRNA, docetaxel, or genistein in PC-3 cells tested by ELISA. (Con, control; si, 100 nmol/L p65 siRNA; Gen, 50 μ mol/L genistein; Doc, 2 nmol/L docetaxel; si + Doc, 100 nmol/L p65 siRNA and 2 nmol/L docetaxel; si + Gen, 100 nmol/L p65 siRNA and 50 μ mol/L genistein.) B, the effect of p65 cDNA transfection, genistein, and docetaxel on the induction of apoptosis. (Con, control; Gen, 50 μ mol/L genistein; Doc, 2 nmol/L docetaxel; p65, p65 cDNA transfection; p65 + G, p65 cDNA transfection and 50 μ mol/L genistein; p65 + D, p65 cDNA transfection and 2 nmol/L docetaxel; p65 + D + G, p65 cDNA transfection, 30 μ mol/L genistein, and 2 nmol/L docetaxel).

induced by the chemotherapeutic agent docetaxel and induce more apoptosis *in vivo*, supporting the biological and clinical relevance of our findings. Collectively, our results showed that genistein could sensitize cancer cells to chemotherapeutic agents by blocking NF- κ B activation induced by chemotherapeutic agents both *in vitro* and

in vivo. These results provide experimental evidence showing that novel therapeutic strategies for human cancer could be developed to achieve better treatment outcome by introducing genistein in the therapeutic regimen.

To achieve greater inhibitory effects on cancer cells, combination of two or more chemotherapeutic agents for treatment is commonly considered for designing better therapeutic strategies. However, the combination treatment may result in different levels of systemic toxicities (42, 43). Thus, optimization of combination chemotherapy based on molecular mechanism may improve therapeutic index, and such therapeutic strategies are critically needed for the treatment of patients with cancer. In the present study, our results clearly showed that combination of chemotherapeutic agents with genistein, which is a dietary soy isoflavone with no side effect in humans, could reduce the dose of chemotherapeutic agents with better anticancer activities in different types of cancers including prostate, breast, lung, and pancreatic cancers, suggesting that enhanced anticancer efficacy and reduced cytotoxicity to normal cells could be achieved by optimized combination treatments. Therefore, soy isoflavones could be potentially useful in combination with chemotherapeutic agents for the treatment of different types of cancers in the clinic.

In conclusion, our results clearly show that genistein pretreatment both *in vitro* and *in vivo* inactivates NF- κ B and, in turn, sensitizes cancer cell to growth inhibition and apoptosis induced by chemotherapeutic agents. These phenomena are not cancer cell type specific or chemotherapeutic agent specific, suggesting that soy isoflavone genistein may serve as a potent agent as an adjunct to chemotherapy in the treatment of human cancers. However, further in-depth studies including clinical trials are needed to fully evaluate the value of genistein in combination with chemotherapeutic agents for the treatment of human cancers.

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