

Caspase-dependent apoptosis induction by guggulsterone, a constituent of Ayurvedic medicinal plant *Commiphora mukul*, in PC-3 human prostate cancer cells is mediated by Bax and Bak

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Abstract

The present study was undertaken to gain insights into the molecular mechanism of cell death (apoptosis) by guggulsterone, a constituent of Ayurvedic medicinal plant *Commiphora mukul*, using PC-3 human prostate cancer cells as a model. The viability of PC-3 cells, but not a normal prostate epithelial cell line (PrEC), was reduced significantly on treatment with guggulsterone in a concentration-dependent manner. Guggulsterone-mediated suppression of PC-3 cell proliferation was not due to perturbation in cell cycle progression but caused by apoptosis induction characterized by appearance of subdiploid cells and cytoplasmic histone-associated DNA fragmentation. Guggulsterone-induced apoptosis was associated with induction of multidomain proapoptotic Bcl-2 family members Bax and Bak. Interestingly, the expression of antiapoptotic proteins Bcl-2 and Bcl-xL was initially increased in guggulsterone-treated PC-3 cells but declined markedly following a 16- to 24-hour treatment with guggulsterone. Ectopic expression of Bcl-2 in PC-3 cells failed to confer significant protection against guggulsterone-induced cell death. On the other hand, SV40 immortalized mouse embryonic fibroblasts derived from Bax-Bak double knockout mice were significantly more resistant to guggulsterone-induced cell killing compared with wild-type cells. Guggulsterone treatment

resulted in cleavage (activation) of caspase-9, caspase-8, and caspase-3, and guggulsterone-induced cell death was significantly attenuated in the presence of general caspase inhibitor as well as specific inhibitors of caspase-9 and caspase-8. In conclusion, the present study indicates that caspase-dependent apoptosis by guggulsterone is mediated in part by Bax and Bak. [Mol Cancer Ther 2005;4(11):1747–54]

Introduction

Prostate cancer is the second leading cause of cancer-related deaths among men in the United States (1). Prostate carcinogenesis is a multistep process involving progression from localized, low-grade lesions to large, high-grade, metastatic carcinomas. Molecular mechanism underlying onset or progression of prostate cancer is not fully defined, but age, race, diet, and androgen secretion and metabolism are the identifiable risk factors associated with this malignancy (2, 3). Therapeutic options exist for localized disease, which include surgery, radiation therapy, and hormonal therapy. Androgen ablation is a frequently prescribed treatment option for prostate cancer (4). This treatment modality, however, is palliative and has limited scope, especially for hormone-refractory cancers (4). Moreover, chemotherapy and radiation therapy are largely ineffective against advanced prostate cancer (5, 6). Prostate cancer is usually diagnosed in the sixth or seventh decades of life, which allows a large window of opportunity for intervention to prevent or slow progression of the disease. Therefore, clinical development of agents that are nontoxic to normal cells but can delay onset and/or progression of human prostate cancer could have a significant effect on disease-related cost, morbidity, and mortality for a large segment of population.

Guggulsterone [4,17(20)-(cis)-pregnadiene-3,16-dione; see Fig. 1A for structure of guggulsterone] is a plant sterol derived from the gum resin (*guggulu*) of the tree *Commiphora mukul* that has been used extensively in Indian Ayurvedic medicine for the treatment of different ailments, including bone fracture, arthritis, inflammation, cardiovascular disease, and lipid disorders (7–11). Recent studies have shown that guggulsterone is an antagonist of bile acid farnesoid X receptor (12, 13). In addition, guggulsterone regulates cholesterol homeostasis by increasing the transcription of bile salt export pump (14).

Recently, Shishodia and Aggarwal (15) showed that guggulsterone is a potent suppressor of nuclear factor- κ B

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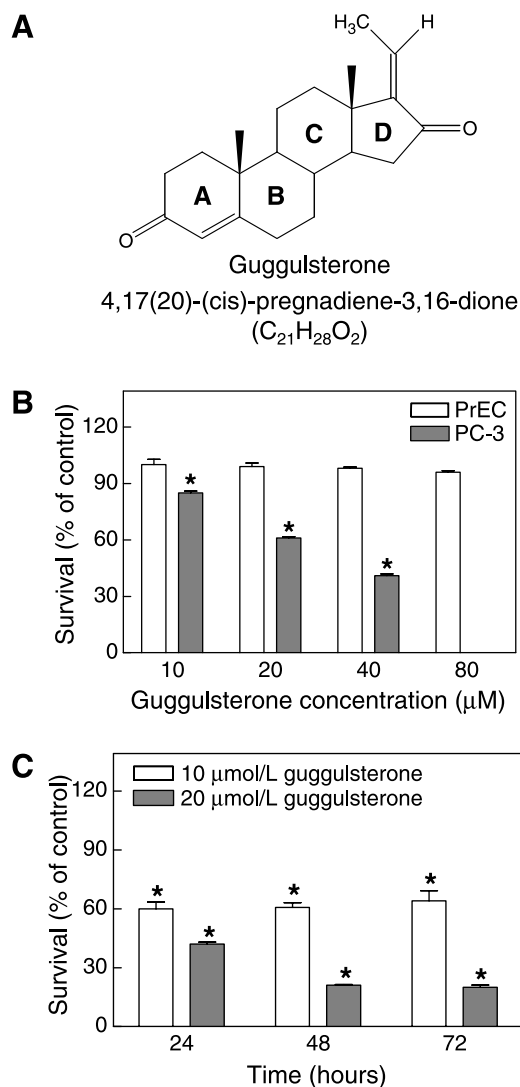


Figure 1. **A**, structure of guggulsterone. **B**, effect of guggulsterone on survival of PrEC (clear columns) and PC-3 (shaded columns) cells determined by sulforhodamine B assay. **C**, effect of guggulsterone on survival of PC-3 cells determined by trypan blue dye exclusion assay. Cells were treated with different concentrations of guggulsterone for 24 h (sulforhodamine B assay) or for 24, 48, or 72 h (trypan blue dye exclusion assays). Columns, mean of three determinations; bars, SE. *, $P < 0.05$, significantly different compared with DMSO-treated control (one-way ANOVA followed by Dunnett's test). Similar results were observed in two independent experiments.

(NF- κ B) activation in tumor cells. NF- κ B is a transcription factor belonging to the Rel family of proteins that are involved in regulation of various genes, including inflammatory cytokines, chemokines, cell adhesion molecules, growth factors, and IFNs (16, 17). NF- κ B activation is considered a prosurvival signal because this transcription factor regulates gene expression of several antiapoptotic proteins, including cIAP1, XIAP, Bfl-1/A1, Bcl-2, cFLIP, and survivin (18–25). Interestingly, NF- κ B is constitutively activated in a variety of hematologic and solid tumor cells, including prostate cancer cells (26–31).

Because guggulsterone inhibits NF- κ B activation (15), we hypothesized that this phytochemical might inhibit growth of cancer cells by causing cell death. In the present study, we tested this hypothesis using PC-3 human prostate cancer cells as a model. We show that guggulsterone suppresses proliferation of PC-3 human prostate cancer cells, but not a normal prostate epithelial cell line, by causing apoptosis induction in association with induction of multidomain proapoptotic Bcl-2 family members Bax and Bak and down-regulation of antiapoptotic proteins Bcl-2 and Bcl-xL leading to activation of caspases. Selectivity of guggulsterone toward cancer cells is intriguing and warrants its clinical development as a potential chemopreventive or therapeutic agent for prostate cancer.

Materials and Methods

Reagents

Z-Guggulsterone was purchased from Steraloids (Newport, RI). Tissue culture medium and fetal bovine serum were from Invitrogen (Grand Island, NY), propidium iodide was from Sigma (St. Louis, MO), and RNase A was from Promega (Madison, WI). Antibodies against Bak (clone G-23), Bax (clone N-20), and Bcl-xL (clone H-5) were from Santa Cruz Biotechnology (Santa Cruz, CA), antibody against caspase-9 was from BD PharMingen (Palo Alto, CA), anti-caspase-8 antibody was from Biosource (Camarillo, CA), antibody specific for detection of cleaved caspase-3 was from Cell Signaling Technology (Beverly, MA), antibody against Bcl-2 (clone 124) was from DAKO (Carpinteria, CA), and anti-actin antibody was from Oncogene Research Products (Boston, MA). The caspase inhibitors zVAD-fmk (general caspase inhibitor), zIETD-fmk (caspase-8), and zLEHD-fmk (caspase-9) were from Enzyme Systems (Dublin, CA).

Cell Culture and Cell Survival Assays

Monolayer cultures of PC-3 cells were maintained in F-12K nutrient mixture (Kaighn's modification) supplemented with 7% (v/v) non-heat-inactivated fetal bovine serum and antibiotics. Normal prostate epithelial cell line PrEC (Clonetics, San Diego, CA) was maintained in PrEBM (Cambrex, Walkersville, MD). The culture conditions for PC-3/neo and PC-3/Bcl-2 cells have been described by us previously (32, 33). The PC-3/neo and PC-3/Bcl-2 cells were maintained similarly, except that G418 (500 μ g/mL) was added to the cultures. The mouse embryonic fibroblasts (MEF) derived from wild-type (WT), Bax or Bak single knockout, and Bax-Bak double knockout mice and immortalized by transfection with a plasmid containing SV40 genomic DNA were generously provided by Dr. Stanley J. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA) and maintained as described previously (34). Each cell line was maintained in an atmosphere of 95% air and 5% CO₂ at 37°C. The effect of guggulsterone on cell viability was determined by sulforhodamine B and trypan blue dye exclusion assays as described previously (32).

Analysis of Cell Cycle Distribution

Effect of guggulsterone treatment on cell cycle distribution was determined by flow cytometry following staining with

propidium iodide as described previously (35, 36). Briefly, cells (5×10^5) were seeded in T25 flasks and allowed to attach by overnight incubation. The medium was replaced with fresh complete medium containing desired concentrations of guggulsterone. Stock solution of guggulsterone was prepared in DMSO and diluted with complete medium. An equal volume of DMSO (final concentration, 0.1%) was added to the controls. Following incubation at 37°C for 24 or 48 hours, floating and attached cells were collected, washed with PBS, and fixed with 70% ethanol. Fixed cells were then treated with RNase A and propidium iodide, and the stained cells were analyzed using a Coulter Epics XL flow cytometer (Miami, FL) as described previously (35, 36). Cells in different phases of the cell cycle were computed for control (DMSO-treated) and guggulsterone-treated cultures.

Detection of Apoptosis

Apoptosis induction in guggulsterone-treated cells was assessed by fluorescence microscopic analysis of cells with condensed and segmented DNA following staining with 4',6-diamidino-2-phenylindole, flow cytometric analysis of cells with sub-G₀-G₁ DNA content following staining with propidium iodide, or analysis of cytoplasmic histone-associated DNA fragmentation. For 4',6-diamidino-2-phenylindole staining, 2×10^4 cells were grown on coverslips and allowed to attach overnight. Cells were then exposed to DMSO (control) or desired concentration of guggulsterone for 24 hours and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. After washing thrice with PBS, cells were permeabilized with 0.2% Triton X-100 for 15 minutes. After rinsing with PBS, cells were stained with 4',6-diamidino-2-phenylindole (1 µg/mL) for 15 minutes. Nuclear condensation and fragmentation was examined under a fluorescence microscope at $\times 20$ magnification. For analysis of cells with sub-G₀-G₁ DNA content, cells were treated as described above for analysis of cell cycle distribution. Cytoplasmic histone-associated DNA fragmentation was determined as described previously (37). In some experiments, cells were pretreated with 80 µmol/L pan-caspase inhibitor zVAD-fmk, 40 µmol/L caspase-9-specific inhibitor zLEHD-fmk, or 40 µmol/L caspase-8-specific inhibitor zIETD-fmk for 2 hours before guggulsterone treatment and assessment of apoptosis.

Immunoblotting

Control and guggulsterone-treated cells were lysed as described previously (32, 33). The cell lysate was cleared by centrifugation at $21,000 \times g$ for 15 minutes, and the supernatant fraction was used for immunoblotting of Bcl-2 family proteins and analysis of caspase cleavage. Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk in TBS containing 0.05% Tween 20, the membrane was incubated with the desired primary antibody for 1 hour at room temperature or for overnight at 4°C. The membrane was then treated with appropriate secondary antibody, and the immunoreactive bands were visualized by enhanced chemiluminescence method. Each membrane was stripped and reprobated with anti-actin antibody to normalize for differences in protein loading.

Results

Guggulsterone Reduced Viability of PC-3 Cells

The effect of guggulsterone on PC-3 cell viability was assessed by sulforhodamine B (Fig. 1B) and trypan blue dye exclusion (Fig. 1C) assays. As can be seen in Fig. 1B, the viability of PC-3 cells was reduced significantly on treatment with guggulsterone in a concentration-dependent manner. For example, a 24-hour treatment of PC-3 cells with 20 and 40 µmol/L guggulsterone caused ~40% and 58% reduction in cell viability, respectively, compared with DMSO-treated control (Fig. 1B). Next, we raised the question of whether guggulsterone-mediated suppression of PC-3 cell growth was selective to cancer cells, which is a highly desirable feature of potential cancer preventive and therapeutic agents. We addressed this question by determining the effect of guggulsterone treatment on viability of a normal prostate epithelial cell line (PrEC). The PrEC cell line has been used extensively as a representative normal prostate epithelial cell line (38–40). Proliferating PrEC exhibit features most consistent with the prostate epithelial origin (38). As can be seen in Fig. 1B (clear columns), the viability of PrEC was not significantly affected by guggulsterone treatment even at concentrations (e.g., 40 and 80 µmol/L) that were cytotoxic to the PC-3 cell line (Fig. 1B). Trypan blue dye exclusion assay confirmed that guggulsterone treatment inhibited proliferation of PC-3 cells in a concentration- and time-dependent manner (Fig. 1C). Collectively, these results indicated that PC-3 cell line, but not a normal prostate epithelial cell line, was sensitive to growth inhibition by guggulsterone.

Guggulsterone Induced Apoptosis in PC-3 Cells

To gain insights into the mechanism of guggulsterone-mediated suppression of PC-3 cell proliferation, we determined its effect on cell cycle distribution by flow cytometry following staining with propidium iodide. As can be seen in Table 1, guggulsterone treatment caused a less than impressive increase in G₀-G₁-phase cells and a slight reduction in S-phase cells at 20 µmol/L concentration, but the G₂-M fraction did not differ significantly between control and guggulsterone-treated PC-3 cultures. As shown in Fig. 2A, the guggulsterone-treated PC-3

Table 1. Effect of guggulsterone on PC-3 cell cycle distribution

Treatment	% Cells		
	G ₀ -G ₁	S	G ₂ -M
DMSO (control)	57 ± 1	17 ± 1	22 ± 1
Guggulsterone (10 µmol/L)	59 ± 1	16 ± 1	22 ± 2
Guggulsterone (20 µmol/L)	61 ± 1*	13 ± 1*	23 ± 2

NOTE: Cells were treated with DMSO or desired concentrations of guggulsterone for 24 hours at 37°C. Both floating and attached cells were collected and processed for analysis of cell cycle distribution. Results are mean ± SE ($n = 3$). Similar results were observed in two independent experiments.

* $P < 0.05$, significantly different compared with control by one-way ANOVA followed by Dunnett's test.

cultures revealed appearance of cells with subdiploid DNA content, which is a characteristic feature of cells undergoing apoptosis. Apoptosis induction by guggulsterone was confirmed by analysis of cytoplasmic histone-associated DNA fragmentation using an ELISA kit, and the results are shown in Fig. 2B. The PrEC cells were included in the analysis for direct comparison. Treatment of PC-3 cells with

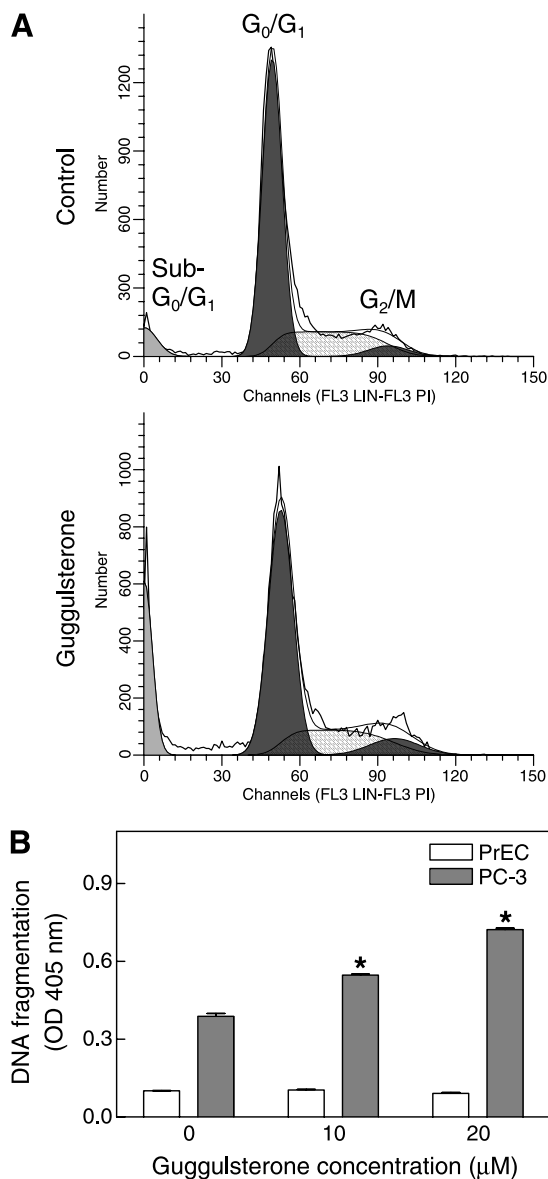


Figure 2. **A**, representative histograms depicting cell cycle distribution in PC-3 cells treated with DMSO (control) or 20 μmol/L guggulsterone for 48 h. **B**, ELISA-based quantitation of cytoplasmic histone-associated DNA fragmentation in PC-3 (shaded columns) or PrEC (clear columns) cells following treatment with DMSO (control) or guggulsterone (10 or 20 μmol/L) for 24 h. Similar results were observed in two independent experiments. Representative of a single experiment. Columns, mean of two determinations; bars, data scatter. *, $P < 0.05$, significantly different compared with DMSO-treated control (one-way ANOVA followed by Dunnett's test).

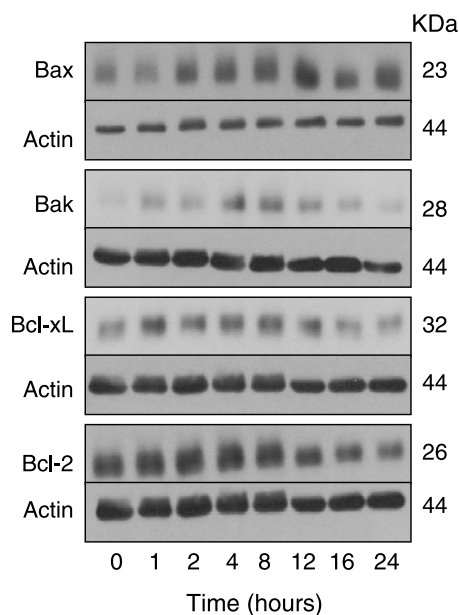


Figure 3. Immunoblotting for Bax, Bak, Bcl-xL, and Bcl-2 using lysates from PC-3 cells treated with 20 μmol/L guggulsterone for the indicated time periods. The blots were stripped and reprobbed with anti-actin antibody to normalize for differences in protein loading. Change in protein level was quantified by densitometric scanning of the immunoreactive bands and corrected for actin loading control. Immunoblotting for each protein was done at least twice using independently prepared lysates, and the results were comparable.

guggulsterone resulted in a concentration-dependent and statistically significant increase in cytoplasmic histone-associated DNA fragmentation compared with control (Fig. 2B). For instance, the cytoplasmic histone-associated DNA fragmentation was increased by ~1.9-fold on a 24-hour treatment of PC-3 cells with 20 μmol/L guggulsterone compared with DMSO-treated control (Fig. 2B, shaded columns). Consistent with the results of cell survival assays, guggulsterone treatment failed to cause DNA fragmentation in PrEC cells (Fig. 2B, clear columns). Collectively, these results indicated that guggulsterone-mediated inhibition of PC-3 cell proliferation was due to apoptosis induction.

Effect of Guggulsterone Treatment on Levels of Bcl-2 Family Proteins

The Bcl-2 family proteins play critical roles in regulation of apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of the cell death process (41–43). To gain insights into the mechanism of apoptosis induction in our model, we determined the effect of guggulsterone treatment on levels of Bcl-2 family proteins by immunoblotting, and the results are shown in Fig. 3. The guggulsterone treatment caused a rapid and marked increase in the level of Bax protein. The guggulsterone-mediated induction of Bax protein expression was evident as early as 2 hours after treatment and persisted for the duration of the experiment (24 hours after treatment). The guggulsterone treatment also caused an increase in the protein level of Bak that peaked between 8 and 12 hours

and declined thereafter. Similarly, the levels of antiapoptotic proteins Bcl-xL and Bcl-2 were initially increased after treatment with guggulsterone (2–12 hours) but declined below control level at 16- and 24-hour time points (Fig. 3). These results suggested that guggulsterone-induced cell death might be regulated by Bcl-2 family proteins.

Effect of Bcl-2 Overexpression on Guggulsterone-Induced Apoptosis

To further examine the role of Bcl-2 in regulation of guggulsterone-induced cell death, we compared sensitivities of PC-3 cells stably transfected with Bcl-2 (PC-3/Bcl-2) and empty vector (PC-3/neo) to apoptosis induction by guggulsterone. As can be seen in Fig. 4A, the level of Bcl-2 protein was ~15-fold higher in PC-3/Bcl-2 cells compared

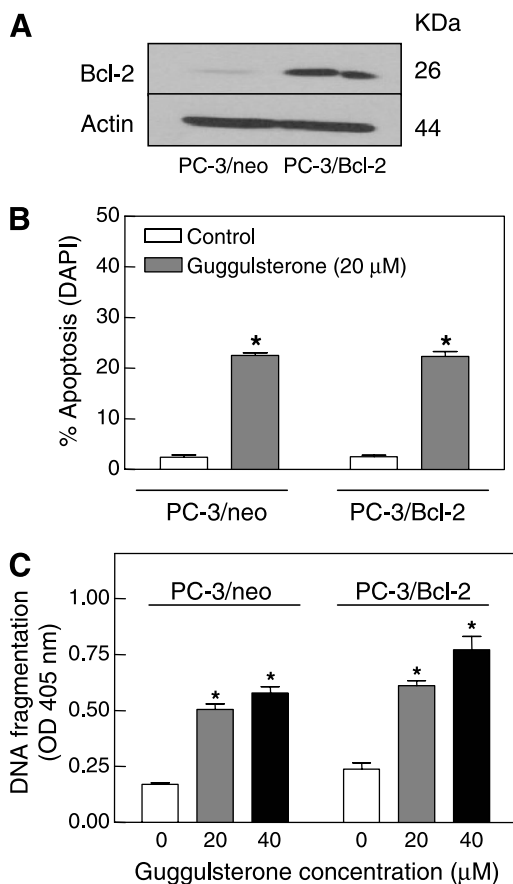


Figure 4. **A**, immunoblotting for Bcl-2 using lysates from PC-3/neo and PC-3/Bcl-2 cells. The blot was stripped and reprobbed with anti-actin antibody to ensure equal protein loading. **B**, analysis of apoptotic cells with condensed and fragmented DNA by 4',6-diamidino-2-phenylindole (DAPI) assay in PC-3/neo and PC-3/Bcl-2 cells following a 24-h treatment with DMSO (control) or 20 μmol/L guggulsterone. *Columns*, mean of three determinations; *bars*, SE. *, $P < 0.05$, significantly different compared with DMSO-treated control (paired t test). **C**, ELISA-based quantitation of cytoplasmic histone-associated DNA fragmentation in PC-3/neo and PC-3/Bcl-2 cells following a 24-h treatment with DMSO (control) or guggulsterone (20 or 40 μmol/L). *Columns*, mean of three determinations; *bars*, SE. *, $P < 0.05$, significantly different compared with DMSO-treated control (one-way ANOVA followed by Dunnett's test).

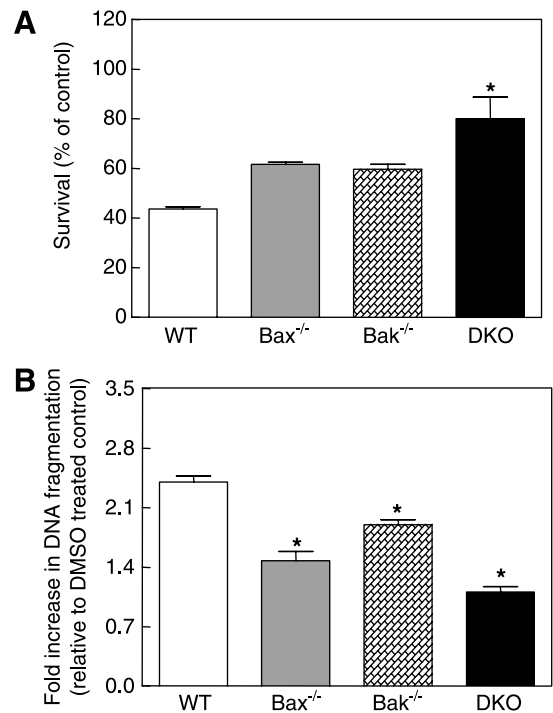


Figure 5. **A**, sulforhodamine B assay for the effect of guggulsterone treatment on survival of MEFs derived from WT, Bax knockout ($Bax^{-/-}$), Bak knockout ($Bak^{-/-}$), and Bax-Bak double knockout (DKO) mice and immortalized by transfection with SV40 genomic DNA. Cells were treated with 40 μmol/L guggulsterone for 24 h. *Columns*, mean of three determinations; *bars*, SE. *, $P < 0.05$, significantly different compared with DMSO-treated control (one-way ANOVA followed by Bonferroni's multiple comparison test). **B**, ELISA-based quantitation of cytoplasmic histone-associated DNA fragmentation in MEFs derived from WT, Bax knockout, Bak knockout, and double knockout mice following a 24-h treatment with DMSO or 20 μmol/L guggulsterone. Data shown are relative to respective DMSO-treated control. Similar results were observed in replicate experiments. *Columns*, mean of three or four determinations; *bars*, SE. *, $P < 0.05$, significantly different compared with WT (one-way ANOVA followed by Bonferroni's multiple comparison test).

with PC-3/neo. Next, we determined the effect of Bcl-2 overexpression on guggulsterone-induced cell death by 4',6-diamidino-2-phenylindole assay, and the results are shown in Fig. 4B. A 24-hour treatment with 20 μmol/L guggulsterone caused a statistically significant increase in fraction of apoptotic cells with condensed and fragmented DNA in both vector-transfected control and Bcl-2-overexpressing PC-3 cells (Fig. 4B). In agreement with these results, guggulsterone treatment caused a dose-dependent and statistically significant increase in cytoplasmic histone-associated DNA fragmentation not only in PC-3/neo cells but also in PC-3 cells stably transfected with Bcl-2 (Fig. 4C). Collectively, these results indicated that apoptosis induction by guggulsterone was not regulated by Bcl-2.

Bak-Bax Double Knockout MEFs Were Resistant to Guggulsterone-Induced Apoptosis

Because guggulsterone treatment caused a marked increase in the protein levels of Bax and Bak (Fig. 3), we determined their roles in guggulsterone-induced cell death

using SV40 immortalized MEFs derived from WT and Bax and/or Bak knockout mice. As can be seen in Fig. 5A, the MEFs derived from Bax or Bak single knockout mice were relatively less sensitive to growth inhibition by guggulsterone compared with the MEFs derived from WT mice as judged by sulforhodamine B assay, although the differences in cell survival between WT and Bax or Bak single knockout MEFs did not reach statistical significance. On the other hand, the MEFs derived from Bax-Bak double knockout mice were statistically significantly more resistant to cell killing by guggulsterone compared with WT MEFs. For instance, the viability of WT MEFs was reduced by ~56% on a 24-hour treatment with 40 $\mu\text{mol/L}$ guggulsterone. A similar treatment with guggulsterone caused a reduction of only ~20% in viability of double knockout MEFs (Fig. 5A).

Consistent with these results, the double knockout MEFs were significantly more resistant to guggulsterone-induced cytoplasmic histone-associated DNA fragmentation compared with the MEFs derived from WT mice (Fig. 5B). For instance, relative to DMSO-treated control, the cytoplasmic histone-associated DNA fragmentation in WT MEFs was increased by ~2.4-fold on a 24-hour treatment with 20 $\mu\text{mol/L}$ guggulsterone. On the other hand, a similar treatment with guggulsterone caused an increase of only ~10% in DNA fragmentation over DMSO-treated control in the MEFs derived from Bax-Bak double knockout mice (Fig. 5B).

Involvement of Caspases in Guggulsterone-Induced Apoptosis

Caspases are aspartate-specific cysteine proteases that play critical roles in execution of apoptosis program (44, 45). Activation of caspases results in cleavage and inactivation of key cellular proteins (44, 45). Next, we explored the possibility of whether the guggulsterone-induced cell death was mediated by caspases. As can be seen in Fig. 6A, treatment of PC-3 cells with 20 $\mu\text{mol/L}$ guggulsterone resulted in cleavage of procaspase-9 that was evidenced by appearance of 37-kDa cleaved intermediate. In addition, guggulsterone treatment caused a decrease in the level of procaspase-8 (this antibody did not recognize cleaved intermediates even after overnight exposure). Immunoblotting using an antibody specific for detection of 19-kDa cleaved caspase-3 intermediate revealed cleavage of procaspase-3 following treatment with guggulsterone for 12 to 24 hours (Fig. 6A). We used pharmacologic inhibitors of caspases to confirm their involvement in guggulsterone-induced apoptosis. As shown in Fig. 6B, the guggulsterone-induced cytoplasmic histone-associated DNA fragmentation was attenuated in the presence of pan-caspase inhibitor zVAD-fmk and specific inhibitors of caspase-9 (zLEHD-fmk) and caspase-8 (zIETD-fmk). These results pointed toward involvement of both caspase-8 and caspase-9 pathways in execution of guggulsterone-induced apoptosis.

Discussion

Our interest in guggulsterone stemmed from a recent study showing its efficacy against NF- κ B activation in

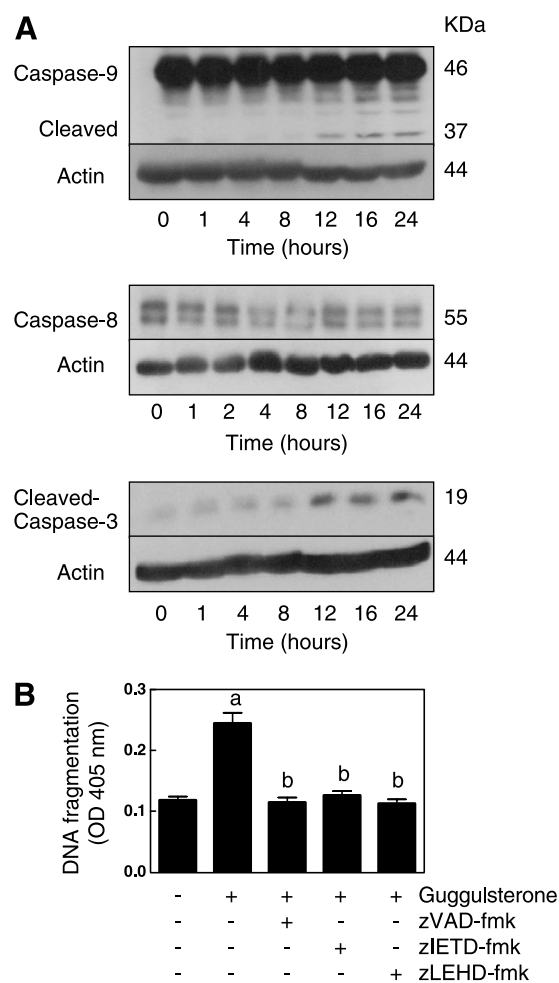


Figure 6. **A**, immunoblotting for caspase-9 (full-length and cleaved intermediate), caspase-8 (full-length), and cleaved caspase-3 using lysates from PC-3 cells treated with 20 $\mu\text{mol/L}$ guggulsterone for the indicated time periods. The blots were stripped and reprobbed with anti-actin antibody to ensure equal protein loading. Immunoblotting for each protein was done at least twice using independently prepared lysates, and the results were comparable. **B**, effect of general caspase inhibitor zVAD-fmk, caspase-8-specific inhibitor zIETD-fmk, and caspase-9-specific inhibitor zLEHD-fmk on guggulsterone-induced cytoplasmic histone-associated DNA fragmentation. PC-3 cells were pretreated for 2 h with desired caspase inhibitor and then exposed to 40 $\mu\text{mol/L}$ guggulsterone for 24 h in the presence of the inhibitor. *Columns*, mean of three determinations; *bars*, SE. *a*, $P < 0.05$, significantly different compared with DMSO-treated control (one-way ANOVA followed by Bonferroni's test for multiple comparisons); *b*, $P < 0.05$, significantly different compared with guggulsterone alone treatment group (one-way ANOVA followed by Bonferroni's test for multiple comparisons).

different tumor cells (15). Furthermore, guggulsterone treatment suppressed DNA binding of NF- κ B induced by tumor necrosis factor, phorbol ester, cigarette smoke condensate, hydrogen peroxide, and interleukin-1 (15). Because guggulsterone treatment also suppressed expression of gene products involved in regulation of cell death, including XIAP, Bcl-2, and cFLIP (15), we reasoned that this phytochemical, which has been used extensively in Indian

Ayurvedic medicine (7–11), might cause apoptotic cell death in tumor cells with constitutively active NF- κ B, such as PC-3 human prostate cancer cells. Indeed, the present study indicates that guggulsterone suppresses proliferation of PC-3 cell by causing apoptosis that is characterized by appearance of subdiploid cells, cytoplasmic histone-associated DNA fragmentation, and cleavage of executioner caspase-3. On the other hand, a normal prostate epithelial cell line (PrEC) seems resistant to growth inhibition and apoptosis induction by guggulsterone even at concentrations that are cytotoxic to the PC-3 cells. Although further studies are needed to elucidate the mechanism of differential response of normal and cancer cells to guggulsterone, selectivity toward cancer cells warrants further preclinical and clinical evaluation of guggulsterone for its efficacy against prostate cancer.

Another objective of the present study was to gain insights into the mechanism of apoptosis induction by guggulsterone. The Bcl-2 family proteins have emerged as critical regulators of the mitochondria-mediated apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of the cell death process (41–43). Differential interaction among Bcl-2 protein family members as well as their association with other cellular proteins regulates cell death (41–43). For example, Bcl-2 normally blocks apoptosis by forming heterodimer complex with proapoptotic proteins, such as Bax (42, 43, 46, 47). Mutations in *Bak* and *Bax* genes have been shown to cause resistance to apoptosis induction by certain stimuli (48–50). The present study reveals that guggulsterone treatment causes a marked increase in the levels of Bax and Bak protein. It is interesting to note, however, that the Bax or Bak single knockout MEFs are only slightly more resistant to guggulsterone-induced cell death compared with the WT MEFs. On the other hand, the MEFs derived from Bak-Bax double knockout mice are significantly more resistant to cell death caused by guggulsterone in comparison with WT MEFs. It is interesting to note that Bcl-2 overexpression fails to offer protection against guggulsterone-induced apoptosis. Thus, it seems reasonable to conclude that multidomain proapoptotic Bcl-2 family members Bax and Bak play an important role in execution of guggulsterone-induced cell death.

Caspase activation leads to cleavage and inactivation of key cellular proteins, such as poly(ADP-ribose) polymerase (44, 45). The guggulsterone treatment causes cleavage of caspase-3 that coincides with cleavage of caspase-9 and caspase-8. Caspase-3 is an executioner caspase that can be activated by a mitochondrial pathway involving caspase-9 or a death receptor pathway involving caspase-8 (44, 45). The results of the present study indicate that guggulsterone-induced apoptosis in PC-3 cells is probably mediated by both caspase-9 and caspase-8 because specific inhibitors of these caspases are able to significantly inhibit the cell death caused by guggulsterone. Involvement of both caspase-9 and caspase-8 pathways in apoptosis induction has also been suggested in other systems (33, 37).

In conclusion, the results of the present study indicate that guggulsterone inhibits proliferation of PC-3 cells in culture by causing apoptosis, whereas a normal prostate epithelial cell line is resistant to growth inhibition and apoptosis induction by this phytochemical. In addition, we provide experimental evidence to implicate Bak and Bax in regulation of guggulsterone-induced apoptosis. These observations provide rationale for further preclinical and clinical evaluation of guggulsterone for its efficacy against prostate cancer.

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References

- Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. *CA Cancer J Clin* 2004;54:8–29.
- Ross RK, Henderson BE. Do diet and androgens alter prostate cancer risk via a common etiologic pathway? *J Natl Cancer Inst* 1994;86:252–4.
- Whittemore AS, Kolonel LN, Wu AH, et al. Prostate cancer in relation to diet, physical activity, and body size in Blacks, Whites, and Asians in the United States and Canada. *J Natl Cancer Inst* 1995;87:652–61.
- Laufer M, Denmeade SR, Sinibaldi VJ, Carducci MA, Eisenberger MA. Complete androgen blockade for prostate cancer: what went wrong? *J Urol* 2000;164:3–9.
- Ramakrishna NR, DeWeese TL. In: Chung LWK, Isaacs WB, Simons JW, editors. *Prostate cancer: biology, genetics, and the new therapeutics*. New Jersey: Humana Press; 2001. p. 387–413.
- Gilligan T, Kantoff PW. Chemotherapy for prostate cancer. *Urology* 2002;60:94–100.
- Gujral ML, Sareen K, Tangri KK, Amma MK, Roy AK. Antiarthritic and anti-inflammatory activity of gum guggul (*Balsamodendron mukul* Hook). *Ind J Physiol Pharmacol* 1960;4:267–73.
- Sharma JN. Comparison of the anti-inflammatory activity of *Commiphora mukul* (an indigenous drug) with those of phenylbutazone and ibuprofen in experimental arthritis induced by mycobacterial adjuvant. *Arzneimittelforschung* 1977;27:1455–7.
- Sinal CJ, Gonzalez FJ. Guggulsterone: an old approach to a new problem. *Trends Endocrinol Metab* 2002;13:275–6.
- Urizar NL, Moore DD. GUGULIPID: a natural cholesterol-lowering agent. *Annu Rev Nutr* 2003;23:303–13.
- Tripathi YB, Tripathi P, Malhotra OP, Tripathi SN. Thyroid stimulatory action of Z Guggulsterone: mechanism of action. *Planta Med* 1988;4:271–7.
- Urizar NL, Liverman AB, Dodds DT, et al. A natural product that lowers cholesterol as an antagonist ligand for FXR. *Science* 2002;296:1703–6.
- Wu J, Xia C, Meier J, Li S, Hu X, Lala DS. The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. *Mol Endocrinol* 2002;16:1590–7.
- Cui J, Huang L, Zhao A, et al. Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. *J Biol Chem* 2003;278:10214–20.
- Shishodia S, Aggarwal BB. Guggulsterone inhibits NF- κ B and I κ B α kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. *J Biol Chem* 2004;279:47148–58.
- Baeuerle PA, Henkel T. Function and activation of NF- κ B in the immune system. *Annu Rev Immunol* 1994;12:141–79.
- Miyamoto S, Verma IM. Rel/NF- κ B/I κ B story. *Adv Cancer Res* 1995;66:255–92.
- Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF- κ B control. *Proc Natl Acad Sci U S A* 1997;94:10057–62.
- You M, Ku PT, Hrdlickova R, Bose HR. ch-IAP1, a member of

- the inhibitor-of-apoptosis protein family, is a mediator of the anti-apoptotic activity of the ν -Rel oncoprotein. *Mol Cell Biol* 1997;17:7328–41.
20. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)- κ B-regulated X-chromosome-linked IAP gene expression protects endothelial cells from tumor necrosis factor α -induced apoptosis. *J Exp Med* 1998;188:211–6.
 21. Schwenzler R, Siemienski K, Liptay S, et al. The human tumor necrosis factor (TNF) receptor-associated factor 1 gene (TRAF1) is up-regulated by cytokines of the TNF ligand family and modulates TNF-induced activation of NF- κ B and c-Jun N-terminal kinase. *J Biol Chem* 1999;274:19368–74.
 22. Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev* 1999;13:400–11.
 23. Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF- κ B that blocks TNF α -induced apoptosis. *Genes Dev* 1999;13:382–7.
 24. Zhu L, Fukuda S, Cordis G, Das DK, Maulik N. Anti-apoptotic protein survivin plays a significant role in tubular morphogenesis of human coronary arteriolar endothelial cells by hypoxic preconditioning. *FEBS Lett* 2001;508:369–74.
 25. Kreuz S, Siegmund D, Scheurich P, Wajant H. NF- κ B induces upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol Cell Biol* 2001;21:3964–73.
 26. Bargou RC, Emmerich F, Krappmann D, et al. Constitutive nuclear factor- κ B-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997;100:2961–9.
 27. Shattuck-Brandt RL, Richmond A. Enhanced degradation of I- κ B α contributes to endogenous activation of NF- κ B in Hs294T melanoma cells. *Cancer Res* 1997;57:3032–9.
 28. Giri DK, Aggarwal B. Constitutive activation of NF- κ B causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. Autocrine role of tumor necrosis factor and reactive oxygen intermediates. *J Biol Chem* 1998;273:14008–14.
 29. Reuther JY, Reuther GW, Cortez D, Pendergast AM, Baldwin AS. A requirement for NF- κ B activation in Bcr-Abl-mediated transformation. *Genes Dev* 1998;12:968–81.
 30. Dong G, Chen Z, Kato T, Van Waes C. The host environment promotes the constitutive activation of nuclear factor- κ B and proinflammatory cytokine expression during metastatic tumor progression of murine squamous cell carcinoma. *Cancer Res* 1999;59:3495–504.
 31. Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of I- κ B kinase α and NF- κ B in prostate cancer cells is inhibited by ibuprofen. *Oncogene* 1999;18:7389–94.
 32. Xiao D, Choi S, Johnson DE, et al. Diallyl trisulfide-induced apoptosis in human prostate cancer cells involves c-Jun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2. *Oncogene* 2004;23:5594–606.
 33. Singh SV, Srivastava SK, Choi S, et al. Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. *J Biol Chem* 2005;280:19911–24.
 34. Choi S, Singh SV. Bax and Bak are required for apoptosis induction by sulforaphane, a cruciferous vegetable-derived cancer chemopreventive agent. *Cancer Res* 2005;65:2035–43.
 35. Singh SV, Herman-Antosiewicz A, Singh AV, et al. Sulforaphane-induced G₂-M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. *J Biol Chem* 2004;279:25813–22.
 36. Xiao D, Herman-Antosiewicz A, Antosiewicz J, et al. Diallyl trisulfide-induced G(2)-M phase cell cycle arrest in human prostate cancer cells is caused by reactive oxygen species-dependent destruction and hyperphosphorylation of Cdc25C. *Oncogene* 2005;24:6256–68.
 37. Singh AV, Xiao D, Lew KL, Dhir R, Singh SV. Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. *Carcinogenesis* 2004;25:83–90.
 38. Campbell CL, Savarese DMF, Quesenberry PJ, Savarese TM. Expression of multiple angiogenic cytokines in cultured normal human prostate epithelial cells: predominance of vascular endothelial growth factor. *Int J Cancer* 1999;80:868–74.
 39. Chen L, Hodge GB, Guarda LA, Welch JL, Greenberg NM, Chai KX. Down-regulation of prostatic serine protease: a potential invasion suppressor in prostate cancer. *Prostate* 2001;48:93–103.
 40. Garraway LA, Lin D, Signoretti S, et al. Intermediate basal cells of the prostate: *in vitro* and *in vivo* characterization. *Prostate* 2003;55:206–18.
 41. Reed JC. Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. *Semin Hematol* 1997;34:9–19.
 42. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol* 1998;16:395–419.
 43. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281:1322–6.
 44. Thornberry N, Lazebrnick Y. Caspases: enemies within. *Science* 1998;281:1312–6.
 45. Wolf BB, Green DR. Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem* 1999;274:20049–52.
 46. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993;74:609–19.
 47. Kiefer MC, Brauer MJ, Powers VC, et al. Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature* 1995;374:736–9.
 48. Kondo S, Shinomura Y, Miyazaki Y, et al. Mutations of the bak gene in human gastric and colorectal cancers. *Cancer Res* 2000;60:4328–30.
 49. Ionov Y, Yamamoto H, Krajewski S, Reed JC, Perucho M. Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. *Proc Natl Acad Sci U S A* 2000;97:10872–7.
 50. LeBlanc H, Lawrence D, Varfolomeev E, et al. Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* 2002;8:274–81.

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