Research Article

Gossypol Induces Apoptosis by Activating p53 in Prostate Cancer Cells and Prostate Tumor–Initiating Cells

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Abstract

Prostate cancer continues to represent a burgeoning medical problem in the United States. Recent studies suggest that gossypol, a bioactive phytochemical produced by cotton plants, is a promising agent against prostate cancer. The current studies were undertaken to examine the chemotherapeutic efficacy of gossypol on human prostate cancer cell lines and prostate tumor–initiating cells. Gossypol reduced the viability of three prostate cancer cell lines (LAPC4, PC3, and DU145) with an IC50 between 3 and 5 μmol/L. Additionally, gossypol was effective at inhibiting prostate tumor–initiating cell-driven tumor growth in a nontobese diabetic/severe combined immunodeficient xenograft model. Our integrated molecular profiling approach encompassing proteomics, activated transcription factors, and genomics suggests that the decrease in viability was associated with increased DNA damage and the induction of apoptosis. Exposure of DU145 cells to gossypol (1–10 μmol/L) resulted in the activation of 13 proteins and 7 transcription factors, and the expression of 17 genes involved in the mitochondrial pathway of apoptosis. These studies show for the first time that gossypol treatment induces DNA damage and activates p53. Collectively, these data support the use of gossypol as a novel agent for prostate cancer. Mol Cancer Ther; 9(2); 461–70. ©2010 AACR.

Introduction

Prostate cancer continues to be the most common malignancy diagnosed in American men and the second leading cause of male cancer mortality (1). Current estimates indicate that over 2 million American men have been diagnosed with prostate cancer (2). Although localized forms of the disease can often be successfully treated by surgery or radiotherapy, a significant proportion of patients having undergone such interventions are at risk of disease recurrence. Androgen deprivation therapy (ADT) can prolong the life expectancy of these patients; however, ADT is associated with several distressing side effects, including loss of libido, bone density, and muscle mass along with erectile dysfunction and cardiovascular morbidity (3). Moreover, nearly all the patients undergoing ADT are expected to fail hormone therapy and progress to an androgen-independent phenotype (androgen-independent prostate cancer; ref. 4). As currently available treatment options for androgen-independent prostate cancer are lacking, there is a growing need for novel therapeutics that are effective against this fatal disease with minimal toxicity.

Gossypol, a yellowish compound produced by cotton plants (Gossypium species), has a long history of use in Chinese folk medicine and has been extensively studied for its use as a male contraceptive agent (5). In recent years, however, this bioactive compound has generated interest in the scientific community as a promising agent against cancer. For instance, studies conducted by three independent laboratory have shown that gossypol specifically targets cancer cells and is not toxic to primary normal cells or noncancerous cells (6–8). Clinical trials indicate gossypol is apparently safe in doses up to 70 mg/day (9) and pharmacokinetic studies revealed that this compound can reach peak plasma concentrations in micromolar ranges [0.79 ± 0.4 μg/mL (1.4 μmol/L); ref. 5]. Although cell cultures (10, 11), animal studies (12, 13), and clinical trials (14, 15) have shown that gossypol inhibits cell proliferation and prevents metastases of many types of cancers (15), the ability of this compound to modulate prostate cancer has not been well studied. Accordingly, the primary objective of this study was to access the chemotherapeutic effects of gossypol on human prostate cancer cell lines. Furthermore, to decipher the mechanism by which this compound exerts its anticancer effects, we chose DU145 as our model and used an integrated molecular profiling approach in which we screened 650 proteins (377 pan-specific proteins and
273 phospho-specific proteins), 345 transcription factors, and the entire human genome.

Increasing evidence suggests that prostate tumors originate from a subpopulation of stem cell-like cells called prostate tumor-initiating cells (pTIC). These pTICs are thought to be responsible for tumorigenesis, tumor differentiation, and tumor maintenance. Patrawala et al. (16, 17) have previously shown that CD44+/hi cells, isolated from various human prostatic cancer cell lines, are enriched for pTICs and confirmed that this subpopulation of cells are highly tumorigenic and metastatic. Similarly, we have shown that as little as 100 CD44+/hi cells isolated from DU-145 prostate carcinoma cell lines were sufficient for tumor formation when engrafted into the flanks of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (18). We and others have documented that the CD44+/hi subpopulation of cells overexpress genes known to play a role in stem cell self-renewal, such as OCT-3/4 and Bmi1 (16, 18). The existence of a sinister subset of pTICs suggests that successful elimination of prostate cancer requires an anticancer therapy that targets this subpopulation of cells as well as the differentiated tumor cells. Therefore, our second objective was to assess the efficacy of gossypol on pTICs.

In this study, we show for the first time that gossypol is effective at reducing the viability of three prostate cancer cell lines (LAPC4, PC3, and DU145) and inhibiting tumor growth in a NOD/SCID xenograft model. Furthermore, the growth of pTICs isolated from DU145 (CD44+/hi) are also inhibited. Our integrated molecular profiling approach suggests that the inhibitory effect of gossypol is attributable to the induction of DNA damage, which consequently leads to the stabilization of p53 and the activation of the mitochondrial pathway of apoptosis.

Materials and Methods

Cell Culture and Chemicals

Human prostate cancer cell lines DU145 and PC-3 were purchased from the American Type Culture Collection and LAPC4 cells were a kind gift from Dr. Charles Sawyers from the Memorial Sloan-Kettering Cancer Center, New York, NY. The DU145, LAPC4, and PC3 cell lines were cultured in DMEM, Iscove’s DMEM, and F-12 Káihõ’s, respectively. All cell lines were supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin/streptomycin, and 2 mmol/L L-glutamine. Sorted cells were cultured in a serum-free medium as previously described (18). Gossypol (>98% pure) was purchased from LKT Laboratories, Inc., and reconstituted in DMSO (Sigma). For analytic purposes, a standard stock solution of gossypol (10 mmol/L) was prepared and stored at −20°C. The final concentration of DMSO in all cell culture experiments was 0.1%. Control cells were grown in the same concentrations of DMSO.

Cell Proliferation and Cytotoxicity Assay

Prostate cancer cell lines (DU145, LAPC4, and PC3) were seeded at a density of 5 × 10⁴ per well in 96-well tissue culture dishes and allowed to attach overnight. Triplicate samples of growing cells were treated with gossypol for time and concentrations as indicated. Cell proliferation was accessed by CellTiter-Glo assay (Promega). To evaluate the cytotoxicity of gossypol on prostate cancer cell lines, CytoTox-ONE membrane integrity assay was used. For this assay, prostate cancer cells were seeded at a density of 10,000 cells per 100 μL per well in 96-well plates. After 24 h, cells were treated with either gossypol or 0.1% DMSO as vehicle control for 48 h. Cytotoxicity was determined according to the manufacturer’s recommendations. Briefly, lactate dehydrogenase (LDH) leakage was measured as the ratio of treatment-induced LDH to maximum LDH release. Samples were measured in triplicate and the measurement was repeated in at least three independent experiments.

Fluorescence-Activated Cell Sorting of CD44+/lo and CD44+/hi Prostate Cancer Cells

Trypsinized cells were washed twice with fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% bovine serum albumin) and stained with 5 μL of phycoerythrin conjugated anti-CD44 (Invitrogen) per million cells at 4°C for 15 min. After staining, cells were washed twice with FACS buffer, resuspended at 2 × 10⁶ cells/mL, and were sorted using a MoFlo high-speed cell sorter (DAKO Cytomation). Live cells were gated on the basis of forward and side scatter, and only the top 5% (CD44+/hi) and the bottom 5% (CD44−/lo) of stained cells were used for experiments.

Mouse Xenograft Studies. Eighteen male NOD/SCID mice were purchased at 4 wk of age from National Cancer Institute’s (NCI) Animal Genetics and Production Facility and housed in the Association for Assessment and Accreditation of Laboratory Care-accredited animal facility at NCI. Animal care was provided in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council, 1996). The mice were injected s.c. at 6 wk of age, with 100 CD44+/hi DU145 cells as previously described (18), and were randomly divided into three groups (six mice per group). Group 1 was the positive control (no test agent), whereas groups 2 and group 3 received low dose (5 mg/kg) and high dose (15 mg/kg) of gossypol by oral gavage thrice weekly. Mice were monitored weekly for palpable tumor formation for 106 d.

Quantitative Apoptotic Cell Death Assay

Cell death was measured by fluorescein-conjugated Annexin V (Annexin V-FITC) and propidium iodide (PI) staining. DU145 cells in log phase growth were plated into six-well plates at a density of 1 × 10⁶ cells per well. Twenty-four hours later, cells were treated with gossypol (1, 5, and 10 μmol/L) for the indicated periods of time. After the treatment, the attached cells were trypsinized,
washed twice with cold PBS, and resuspended in binding buffer (1 × 10^5 cells/mL). Five microliters of Annexin V-FITC and PI were added to suspended cells, incubated for 15 min at room temperature in the dark, washed twice with PBS, and analyzed by flow cytometry.

Caspase Activity Assay. DU145 cells were seeded at a density of 2.5 × 10^3 cells per well in a 96-well plate and cultured for 24 h. After incubation with 10 μmol/L gossypol for the indicated times, caspase catalytic activity of caspase-3/7, caspase-8, and caspase-9 were determined by means of Caspase-Glo 3/7, Caspase-Glo 8, and Caspase-Glo 9 Assay kit (Promega) according to manufactures instructions. Briefly, after treatment with gossypol, 100 μL culture supernatant were transferred into an white-walled 96-well plate. Equal volume of caspase substrate was added and samples were incubated at room temperature for 1 h. Culture medium was used as a blank and luminescence was measured using an Infinite M200 luminometer (Tecan US).

Comet Assay for Detecting DNA Damage
An alkali comet assay, used for detecting DNA damage, was done according to instructions provided by the manufacturer (Trevigen). A suspension of 10,000 unsorted and sorted (CD44^hi and CD 4^−/lo) cells either treated with or not treated with 10 μmol/L gossypol for 48 h were gently mixed with 100 μL of low-melting-point agrose. Seventy-five microliters of the cells/agrose mixture were directly added to the comet slides and were allowed to gel on ice for 10 min. After the solidification of agarose, the slide was immersed in lysis solution at 4°C for 1 h. Following lysis, electrophoresis (1 V/cm) was done in alkali solution [0.3 mol/L NaOH, 1 mmol/L EDTA (pH >13)] for 20 min at 4°C. The slides were immersed in ethanol (5 min), air-dried, and stained with SYBR Green and were analyzed immediately at ×200 in a fluorescence microscope (Olympus 70) under green light. An image analysis system (Image J), provided by the NIH^4 was used to quantify DNA damage. The DNA damage in nontreated (n = 32 for CD44^hi, n = 38 for CD 44^−/lo, and n = 62 for unsorted cells) and gossypol-treated cells (n = 22 for CD44^hi, n = 56 for CD 44^−/lo, and n = 95 for unsorted cells) was quantified as tail moment (the mean migration distance in the tail). One-way ANOVA was used to analyze normally distributed tail migration and head diameter. Overall differences were considered significant when P < 0.05.

Statistical Analysis
Data are presented as means ± SD. Statistical analysis was done using one-way ANOVA for multiple samples. P value of <0.05 were considered statically significant.

Cell viability, DNA damage, and caspase activity assay were presented graphically in the form of histograms, using Microsoft Excel computer program.

Protein Microarray, Transcription Factor/DNA Binding Array, and Gene Array
See the Supplemental Experimental Procedures.

Results
Growth-Inhibitory Effect of Gossypol on Human Prostate Cancer Cell Lines
Gossypol inhibits proliferation and prevents the metastases of Dunning prostate cells in xenograft Copenhagen rats (19). However, the oncostatic effects of gossypol have not been thoroughly investigated in other human prostate cancer cell lines and the molecular basis underlying these anticancer properties is still poorly understood. Hence, we first sought to access the effect of

4 http://rsb.info.nih.gov/ij/
gossypol on the viability of three prostate cancer cell lines, DU145, PC3, and LAPC4. As shown in Fig. 1A, treatment with gossypol for 72 hours induced a dose-dependent decrease in cell viability compared with non-treated cells. Although the IC_{50} of gossypol on these three cell lines ranged from 3 to 5 μmol/L (3 μmol/L for PC3, 4 μmol/L for LAPC4, and 5 μmol/L for DU145), the IC_{90} was 10 μmol/L on all three cell lines. These results show that gossypol can dramatically inhibit the growth of prostate cancer cells.

A reduction in cell number can either be the consequence of gross injury to the cells, cytotoxicity, or the consequence of an actively driven biochemical process such as cell cycle arrest or apoptosis. To ensure that the growth-inhibitory effect of gossypol on prostate cancer cells was not due to cytotoxicity, LDH leakage in response to 1, 5, and 10 μmol/L of gossypol was done. As depicted by Fig. 1B, even at high concentrations (10 μmol/L), gossypol exerted no cytotoxicity on DU145 cells. Taken together, these experiments suggest that gossypol can inhibit the growth of prostate cancer cells and that this inhibition is not due to cytotoxicity.

**Proteins Modulated by Gossypol**

The effects of biological disruption are usually observed within the networks of biochemical intracellular systems. Using an integrated molecular profiling platform (proteomics, activated transcription factors, and genomics), we examined the effects of gossypol on the three systems important to the biological functions of cells. Because gossypol was equally potent across all three cell lines (Fig. 1A) examined, we selected DU145 as a model to determine gossypol's growth-inhibitory effects. Cells exposed to 10 μmol/L gossypol for 15 or 60 minutes were subjected to kinexus antibody microarray, which screens 273 phospho-specific proteins and 377 pan-specific proteins. Of the 650 proteins, we found a 2-fold or greater downregulation in phosphorylation or expression of 96 proteins at either 15- or 60-minute time point. As shown in Supplementary Fig. S1, the majority of the proteins involved with the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway, Janus-activated kinase/signal transducers and activators of transcription signaling pathway, and cell structure were inactivated. Gossypol activated proteins that are involved in the mitochondrial apoptotic pathway (Fig. 2A) and increased the...
phosphorylation of p53 at serine-392, which is phosphorylated in response to DNA damage (20). To validate these findings, the activity of initiator caspases (caspase-8 and 9) and the effector caspases (caspase-3/7) were accessed. As shown in Fig. 2B, addition of gossypol to DU145 cells lead to a time-dependent increase in caspase-9 and caspase-3/7 but had no effect on caspase-8. These results suggest that gossypol induces DNA damage and triggers apoptosis by using the mitochondrial pathway.

Transcription Factors (Protein/DNA) Altered by Gossypol

The next phase of our integrated molecular profiling was to identify transcription factors that were modulated by gossypol; for this, we used a Panomics TransSignal protein/DNA combo array. Exposing DU145 cells to 10 μmol/L gossypol for either 30 or 90 minutes showed that gossypol altered the binding of 17 of 345 transcription factors (5%); 3 decreased and 14 increased DNA binding (Supplementary Fig. S2; Fig. 2C). Interestingly, 50% (7 of 14) of the transcription factors that show increased DNA binding are known to transcribe genes that participate in apoptosis (Fig. 2C); the most noteworthy being p53. The ability of gossypol to increase the DNA binding of p53 correlates well with our protein array data that showed an increase in activation (phosphorylation) of this protein (Fig. 2B).

Gene Expression Changes Induced by Gossypol

To finalize our integrated molecular profiling approach, gene expression changes were monitored after
exposure to 10 μmol/L gossypol using DU145 cells. By using Ingenuity System’s Pathways Analysis software, we focused our analysis to the transcripts involved in apoptotic pathways. Figure 3A reveals that treating DU145 cells with gossypol for 0, 1, 4, and 8 hours increased the expression of proapoptotic genes such as Bax, Bak, AIE, NOXA1, CAD, PARP, and cytochrome c and decreased the expression of antiapoptotic genes such as Bcl-2 and Bcl-xL. Additionally, analysis of the caspases involved in apoptosis showed an increase in transcripts of caspase-9 and caspase-7 and a decrease in transcripts of caspase-8 and caspase-10, thus confirming the involvement of the mitochondrial pathway. In addition, consistent with our protein and transcription factor array data, we noticed that an increase expression of p53 occurred within 1 hours after gossypol treatment, indicating that DNA damage might be responsible for gossypol-induced apoptosis. Therefore, we enriched our data for genes involved in DNA damage and found six genes (BRCA-1, FANCA, NHN, RFC1, FANCD2, and p53) upregulated (Fig. 3B). To verify these results, we performed a comet assay to detect DNA damage. As shown in Fig. 3C and D, exposing DU145 cells to gossypol for 48 hours lead to a marked increase in the mean tail moment, thus confirming DNA damage.

**Gossypol Induces Apoptosis of DU145 Cells**

Because apoptosis seemed to be the major mechanism by which gossypol reduced viability, DU145 cells were examined using Annexin V and PI staining. As shown in Fig. 4, gossypol treatment of different doses (1, 5, and 10 μmol/L) for 72 hours resulted in 15%, 26%, and 52% apoptosis compared with the vehicle (14%). Similarly, exposing DU145 cells to 10 μmol/L of gossypol for 48, and 72 hours resulted in 5%, 12%, and 52% apoptosis, respectively (data not shown).

**Gossypol Inhibits Growth and Induces Apoptosis of Both pTICs (CD44+/hi) and Bulk Tumor (CD44−/lo) Cells**

Several studies suggest that pTICs are responsible for tumor growth and maintenance (16, 18). We have previously shown that CD44+/hi cells isolated from DU145 cells are enriched for pTICs. Indeed, injecting as little as 100 CD44+/hi cells into NOD/SCID mice was sufficient to induce tumors (21). To assess whether gossypol induces growth-inhibitory effects on this subpopulation of cells, we isolated the top 5% (CD44+/hi) and the bottom 5% (CD 44−/lo) of cell fractions from cultured parental prostate cancer cell lines by FACS. As shown in Fig. 5A, gossypol inhibited the growth of both CD44+/hi and CD 44−/lo cells. Gossypol at 5 μmol/L reduced cellular viability to 10% to 28% for CD44+/hi cells in all cell lines. At the same concentration, for the CD44+/hi cells, gossypol reduced cellular viability to 18% and 20% for PC3 and DU145, respectively. However, increasing the concentration to 10 μmol/L reduced the viability of all three cell lines to 1% to 3% in CD44+/hi and CD 44−/lo cells. To determine whether the decrease in cell number of CD44+/hi and CD 44−/lo cells was due to the ability of gossypol to induce apoptosis, the ability of gossypol to modulate the activity of initiator caspases (caspase-8 and caspase-9) and effector caspases (caspase-3/7) was evaluated. Similar to our observation in unsorted DU145 cells, CD44+/hi and CD 44−/lo cells exposed to 10 μmol/L gossypol resulted in a time-dependent increase in caspase-3/7 activity and caspase-9 activity, but had no effect of the caspase-8 activity (Fig. 5B).

**Gossypol Induces DNA Damage in Both pTICs (CD44+/hi) and Bulk Tumor (CD44−/lo) Cells**

Our results on unsorted DU145 cells suggest that the apoptotic effect of gossypol was mainly due to the induction of DNA damage. To investigate whether gossypol also induced DNA damage in CD44+/hi and CD 44−/lo cells, we performed an alkaline comet assay. Cells treated with gossypol show statically significant longer tails (Fig. 5C, right) than control (nontreated) cells (Fig. 5C, left). In addition, the tail length observed for both CD44+/hi and CD 44−/lo cells were approximately the same (Fig. 5D), indicating that gossypol is equally potent on both cell types.

**Gossypol Inhibits Tumor Incidence in a NOD/SCID Xenograft Model**

The in vitro data prompted us to investigate the efficacy of DU145 in a pTIC-driven tumor-initiation study. NOD/SCID mice were injected s.c. with 100 CD44+/hi cells and gossypol was administered by oral gavage thrice weekly over the course of the entire experiment (up to 106 days) at two concentrations (5 and 15 mg/kg). As shown in Fig. 6, gossypol reduced both tumor incidence and tumor
Figure 5. Effects of gossypol on PTICs (CD44<sup>+</sup>/hi) and bulk tumor (CD44<sup>−</sup>/lo) cells isolated from DU145. A, prostate cancer cells were FACS sorted into two populations: CD44<sup>+</sup>/hi and CD44<sup>−</sup>/lo. R3 and R4, the lowest and highest 5% of CD44-expressing cells, respectively. Cell viability was measured using Promega’s Cell-Titer Glo assay. Y-axis, percent viability normalized to nontreated (0.1% DMSO) control. B, activity of initiator caspase (caspase-8 and caspase-9) and effector caspase (caspase-3/7) in response to 10 μmol/L gossypol accessed at various time points (6–48 h) in CD44<sup>+</sup>/hi (top right) and CD44<sup>−</sup>/lo (bottom right) cells isolated from DU145. After treatment, the cells were lysed and caspase-3/7, caspase-8, and caspase-9 activity was measured using Caspase-Glo assay kit. All experiments were done in triplicate and were done at least thrice. C, a representative comet assay image showing DNA damage induced by gossypol on CD44<sup>+</sup>/hi and CD44<sup>−</sup>/lo cells. D, a histogram summarizing the mean tail moment of DNA.
Injections were supplemented with an equal volume of Matrigel. Each group contained six animals and was monitored for palpable tumor formation weekly. The average latency period for vehicle treated was 61 days, whereas the average latency period was 78 and 90 days for the 5 and 15 mg/kg doses, respectively.

**Discussion**

Prostate cancer causes significant morbidity and mortality, and is a major public problem in the United States. Although ADT can prolong the life expectancy of these patients, acquired drug resistance as well as the induction of side effects remains a major obstacle in the clinical settings. Therefore, attention has been focused on natural products as potential sources of novel anticancer drugs over the last few decades (22–25). Accordingly, in this study, we evaluated the effect of gossypol on the growth of human prostate cancer cell lines. These results indicate that gossypol acts as an inhibitor of prostate cancer cell growth as shown by its ability to reduce the viability of three prostate cancer cell lines (LAPC4, PC3, and DU145) and the putative prostate cancer stem cells isolated from DU145 (CD44+/hi) in culture, and its ability to inhibit tumor growth in a NOD/SCID xenograft model. Furthermore, these investigations provide a plausible molecular basis for the development of naturally occurring anticancer agents for better management of androgen-independent prostate cancer.

Humans have consumed gossypol and gossypol-containing drugs and food for a long period of time with few adverse consequences. Chinese contraceptive studies conducted by Qian et al. (5) found that gossypol given orally at a dose of 60 to 70 mg per day for 35 to 42 days caused a gradual increase in the percentage of nonmotile spermatozoa in the ejaculate in 25 (100%) volunteers, suggesting that gossypol may act on epididymal or testicular spermatozoa. The side effects of this dosage were reversible and generally of mild degree, mainly including decrease or increase in appetite, fatigue, dryness of mouth, and a seemingly decreased libido. A preliminary clinical study conducted by Stein et al. (15) reported that oral gossypol at lower doses (20 mg daily) can safely be given to patients with advance cancer and the main subjective toxicity seemed to be emesis. In our study, mice fed gossypol by oral gavage at 5 and 15 mg/kg displayed no signs of toxicity during the 106 days of study. Furthermore, our in vitro studies on prostate cancer cell lines showed that gossypol can inhibit the growth of prostate cancer cells without inducing cytotoxicity as measured by the membrane integrity assay.

Many chemotherapeutic agents have been found to retain the activity of apoptosis (26). The integrated molecular profiling approach used in our study reveals that the growth-inhibiting effects of gossypol seemed to be mostly associated with the induction of apoptosis. We observed that the exposure of DU145 cells to 10 μmol/L gossypol resulted in the activation of 13 proteins (Fig. 2A), 7 transcription factors (Fig. 2C), and the expression of 17 genes (Fig. 3) involved in apoptosis. Furthermore, our observation that gossypol treatment lead to an increase in PI and Annexin V staining and increased activity of caspase-3/7 confirms that gossypol induces apoptosis of prostate cancer cells.

There are two pathways currently proposed to play major roles in regulating apoptosis in mammalian cells: caspase-8-mediated extrinsic pathway and mitochondria-related intrinsic (27). Although both pathways share a common downstream caspase-3/7 protease signaling step, the intrinsic pathway involves the permeabilization of the outer mitochondrial membrane by proapoptotic proteins such as Bax and Bad, resulting in the release of Smac/DIABLO and cytochrome c, which in turn leads to the activation of caspase-9 (28). These studies show that gossypol activated proteins involved in the mitochondrial pathway such as Bad and Smac/Diablo as early as 15 minutes posttreatment. After 1 hour of gossypol treatment, we noticed increased transcript levels of proapoptotic genes such as Bak1, PARP6, AIF, Cyto C, and Nexo and a decrease in transcript levels of Bcl-2 and Bcl-XL. Moreover, an increase in the activation of caspase-9, and not caspase-8, strongly suggests that the intrinsic mitochondrial pathway is engaged in gossypol-induced apoptosis.

Activation of the p53 pathway is required for apoptosis induction by growth factor withdrawal, hypoxia, and DNA damage (29). It is well established that p53 mediates apoptosis by transcribing genes that encode the mitochondrial pathway of apoptosis, such as Bax (29, 30). The current studies reveal that the gossypol-induced apoptotic response in prostate cancer cells is, in part, achieved through DNA damage and p53 activation.
Treating DU145 cells with gossypol lead to an increase in length of the mean tail moment and transcripts of genes involved in DNA damage. Furthermore, we observed that within 15 minutes of exposure to gossypol, p53 was stabilized, as evident by increased phosphorylation at S392 (Fig. 2A). After 30 minutes, we noticed an increase in its ability to bind DNA (Fig. 2C) and in as little as 1 hour, we detected an increase of p53 transcripts (Fig. 3B) for up to 4 hours. These results, taken together, show that gossypol-induced apoptosis in prostate cancer cells is associated with DNA damage and activation of p53. Although gossypol has been reported to induce apoptosis by inhibiting antiapoptotic Bcl-2 family members and interaction with the mitochondrial caspase pathway (31), to our knowledge, this is the first report to show that gossypol induces DNA damage leading to the activation of p53 and apoptosis.

There is a growing literature supporting that prostate cancer is the result of the hierarchical expansion of pTICs, which function as stem-like cells to maintain malignant growth and contribute to drug resistance (16, 18). Although most cancer cells are apparently killed during chemotherapy, a few pTICs can survive and lead to relapse of disease. The current studies indicate that gossypol is a potential antiprostate cancer agent that can target both the tumor-initiating cells (pTICs) and their differentiated progeny (Fig. 5A). Additionally, we showed that mice engrafted with pTIC and treated with gossypol exhibited a marked depression in tumor incidence and latency (Fig. 6). Furthermore, gossypol increased the activation of caspase-3/7 and caspase-9 activity, but had no effect on the activity of caspase-8, suggesting that the mechanism by which gossypol reduced the viability of pTICs is similar to that of the sorted cells (Fig. 5B).

In conclusion, we have shown, for the first time that gossypol inhibits tumor growth in a NOD/SCID xenograft model and reduces the viability of three prostate cancer cell lines (LAPC4, PC3, and DU145). The inhibitory effect can be attributed to the ability of gossypol to induce apoptosis. Gossypol leads to DNA damage with subsequent activation of p53, which in turn activates apoptosis through the mitochondrial pathway. These results provide molecular information for further investigations on the clinical application of gossypol for prostate cancer prevention/therapy. Our findings that gossypol also inhibits the growth of pTICs could be important in devising a target-based therapeutic strategies that have effects on tumor-initiating cells that may be resistant to conventional therapies. Moreover, gossypol may be an appropriate postconventional treatment as a relatively nontoxic means of suppressing residual pTICs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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