

# Natural BH3 mimetic (-)-gossypol chemosensitizes human prostate cancer via Bcl-xL inhibition accompanied by increase of Puma and Noxa

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## Abstract

Antiapoptotic members of the Bcl-2 family proteins are overexpressed in prostate cancer and are promising molecular targets for modulating chemoresistance of prostate cancer. (-)-Gossypol, a natural BH3 mimetic, is a small-molecule inhibitor of Bcl-2/Bcl-xL/Mcl-1 currently in phase II clinical trials as an adjuvant therapy for human prostate cancer. Our objective is to examine the chemosensitization potential of (-)-gossypol in prostate cancer and its molecular mechanisms of action. (-)-Gossypol inhibited cell growth and induced apoptosis through mitochondria pathway in human prostate cancer PC-3 cells and synergistically enhanced the antitumor activity of docetaxel both *in vitro* and *in vivo* in PC-3 xenograft model in nude mouse. (-)-Gossypol blocked the interactions of Bcl-xL with Bax or Bad in cancer cells by fluorescence resonance energy transfer assay and overcame the Bcl-xL protection of FL5.12 model cells on interleukin-3 withdrawal. Western blot and real-time PCR studies showed that a dose-dependent increase of the proapoptotic BH3-only proteins Noxa and Puma contributed to the cell death induced by (-)-gossypol and to the synergistic effects of (-)-gossypol and docetaxel. The

small interfering RNA knockdown studies showed that Noxa and Puma are required in the (-)-gossypol-induced cell death. Taken together, these data suggest that (-)-gossypol exerts its antitumor activity through inhibition of the antiapoptotic protein Bcl-xL accompanied by an increase of proapoptotic Noxa and Puma. (-)-Gossypol significantly enhances the antitumor activity of chemotherapy *in vitro* and *in vivo*, representing a promising new regime for the treatment of human hormone-refractory prostate cancer with Bcl-2/Bcl-xL/Mcl-1 overexpression. [Mol Cancer Ther 2008;7(7):2192–202]

## Introduction

Androgen deprivation therapy is the cornerstone treatment for men with *de novo* or recurrent metastatic prostate cancer (1). Unfortunately, androgen deprivation therapy is primarily palliative, with nearly all patients progressing to an androgen-independent or hormone-refractory state, for which there is currently no effective therapy (1). Despite several hundred clinical studies of both experimental and approved antitumor agents, chemotherapy has limited activity, with an objective response rate of <50% and no demonstrated survival benefit (2). Thus, androgen-independent disease is the main obstacle to improving the survival and quality of life in patients with advanced prostate cancer and has been the focus of extensive studies (3). There is an urgent need for novel therapeutic strategies for the treatment of advanced prostate cancer by specifically targeting the fundamental molecular basis of progression to androgen independence and the resistance of androgen-independent disease to chemotherapy.

Bcl-2 family proteins are crucial regulators of apoptosis and were first isolated as the products of an oncogene (4). This family of proteins includes both antiapoptotic molecules such as Bcl-2, Bcl-xL, and Mcl-1 and proapoptotic molecules such as Bax, Bak, Bid, Bad, Noxa, and Puma (5, 6). Bcl-2 and Bcl-xL are closely related proteins and both are highly overexpressed in many types of cancers (7). Overexpression of Bcl-2 is observed in 30% to 60% of prostate cancer at diagnosis and in ~100% of hormone-refractory prostate cancer (8, 9). The expression level of Bcl-2 protein also correlates with resistance to a wide spectrum of chemotherapeutic agents and radiation therapy (9–12). Bcl-xL is also overexpressed in ~100% of hormone-refractory prostate cancer and is associated with advanced disease, poor prognosis, recurrence, metastasis, and shortened survival (13, 14). The transition of prostate cancer from androgen dependent to androgen independent is accompanied by several molecular genetic changes, including overexpression of Bcl-2 and Bcl-xL (10, 15). Noxa and Puma are proapoptotic BH3-only proteins, work upstream of Bax

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and Bak to promote mitochondrial depolarization and apoptosis. The BH3-only proteins are classified as activator and sensitizer. Puma is an activator that binds directly to Bax and Bak and promotes their activation, whereas Noxa as a sensitizer binds to the prosurvival proteins and displaces Bim or tBid, allowing them to directly activate Bax and Bak (16). Noxa also engages Mcl-1 and is found to promote Mcl-1 degradation (17, 18).

We have been investigating small-molecule inhibitors of the Bcl-2 family proteins as novel therapeutics for cancer. Recently, (-)-gossypol, a natural product from cottonseed with the BH3 mimetic structure, is identified as small-molecule inhibitor of Bcl-2/Bcl-xL/Mcl-1 and potently induces apoptosis in various cancer cell lines (19, 20). (-)-Gossypol is now in phase II clinical trials for hormone-refractory prostate cancer and other types of cancer at multiple centers in the United States, as one of the world's first small-molecule Bcl-2 inhibitors entered into clinical trial.<sup>4</sup> In the current study, we investigated the therapeutic potential of (-)-gossypol in combination with docetaxel in human hormone-refractory prostate cancer cells *in vitro* and *in vivo*. Our hypothesis is that (-)-gossypol may improve the efficacy of chemotherapy by overcoming apoptosis resistance rendered by Bcl-2/Bcl-xL overexpression, thereby making the prostate cancer cells more sensitive to chemotherapy. Our results should not only facilitate the rational design of clinical trials but also refine the selection of patients who will benefit the most from Bcl-2 molecular therapy.

## Materials and Methods

### Cell Culture and Reagents

Human prostate cancer cell lines PC-3, DU-145, and LNCaP and human lung fibroblast cell line WI-38 were obtained from the American Type Culture Collection. PC-3 cells were routinely maintained in RPMI 1640 (HyClone), whereas DU-145, LNCaP, and WI-38 were maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum (HyClone). Murine pro-B lymphoid cell line FL5.12 stably transfected with Bcl-xL or vector were kindly provided by Dr. Gabriel Nunez and were maintained in IMEM (Life Technologies) supplemented with 10% fetal bovine serum and 10% WEHI-3B (D-) conditional medium as a source of interleukin-3 (IL-3; ref. 32). The cell cultures were maintained in a humidified incubator at 37°C and with 5% CO<sub>2</sub>. (-)-Gossypol was purified from natural racemic gossypol. Briefly, racemic gossypol was reacted with L-phenylalanine methyl ester hydrochloride overnight at room temperature and sodium bicarbonate was added to yield gossypol Schiff's base as a yellow solid. After silica gel column chromatography purification, the solution of the resolved (±)-gossypol-phenylalanine methyl ester Schiff's base was hydrolyzed by a mixture of tetrahydrofuran, glacial acetic acid, and hydrochloric acid at room

temperature for 2 h. The solution was extracted with acetic ether four times and then washed and dried. The (-)-gossypol was collected by filtration and evaporation. Some of the (-)-gossypol used for initial *in vitro* studies was kindly provided by Dr. Shaomeng Wang (University of Michigan) and Dr. Dajun Yang through the National Cancer Institute RAID program. For *in vitro* experiments, (-)-gossypol was dissolved in DMSO at 20 mmol/L as a stock solution. For *in vivo* studies, (-)-gossypol was suspended in carboxymethyl cellulose and then sonicated for 30 min and mixed before each administration. Docetaxel (Taxotere) was purchased from Sanofi-Aventis and diluted in PBS for *in vivo* studies.

### MTT-Based Cell Viability Assay

Cell viability was determined by the MTT-based assay using Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's instruction. Cells (5,000 per well) were plated in 96-well culture plates, and various concentrations of (-)-gossypol or docetaxel were added to the cells in triplicates. Four days later, WST-1 was added to each well and incubated for 1.5 h at 37°C. Absorbance was measured with a plate reader at 450 nm with correction at 650 nm. The results are expressed as the percentage of absorbance of treated wells versus that of vehicle control. IC<sub>50</sub>, the drug concentration causing 50% growth inhibition, was calculated via sigmoid curve fitting using GraphPad Prism 5.0 (GraphPad).

### Apoptosis Assays

For the detection of apoptotic cells using 4',6-diamidino-2-phenylindole staining, PC-3 cells were plated in six-well plates and treated with various concentrations of (-)-gossypol and then stained with 3 mmol/L 4',6-diamidino-2-phenylindole for 10 min. The cells with the nuclei showing morphologic characteristics of apoptosis (nuclear karyopyknosis and fragmentation) were counted as positive under a fluorescent microscope as described (22). For mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) assay, PC-3 were cultured in a chamber slide; after wash with PBS, cells were incubated with the MitoCapture solution at 37°C for 15 min according to the manufacturer's protocol (BioVision). The fluorescence was detected and recorded using a Zeiss LSM-510 confocal microscope. For apoptosis analysis of tumors in animal studies, tumor tissues were excised and stained for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling using the ApopTag kit (Chemicon) according to the manufacturer's instructions.

### Fluorescence Resonance Energy Transfer Assay

(-)-Gossypol-mediated disruption of Bcl-xL heterodimerization with Bax and Bad was analyzed with fluorescence resonance energy transfer (FRET) assay as described (23) with modification. Briefly, DU-145 cells were transiently transfected with Bcl-xL-CFP together with either Bax-YFP or Bad-YFP (kindly provided by Dr. Junyin Yuan) using FuGene 6 reagent (Roche). Twenty-four hours later, the cells were treated with increasing doses of (-)-gossypol or DMSO vehicle control for another 15 h. Cells were then harvested and fixed in 2% paraformaldehyde. The cells

<sup>4</sup> <http://ClinicalTrials.gov>

were plated in 96-well black plates ( $0.2 \times 10^6$  per well) and fluorescence was measured in a Tecan multimode plate reader and calculated based on Dr. Yuan's method as described (23).

#### Western Blot Analysis

Cells were washed with PBS and lysed in an ice-cold radioimmunoprecipitation assay lysis buffer: 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete Protease Inhibitor cocktail (100  $\mu\text{g}/\text{mL}$ ) in PBS. Tumor tissues were incubated with 200  $\mu\text{L}$  lysis buffer in an ice-cold French Douncer for 15 min after homogenization by 40 strokes. The cell lysates and homogenates were cleared by centrifugation at  $13,000 \times g$  for 10 min at  $4^\circ\text{C}$ . The supernatants were collected. Protein concentrations were determined with the Bradford method (Bio-Rad); 60  $\mu\text{g}$  protein was electrophoresed by 12% SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking with 5% milk, blots were probed with anti-Mcl-1, anti-Bcl-2, anti-Bim, anti- $\beta$ -actin, and anti-glyceraldehyde-3-phosphate dehydrogenase (purchased from Santa Cruz Biotechnology), anti-Bcl-xL (BD Biosciences), anti-Puma and anti-caspase-3 (Cell Signaling Technology), and anti-Noxa (Calbiochem).

#### Subcellular Fractionation and Immunoblotting

PC-3 cells were harvested and washed with PBS. Cell pellets were suspended at  $3 \times 10^7/\text{mL}$  in mitochondrial resuspension buffer: 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L  $\text{MgCl}_2$ , 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and 100  $\mu\text{g}/\text{mL}$  cocktail. Digitonin was added to 200  $\mu\text{g}/\text{mL}$ . The cells were incubated in digitonin-supplemented mitochondrial resuspension buffer for 5 min on ice. Cell lysates were then centrifuged for 10 min at  $13,000 \times g$ . Proteins from the supernatant (cytosolic fraction) were respun at  $13,000 \times g$  for 10 min to clear any remaining debris, and pellet (membrane fraction) was washed twice with mitochondrial resuspension buffer. The pellet was solubilized in radioimmunoprecipitation assay buffer and incubated on ice for 10 min. The cytosolic and mitochondria extracts were analyzed by SDS-PAGE. Antibodies used for immunoblotting were anti-cytochrome *c* (BD PharMingen), anti-apoptosis-inducing factor (AIF; Santa Cruz Biotechnology), and anti-cyclooxygenase IV (Molecular Probes).

#### Quantitative Real-time PCR

Total RNA was extracted from the treated cells using TRIZOL (Invitrogen) according to the manufacturer's instructions. Reverse transcription was done using SuperScript III First-Strand kits (Invitrogen). Each quantitative real-time PCR ( $95^\circ\text{C}$  for 10 min, 40 cycles of  $95^\circ\text{C}$  for 15 s,  $60^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 10 s) was done using TaqMan Universal PCR Master Mix (Applied Biosystems). Primers were designed as *Mcl-1* forward 5'-CTCATTTCTTTTGGTGCCTTT-3' and reverse 5'-CCAGTCCCCTTTTGTCTTAC-3', *Noxa* forward 5'-CTGCAGGACTGTTCTGTTTC-3' and reverse 5'-TTCTGCCGGAAGTTCAGTTT-3', and *Puma* forward 5'-TCCTCAGCCCTCGCTCTCGC-3' and reverse 5'-CCGATGCTGAGTCCATCAGC-3'.

#### Small Interfering RNA Transfections

Small interfering RNA (siRNA) oligonucleotides were purchased from Dharmacon with the sequences for *Noxa* (NM\_021127): 5'-CUUCCGGCAGAAACUUCUG-3' (24) and *Puma* (NM\_014417): 5'-UCUCAUCAUGGGACUCUG-3' (25); PC-3 cells were transfected 24 h after being seeded in six-well plates. siRNA (100 pmol) were dissolved in 200  $\mu\text{L}$  serum-free, antibiotic-free medium; Lipofectamine 2000 transfection reagent (5  $\mu\text{L}$ ; Invitrogen) was dissolved in 200  $\mu\text{L}$  of the same medium; the above two solutions were mixed and allowed to stand at room temperature for 20 min. The resulting 400  $\mu\text{L}$  transfection complexes were then added to each well containing 1.6 mL medium. Six hours later, the cultures were replaced with fresh medium supplemented with 10% fetal bovine serum and antibiotics. (-)-Gossypol was added to the cells 24 h after transfection as indicated. For Western blot, cells were collected after an additional 24 h; for cell survival assay, cells were collected after 4 days.

#### Animal Tumor Model and *In vivo* Experiments

Double-blinded *in vivo* experiments were carried out with 5- to 6-week-old male athymic NCr-*nu/nu* nude mice purchased from the National Cancer Institute. After alcohol preparation of the skin, mice were inoculated s.c. with 0.1 mL PC-3 cell suspension ( $5 \times 10^6$  cells) on both flanks using a sterile 22-gauge needle. When tumors reached 100  $\text{mm}^3$ , the mice were randomized into six groups with 5 to 8 mice per group. Group 1 was given carboxymethyl cellulose as vehicle control; group 2 was given docetaxel 7.5 mg/kg i.v. once weekly  $\times$  3 weeks; groups 3 and 4 were given (-)-gossypol 10 and 20 mg/kg p.o. q.d. 7 weeks, respectively; groups 5 and 6 were given a combination of docetaxel 7.5 mg/kg i.v. once weekly  $\times$  3 weeks and (-)-gossypol 10 or 20 mg/kg p.o. q.d. 7 weeks, respectively. The tumor sizes and animal body weights were measured twice weekly. Three weeks after the first treatment, one mouse from each group was sacrificed and the tumors were dissected. Tumor tissues with a size of  $\sim 8 \text{ mm}^3$  were prepared for Western blot as described above. All animal experiments were done according to the protocol approved by University of Michigan Guidelines for Use and Care of Animals.

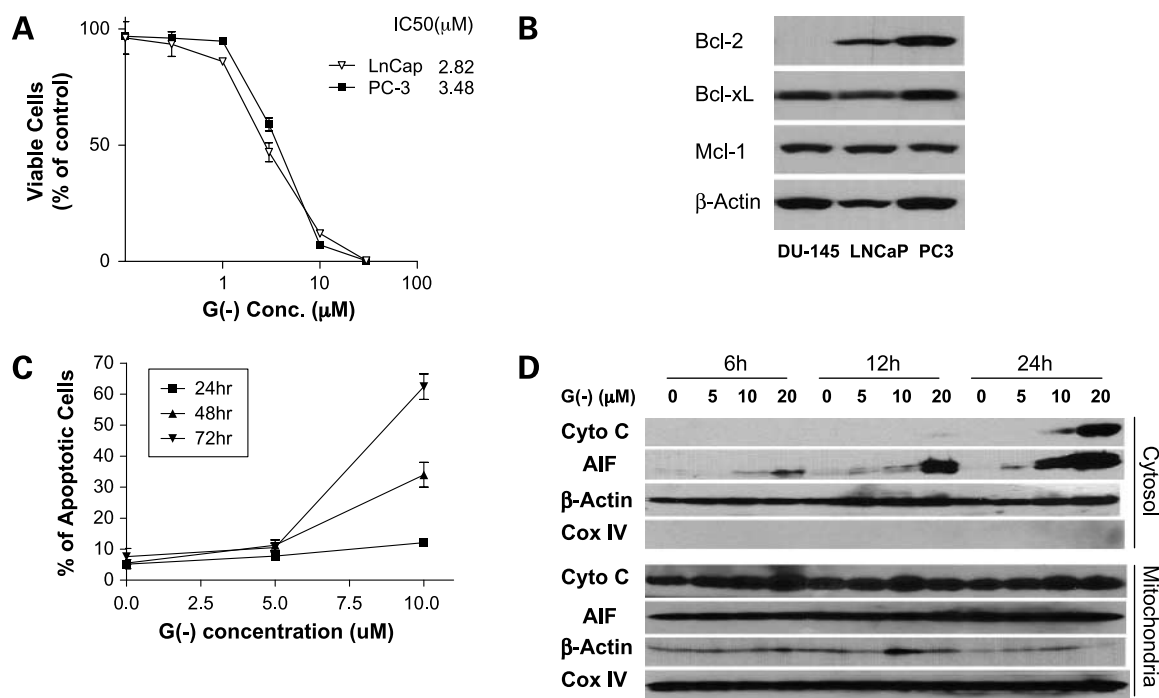
#### Statistical Analysis

Two-tailed Student's *t* test and two-way ANOVA were employed to analyze the *in vitro* and *in vivo* data, respectively, using Prism 5.0 software (GraphPad Prism).

## Results

### (-)-Gossypol Induces Apoptosis through Mitochondria Pathway in Human Prostate Cancer Cells

To evaluate the antitumor activity of (-)-gossypol in prostate cancer cells, we carried out the MTT-based cell viability assay. Human prostate cancer cell lines PC-3 (p53-null) and LNCaP (p53 wild-type) as well as human lung fibroblast cell line WI-38 were exposed to (-)-gossypol. As shown in Fig. 1A, (-)-gossypol potently inhibited the growth of PC-3 and LNCaP cells but had minimal effect on normal fibroblast WI-38 cells. The  $\text{IC}_{50}$  was  $3.5 \mu\text{mol}/\text{L}$



**Figure 1.** (-)-Gossypol causes cell death and induces apoptosis through mitochondria pathway in human prostate cancer cell line PC-3. **A**, MTT-based cell viability assay of (-)-gossypol in PC-3 and LNCaP cells. Cells were seeded in 96-well plates and treated in triplicates. Mean  $\pm$  SE normalized to their respective controls ( $n = 3$ ). **B**, Western blot analysis showed the difference of Bcl-2, Bcl-xL, and Mcl-1 protein levels in three prostate cell lines (60  $\mu$ g/lane). **C**, apoptotic cells were counted with 4',6-diamidino-2-phenylindole staining. Cells were counted in five different fields. Mean  $\pm$  SE. **D**, cytochrome *c* and AIF release from mitochondria to cytosol. PC-3 cells were treated with (-)-gossypol for indicated times and then subjected to a digitonin-based subcellular fractionation as described in Materials and Methods. Cytosolic fractions ( $\sim 120$   $\mu$ g/lane) and mitochondria fractions ( $\sim 60$   $\mu$ g/lane) were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. The cyclooxygenase IV marker was used to determine cross-contamination of cytosolic fractions with mitochondrial proteins.

for PC-3 cells, 2.8  $\mu$ mol/L for LNCaP cells, and 17.4  $\mu$ mol/L for WI-38 cells (Fig. 1A). It was noted that the protein expression of Bcl-2, Bcl-xL, and Mcl-1 differed among these cell lines. PC-3 cells have higher levels of Bcl-2, Bcl-xL, and Mcl-1 (Fig. 1B) than LNCaP cells and form good xenograft tumors in nude mice. Therefore, PC-3 is an appropriate cell line for studying the (-)-gossypol activity and mechanism in prostate cancer.

(-)-Gossypol-induced apoptosis was also assessed by 4',6-diamidino-2-phenylindole staining and counting the cells with karyorrhexis, a typical apoptotic change of cells (22). PC-3 cells treated with 10  $\mu$ mol/L (-)-gossypol for 48 and 72 h showed dramatically increased apoptosis (Fig. 1C). Cytochrome *c* and AIF released from the intermembrane space of mitochondria contribute to caspase-dependent and caspase-independent apoptosis (26, 27). We separated the mitochondria from cytosol and examined the cytochrome *c* and AIF release in (-)-gossypol-treated PC-3 (Fig. 1D). Cytochrome *c* translocated from mitochondria to cytosol 24 h after (-)-gossypol treatment. Interestingly, AIF was released from mitochondria into cytosol at 6 h, preceding the cytochrome *c* release, suggesting that AIF-mediated, caspase-independent apoptosis might also be involved in (-)-gossypol-induced cell death. By using MitoCapture staining, we observed the loss of  $\Delta\Psi_m$ , one of the early

events in apoptosis induction via mitochondrial pathway (28), associated with (-)-gossypol-induced apoptosis. As shown in Supplementary Fig. S1,<sup>5</sup> in control cells, consistent with mitochondrial localization, the bright punctuated yellow/orange fluorescence was found mostly in granular structures distributed throughout the cytoplasm. Exposure of PC-3 cells to 20  $\mu$ mol/L (-)-gossypol for 6 h induced a marked change in  $\Delta\Psi_m$  as evidenced by the disappearance of bright yellow/orange fluorescence and an increase of green fluorescence in most cells with a predominantly perinuclear distribution.

#### (-)-Gossypol Sensitizes PC-3 Cells to Docetaxel-Induced Growth Inhibition and Apoptosis

To investigate whether (-)-gossypol sensitizes the prostate cancer cells to chemotherapy, we examined the cytotoxic effect of the combination of (-)-gossypol and docetaxel, a first-line chemotherapy currently used for hormone-refractory prostate cancer. Figure 2A shows that, as the (-)-gossypol dose increased, there was an obvious left shift of the cytotoxicity curves, indicating that the PC-3 cells

<sup>5</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

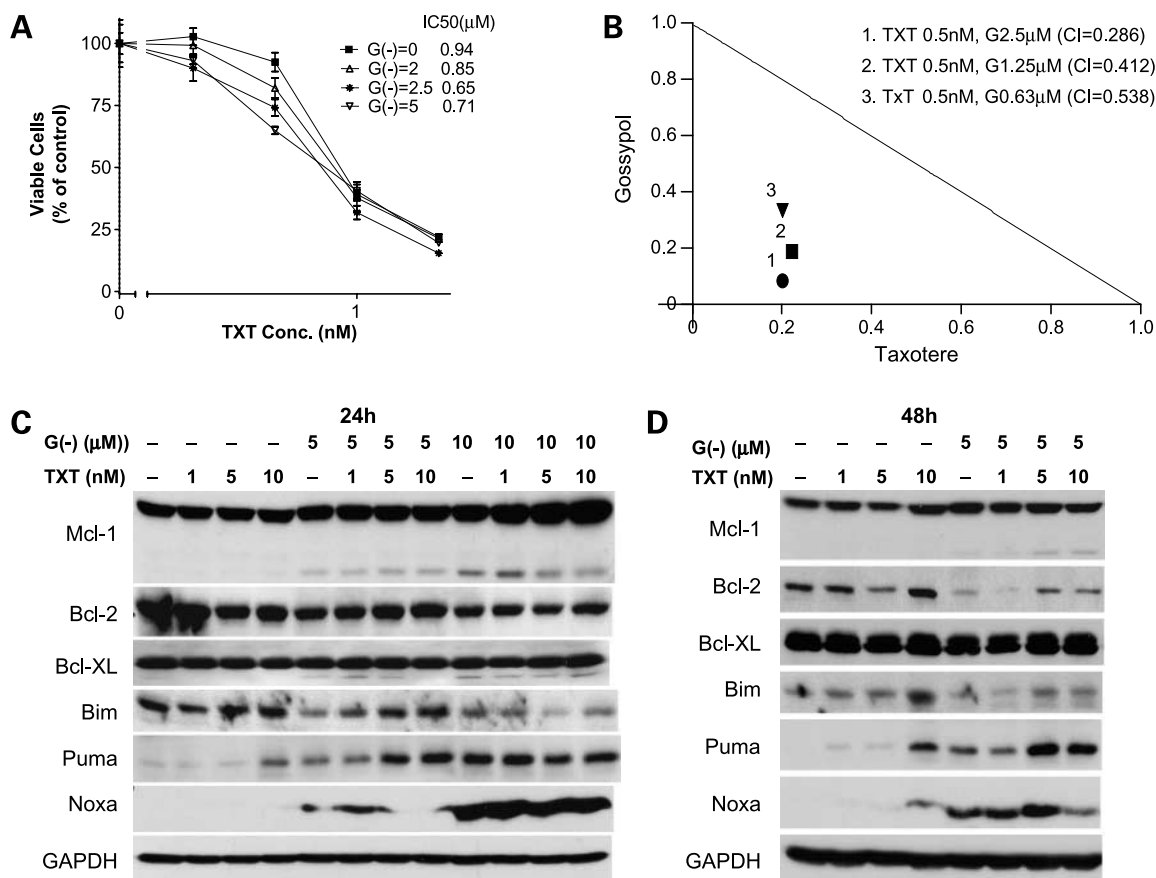
were sensitized to docetaxel by (-)-gossypol. To assess whether the combined effects were synergistic or additive, the combination index value was calculated and isobologram was plotted (Fig. 2B) as we described previously (20). Combination index < 1 is considered synergistic of the combination treatment (20). The combination treatment of 0.5 nmol/L docetaxel with 0.63, 1.25, or 2.5  $\mu$ mol/L (-)-gossypol resulted in synergistic effects (combination indices = 0.286, 0.412, or 0.538, respectively).

We next investigated the changes of several Bcl-2 family members at protein level. As shown in Fig. 2C and D, no significant change was observed in Bcl-2 and Bcl-xL proteins. This is consistent with an earlier report (29) in large cell lymphoma and supports that (-)-gossypol is a functional inhibitor of Bcl-2 and Bcl-xL via binding to the BH3 domain of the proteins without affecting protein levels directly. (-)-Gossypol-treated PC-3 cells showed an increase of Noxa and Puma as well as a decrease of Bim and a dose-dependent increase of Mcl-1 (both full-length and a shortened form; Fig. 2C and D). Combination with

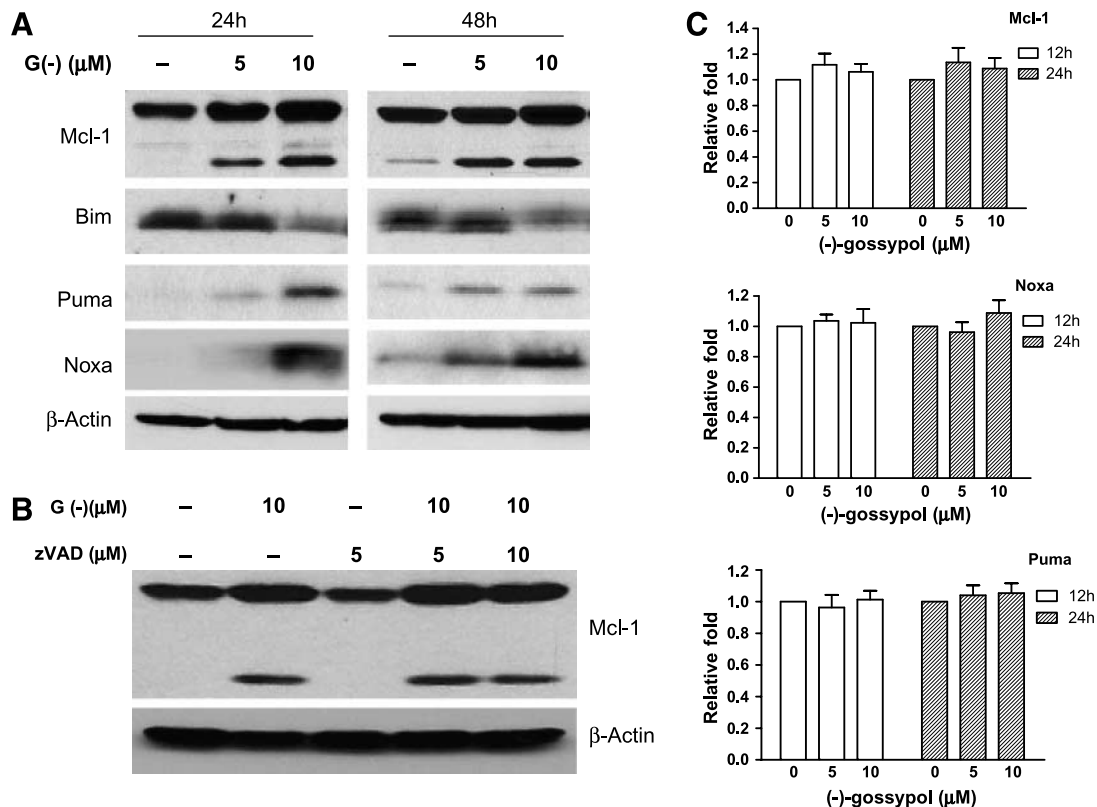
docetaxel reversed the Bim decrease and further increased Puma levels in a time- and dose-dependent manner (Fig. 2C and D), which might contribute to the synergistic effect of the combination therapy.

#### Noxa and Puma Knockdown by siRNA Attenuates (-)-Gossypol Effects on PC-3 Cells

To further investigate the role of Bcl-2 family members in (-)-gossypol-induced apoptosis, we examined the BH3-only proteins Noxa and Puma in (-)-gossypol-treated PC-3 cells by Western blot. Here again, PC-3 cells treated with (-)-gossypol for 24 and 48 h showed a dose-dependent increase of Noxa and Puma proteins as well as Mcl-1 (both full-length and a shortened form; Fig. 3A). This increase was not accompanied by clear and robust increase of their mRNA levels as assessed by quantitative real-time PCR (Fig. 3C), indicating that Mcl-1 increase was post-translational. It is not clear at the present what the shortened form Mcl-1 is. It could be either the alternatively spliced Mcl-1 or the caspase-cleaved Mcl-1, both of which have been reported to be proapoptotic and associated with apoptosis



**Figure 2.** (-)-Gossypol enhances the chemotherapy of docetaxel in human prostate cancer PC-3 cells. **A**, MTT-based cell viability assay of (-)-gossypol docetaxel in PC-3. **B**, IC<sub>50</sub> isobologram of the combination treatments. In the isobologram, a plot on the diagonal line indicates that the combination is simply additive. A plot to the left under the line indicates that the combination is synergistic, whereas a plot to the right above the line indicates that it is antagonistic. Combination index < 1 is considered synergistic of the combination treatment (20). **C** and **D**, Western blot analysis showed the effect of docetaxel and (-)-gossypol on proteins expression in PC-3 cells. Cells were treatment with docetaxel and (-)-gossypol of the indicated doses for 24 h (**C**) or 48 h (**D**) and then collected for Western blot (60  $\mu$ g/lane). Data with (-)-gossypol 10  $\mu$ mol/L treatment for 48 h were not shown because of severe cell death.



**Figure 3.** (-)-Gossypol increases Noxa, Puma, and Mcl-1 and decreases Bim at protein levels. **A**, (-)-gossypol dose-dependently induced Mcl-1, Noxa, and Puma increase and Bim decrease. **B**, (-)-gossypol-induced Mcl-1 increase is caspase independent. PC-3 cells were pretreated with zVAD-fmk for 4 h and then incubated with or without (-)-gossypol for another 24 h before collecting for Western blot analysis. **C**, changes of mRNA levels of Mcl-1, Noxa, and Puma. PC-3 cells were treated with (-)-gossypol at the indicated time points and doses. Quantitative real-time PCR with TaqMan Universal PCR Master Mix (Applied Biosystems) were done on the Mastercycler *realplex* 2 system (Eppendorf). Target gene mRNA levels were normalized to actin mRNA according to the following formula:  $[2^{-C_T^{\text{target}}} - C_T^{\text{actin}}] \times 100\%$ , where  $C_T$  is the threshold cycle. Fold increase was calculated by dividing the normalized target gene expression of the treated sample with that of the untreated control.

induction (30, 31). Interestingly, pan-caspase inhibitor zVAD did not block the Mcl-1 increase nor prevented the increase of the shortened form (Fig. 3B), indicating that the (-)-gossypol-induced Mcl-1 increase was caspase independent, and the shortened form might not be the caspase-cleaved Mcl-1 but is likely to be the alternatively spliced proapoptotic Mcl-1.

To evaluate the role of Noxa and Puma in the (-)-gossypol-induced cell response, we employed RNA interference to knock down Noxa and Puma in PC-3 cells before treatment with (-)-gossypol. Figure 4A shows that Noxa and Puma were effectively knocked down (>95%) by respective siRNA in PC-3 cells. Figure 4B shows that knockdown of Noxa or Puma shifted the (-)-gossypol dose-response curve to the right, with the Noxa/Puma double-knockdown cells most resistant to (-)-gossypol ( $P < 0.001$ , versus control siRNA, two-way ANOVA,  $n = 3$ ). Similar results were observed with colony formation assay (Fig. 4C and D). These data show that Noxa and Puma knockdown rendered PC-3 cells resistant to (-)-gossypol-induced cell death and clonogenic growth, whereas Noxa/Puma double knockdown most effectively attenuated the (-)-gossypol

activity. Our results support that Noxa and Puma are involved in (-)-gossypol-mediated growth inhibition and cell death in PC-3 cells.

#### (-)-Gossypol Dose-Dependently Disrupts Bcl-xL Heterodimerization with Bax and Bad

Using the fluorescence resonance energy transfer assay system described by Dr. Yuan's group (23), we investigated the effect of (-)-gossypol on Bcl-xL heterodimerization with Bax or Bad. (-)-Gossypol dose-dependently inhibited the FRET signal; it inhibited the interaction between Bcl-xL and Bax (Fig. 5A) or Bad (Fig. 5B). (-)-Gossypol (5 μmol/L) blocked 98.5% of interaction between Bcl-xL and Bax and 77% of interaction between Bcl-xL and Bad. The plate-based FRET assay results were also confirmed by FRET analysis under confocal laser scanning microscopy (data not shown). There is a good correlation between (-)-gossypol cellular activity (growth inhibition and apoptosis induction) and the blocking of Bcl-xL-Bax (Pearson's correlation  $r = 0.941$ ) as well as the blocking of Bcl-xL-Bad interaction (Pearson's correlation  $r = 0.994$ ). These data suggest that Bcl-xL might be one of the major molecular targets of (-)-gossypol in cell growth inhibition and

apoptosis induction. These data further support that (-)-gossypol is a potent functional inhibitor of the antiapoptotic protein Bcl-xL.

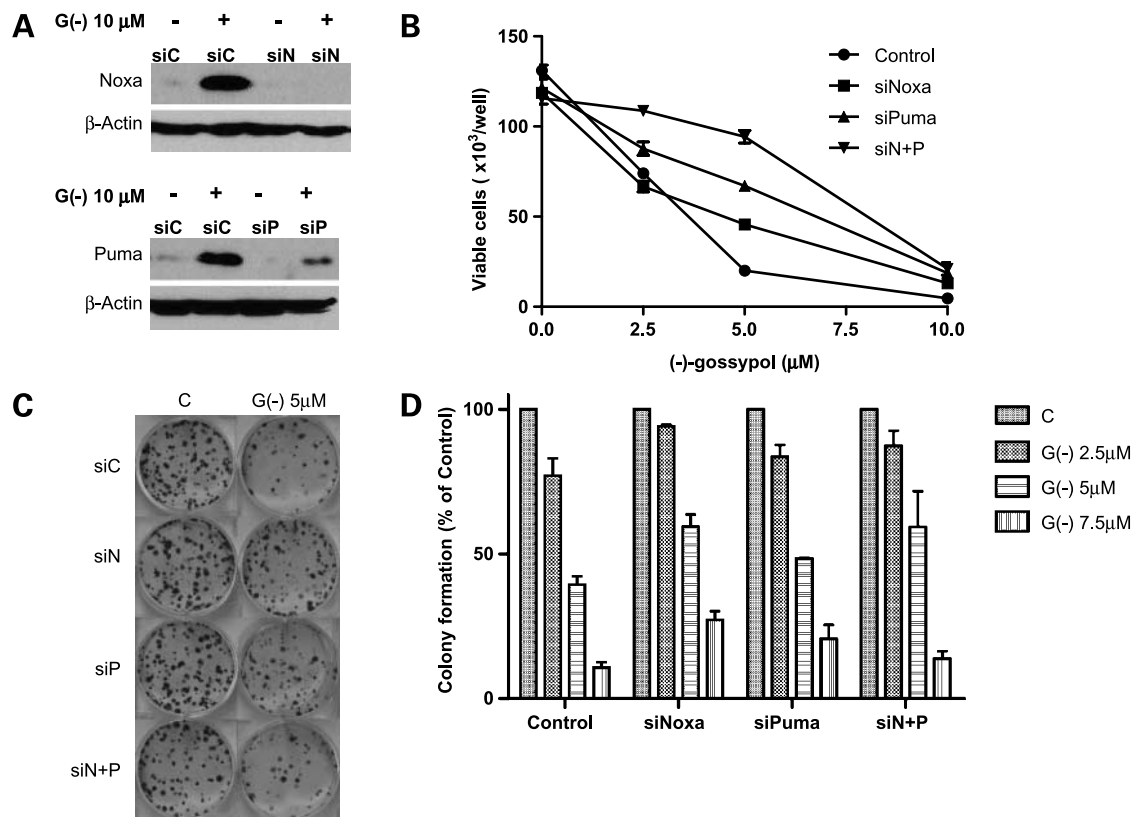
#### (-)-Gossypol Overcomes Bcl-xL Protection of FL5.12 Model Cells from Cell Death Induced by IL-3 Withdrawal

FL5.12 cells depend on IL-3 for growth and survival in culture and rapidly undergo apoptosis on withdrawal of the growth factor IL-3 (32). However, when antiapoptotic Bcl-2 or Bcl-xL genes were transfected into FL5.12 cells, they became resistant to apoptosis induction by IL-3 withdrawal (21, 32, 33). Therefore, the FL5.12 is a model system that is frequently used to analyze the antiapoptotic activity of Bcl-2/Bcl-xL. Parental FL5.12 cells transfected with control vector (FL5.12-neo) undergo apoptosis after IL-3 withdrawal, whereas FL5.12 cells overexpressing Bcl-2 or Bcl-xL are protected (21). As shown in Fig. 5C, FL5.12-neo cells rapidly died on IL-3 withdrawal. FL5.12-Bcl-xL cells remained alive after IL-3 deprivation but did not grow as well as the cells in the presence of growth factor IL-3. Our data are consistent with the literature (21, 32, 33) in that Bcl-xL is a potent inhibitor of apoptosis and thus can protect the FL5.12 cells from cell death induced by IL-3

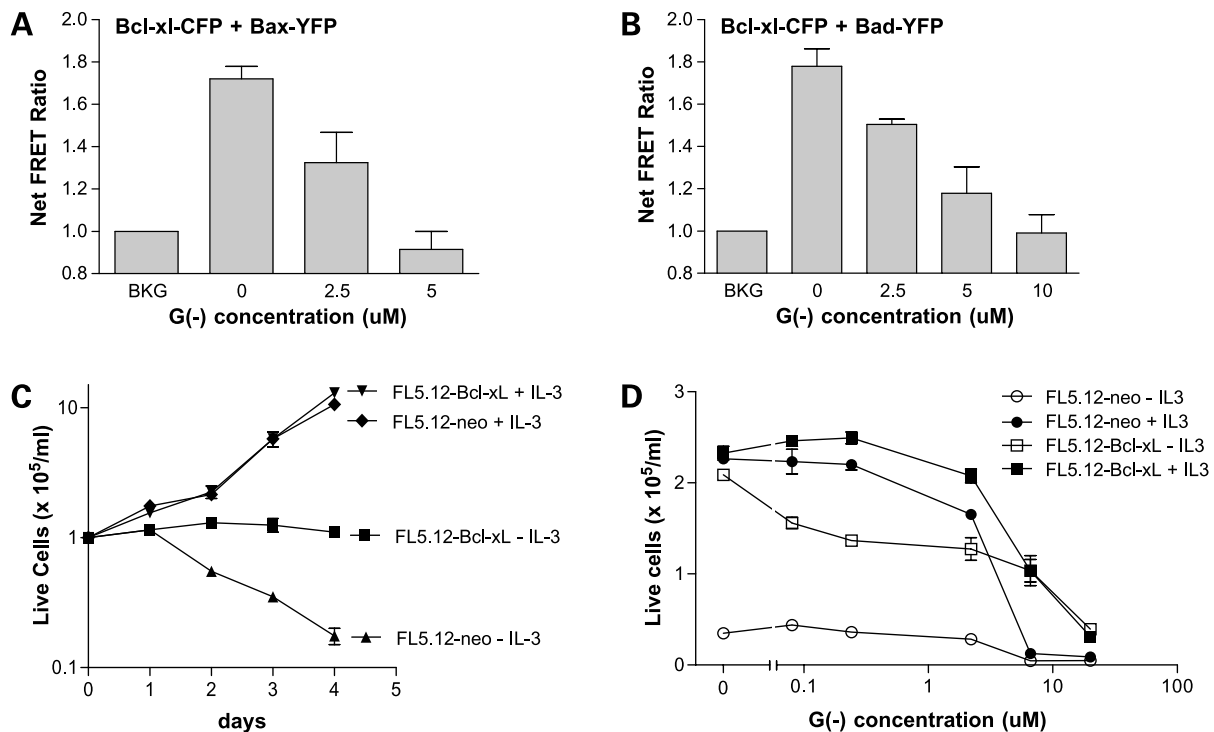
deprivation. On the other hand, because Bcl-xL is not a growth factor on its own, FL5.12-Bcl-xL cells without IL-3 did not grow as fast as those with IL-3 (Fig. 5C). For (-)-gossypol treatment study, the FL5.12 cells were plated in 24-well plates and treated in duplicates with different doses of (-)-gossypol for 2 days, and the live cells were counted by trypan blue exclusion as described (21). As shown in Fig. 5D, the low doses (<6  $\mu\text{mol/L}$ ) of (-)-gossypol did not kill FL5.12-neo and FL5.12-Bcl-xL cells in the presence of IL-3; however, in FL5.12-Bcl-xL cells in the absence of IL-3, whose survival is solely dependent on protection from Bcl-xL, the nontoxic dose of (-)-gossypol potentially killed the FL5.12-Bcl-xL cells, showing that (-)-gossypol can overcome the Bcl-xL protection of FL5.12 cells on IL-3 withdrawal.

#### (-)-Gossypol Enhances Prostate Cancer Response to Docetaxel and Inhibits Tumor Growth *In vivo*

To investigate whether (-)-gossypol can sensitize PC-3 cells to docetaxel chemotherapy *in vivo*, we employed a PC-3 xenograft model in athymic nude mice. As shown in Fig. 6A, oral (-)-gossypol given daily at 20 mg/kg dose significantly improved docetaxel efficacy on PC-3 tumors ( $P < 0.001$  versus docetaxel alone, ANOVA,  $n = 16$ ). Similar



**Figure 4.** Noxa and Puma knockdown by siRNA attenuate the effects of (-)-gossypol on PC-3 cells. PC-3 cells transfected with Noxa and Puma siRNA for 24 h were used in the following assays. **A**, transfected PC-3 cells were treated with 10  $\mu\text{mol/L}$  (-)-gossypol for an additional 24 h and lysed for Western blot; **B**, transfected PC-3 cells were seeded in 24-well plates and treated in triplicates with (-)-gossypol for 4 d, and the viable cells were counted by trypan blue staining. Mean  $\pm$  SD of cells per well ( $\times 10^3$ ). **C**, transfected PC-3 cells were plated in six-well plates (300 per well) and treated in triplicates with (-)-gossypol for colony formation. The plates were cultured for 14 d and stained. Representative pictures of the colonies in the plates. **D**, colonies with >50 cells were counted. Mean  $\pm$  SD ( $n = 3$ ). The assays have been repeated in four independent experiments with similar results.



**Figure 5.** (-)-Gossypol disrupts Bcl-xL heterodimerization with Bax and Bad and overcomes Bcl-xL protection of FL5.12 cells. **A** and **B**, FRET analysis of (-)-gossypol-mediated disruption of Bcl-xL-CFP heterodimerization with Bax-YFP (**A**) or Bad-YFP (**B**). DU-145 cells were transiently transfected with Bcl-xL-CFP together with Bax-CFP or Bad-YFP for 24 h and then treated with (-)-gossypol for 15 h. The cells were counted and plated in a 96-well black assay plate and the fluorescence was measured in a Tecan multimode plate reader at excitation/emission = 430/485 nm (for CFP), 514/535 nm (for YFP), and 430/535 nm (for FRET). Results are net FRET ratios ( $n = 3$ ) normalized with background (set BKG = 1), which is the fluorescence cross-talk (leak-through) from the cells separately transfected with Bcl-xL-CFP and Bax-YFP or Bad-YFP. **C**, FL5.12/Neo and FL5.12/Bcl-xL cells were cultured in the presence or absence of IL-3. The cell numbers were counted daily by trypan blue exclusion. Bcl-xL expression protected the cells from cell death induced by IL-3 withdrawal. **D**, FL5.12/Neo and FL5.12/Bcl-xL cells were treated with the indicated concentrations of (-)-gossypol in the presence or absence of IL-3 for 48 h, and the viable cells were counted. These experiments were repeated two to four times with consistent results. Representative results.

results were shown when 10 mg/kg (-)-gossypol was administered (Supplementary Fig. S2A).<sup>5</sup> Tumor growth delay analysis (Fig. 6B) showed that docetaxel combined with (-)-gossypol was more effective in inhibiting tumor growth compared with either treatment alone ( $P < 0.05$  versus docetaxel alone;  $P < 0.01$  versus (-)-gossypol alone). Supplementary Table S1<sup>5</sup> summarizes the tumor growth inhibition values, calculated from the median tumor size of the treatment group divided by that of the control group on day 32. The combination of docetaxel with (-)-gossypol 10 and 20 mg/kg had tumor growth inhibition values of 37.3% and 29.4%, both of which are  $<42\%$ , a value considered efficacious according to the National Cancer Institute criteria (34). It is worth noting that (-)-gossypol alone at 10 and 20 mg/kg showed similar but limited effects on PC-3 tumor growth ( $P > 0.05$ ); neither is active as single treatment according to National Cancer Institute criteria, consistent with our earlier study (20). The animal body weight loss was moderate, transient, and tolerable and recovered to normal soon after docetaxel treatment was complete (Supplementary Fig. S2B), indicating that the observed toxicity in the combination therapy was reversible and can be managed.

#### (-)-Gossypol Increases Docetaxel-Induced Apoptosis of PC-3 Tumors *In vivo*

Because our *in vitro* study showed that (-)-gossypol increased the docetaxel-induced apoptosis, we also sought to determine whether this is still the case *in vivo*. We randomly picked one mouse from each group at the end of docetaxel treatment (week 3) and took the tumor tissues to perform terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining for apoptosis. Results are shown in Fig. 6C. (-)-Gossypol plus docetaxel induced significantly more apoptosis than either (-)-gossypol or docetaxel alone. Quantification of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells clearly showed that (-)-gossypol enhanced the apoptotic effect of docetaxel in a dose-dependent manner (Fig. 6D). The effects of combination therapy were also evaluated on the proteins related to the apoptosis signaling pathway. Western blot analysis was done on PC-3 tumor xenografts treated with 3 weeks of (-)-gossypol and docetaxel. (-)-Gossypol- and docetaxel-treated tumors showed poly(ADP-ribose) polymerase cleavage [(-)-gossypol 10 mg/kg] and caspase-3 cleavage [(-)-gossypol 20 mg/kg; Supplementary Fig. S2C].

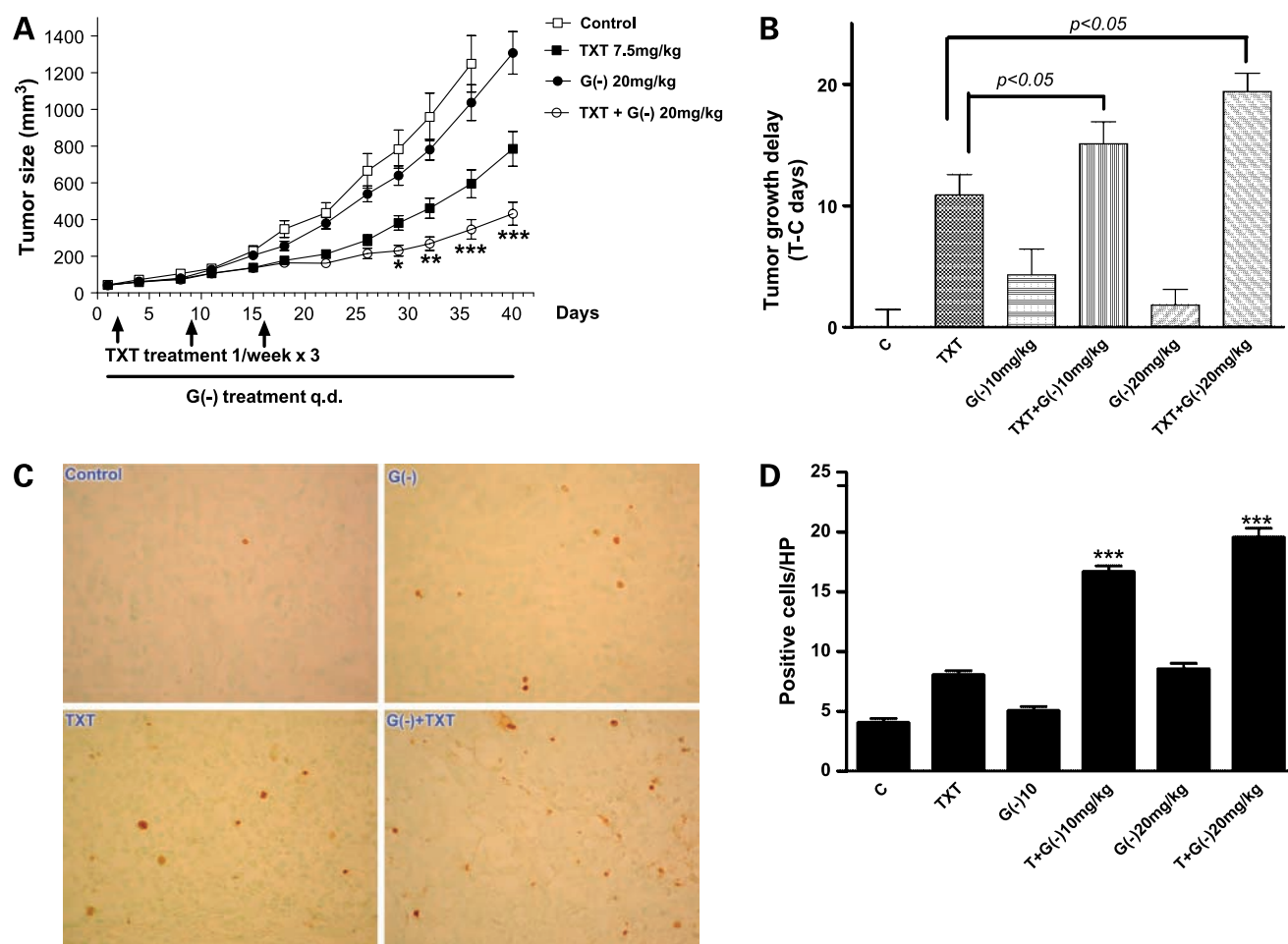


## Discussion

In this study, we have employed (-)-gossypol, a small-molecule inhibitor of Bcl-2/Bcl-xL/Mcl-1, to investigate whether (-)-gossypol potentiates the response of prostate cancer to chemotherapy and whether this potentiation is accompanied with an increase of drug-induced apoptosis. Our *in vitro* and *in vivo* data show that (-)-gossypol inhibits tumor growth and induces apoptosis in human prostate cancer PC-3 cells with high levels of Bcl-2/Bcl-xL proteins. The FRET assay confirmed that (-)-gossypol potently blocks the interactions of Bcl-xL with Bax or Bad in live cells in a dose-dependent manner. In Bcl-xL-transfected model cell lines, (-)-gossypol overcomes the Bcl-xL-mediated protec-

tion of FL5.12 cells on IL-3 withdrawal. The data support that (-)-gossypol exerts its antitumor activity, at least in part, through functional inhibition of the antiapoptotic protein Bcl-xL, although other targets might also be involved. More importantly, (-)-gossypol significantly improved the antitumor activity of current chemotherapeutic agent docetaxel in PC-3 cells both *in vitro* and *in vivo* in a nude mice xenograft model. This enhanced response to chemotherapy is correlated with increased induction of apoptosis *in vivo* by the combination therapy.

(-)-Gossypol has recently been reported as a natural BH3 mimetic small-molecule inhibitor of both Bcl-2 and Bcl-xL and induces apoptosis in multiple cancer cell lines with



**Figure 6.** (-)-Gossypol potentiates docetaxel in inhibiting tumor growth and inducing apoptosis in xenograft model of human prostate cancer PC-3. **A**, PC-3 xenograft tumor growth curves. PC-3 cells ( $5 \times 10^5$ ) were s.c. injected into the flanks on both sides of each mouse. When the tumors reached 50 mm<sup>3</sup>, the mice were randomized into 5 to 8 mice per group and treated with 20 mg/kg (-)-gossypol, 7.5 mg/kg docetaxel, or combination of both. (-)-Gossypol was administered p.o. via oral gavage q.d.; docetaxel was administered i.v. once weekly for 3 wk. The average tumor sizes were shown ( $n = 10-16$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with docetaxel alone, two-way ANOVA ( $n = 14-18$ ). Representative results of at least four independent animal experiments. **B**, tumor growth delay (T-C) analysis. *T* is the median time (in days) required for the treatment group tumors to reach 750 mg, and *C* is the median time (in days) for the control group tumors to reach 750 mg (T-C value for control group is zero). **C** and **D**, tumor tissues from each treatment group were taken at the end of the third week and fixed in 10% formalin. The tumor sections were stained for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling using the ApopTag kit. The apoptotic cells have DNA fragmentation and are stained positive as brown nuclei. **C**, representative pictures: *C*, control; *TXT*, docetaxel; *G(-)*, (-)-gossypol; *TXT + G(-)*, docetaxel + (-)-gossypol. Original magnification,  $\times 400$ . **D**, quantification of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining positive cells. Positive cells were counted under  $\times 400$  magnification at eight different fields, and the average numbers were calculated and plotted. \*\*\*,  $P < 0.001$ , compared with docetaxel alone, *t* test.

high levels of Bcl-2/Bcl-xL (19, 35). These studies show that (-)-gossypol as a potent inducer of apoptosis in these cancer cells is well tolerated and is clinically safe. Oliver et al. (36) showed that (-)-gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-xL-mediated apoptosis resistance. Reports have shown that (-)-gossypol has a significant antitumor activity as a potential novel therapy for the treatment of lymphoma *in vitro* and *in vivo* (29) as well as head and neck cancer cells *in vitro* (37). We have shown previously that (-)-gossypol potently enhanced radiation-induced apoptosis and growth inhibition of human prostate cancer PC-3 cells and improved antitumor activity of X-ray irradiation to PC-3 cells *in vivo*, resulting in tumor regression even in large tumors (20). Interestingly, the (-)-gossypol dose of 1 to 5  $\mu\text{mol/L}$  used in the current study is the dose that can block Bcl-xL-Bax and Bcl-xL-Bad protein-protein interactions in our FRET assay, whereas the same doses of (-)-gossypol potently and specifically overcome the Bcl-xL protection of FL5.12-Bcl-xL cells from cell death induced by IL-3 withdrawal. The same doses of (-)-gossypol also inhibited cell growth of PC-3 and LNCaP cells that have high levels of Bcl-xL in the current study and potently radiosensitized these cells in our earlier publication (20). Taken together, our data support that (-)-gossypol can kill cancer cells, at least in part, through inhibiting the antiapoptotic function of Bcl-xL. Our results show that Bcl-xL is one of the major targets of (-)-gossypol in prostate cancer cell growth inhibition.

The Bcl-2 family of proteins, which includes  $\geq 17$  members in mammalian cells, functions as a "life/death switch" that integrates diverse intercellular and intracellular cues to determine whether a cell should undergo apoptosis in response to diverse damage signals (38). The switch operates through the interactions between the proteins within three subfamilies of the Bcl-2 protein family. The prosurvival subfamily (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and also Bcl-B in humans) protects cells exposed to diverse cytotoxic conditions, whereas two other subfamilies, many members of which were identified as Bcl-2-binding proteins, instead promote cell death (38). They are the Bax-like apoptotic subfamily (Bax, Bak, and Bok) and the BH3-only protein subfamily (includes at least eight members: Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, and Puma; refs. 16, 39). Earlier studies with Noxa- and Puma-knockouts show that Noxa and Puma are critical mediators of the apoptotic responses induced by p53 and cytotoxic drugs. BH3-only proteins Noxa, Puma, and Bim have both overlapping and specialized roles as death initiators in either p53-dependent or p53-independent apoptosis (39). In the current study, (-)-gossypol dose-dependently increased the protein levels of proapoptotic Noxa and Puma independent of p53 (PC-3 is p53 null). This effect on Puma was augmented by combination with docetaxel. Noxa and Puma knockdown by siRNA attenuated the (-)-gossypol-induced cell death and growth inhibition in PC-3 cells, indicating that Noxa and Puma are involved in (-)-gossypol-mediated growth inhibition and cell death in PC-3 cells. Thus, our study provides first proof that (-)-

gossypol exerts its antitumor activity not only through inhibition of antiapoptotic protein Bcl-xL (as well as Bcl-2 and Mcl-1) but also through a p53-independent induction of proapoptotic BH3-only proteins Noxa and Puma.

The molecular mechanism(s) underlying the (-)-gossypol-induced, p53-independent increase of Noxa and Puma are not yet clear. Our data show that (-)-gossypol-induced Noxa and Puma increase appears to be post-translational, without significant change of mRNA levels. The interaction of Mcl-1 with Noxa and Puma in the apoptosis process draws increasing attention recently (40). Mcl-1 stability is tightly regulated; BH3-only proteins Noxa and Bim preferentially bind to Mcl-1 and also target it for proteasomal degradation (41). A recent study (40) using a novel BH3 ligand that selectively binds to Mcl-1 showed that apoptosis can proceed without Mcl-1 degradation. This BH3 mimetic ligand, derived from Bim BH3 mutation, preferentially binds to and stabilizes Mcl-1 and promotes cell death only when Bcl-xL is absent or neutralized (40). Blockage of BH3 domain-containing E3 ubiquitin ligase, Mule, which regulates the basal level of Mcl-1 by targeting it for proteasomal degradation, might be involved in this apparent Mcl-1 stabilization (40). This might account for our results that (-)-gossypol dose-dependently increased Mcl-1 protein level. Based on our data and other reports (42, 43), we propose that the BH3 mimetic (-)-gossypol might work in the same way as this unique BH3 ligand: (a) binds to and inactivates the antiapoptotic function of Bcl-xL (and/or Bcl-2), (b) binds to Mcl-1, blocks and displaces Noxa (and/or Puma), thus blocks Noxa-Mule-mediated Mcl-1 degradation, stabilizes Mcl-1, and increases both full-length and shortened form Mcl-1 (the latter is proapoptotic); and (c) the displaced Noxa and Puma accumulate and promote apoptosis. This mode of action by (-)-gossypol lies upstream of Bax/Bak in the mitochondrial apoptosis pathway. Indeed, a recent report with melanoma cells (43) lends support to this action by (-)-gossypol, which showed that (-)-gossypol was effective in killing cancer cells dependent on Mcl-1/Bcl-xL for survival. This effect was enhanced when the proteasome was blocked and accompanied with increase of Noxa (43). We are currently carrying out detailed studies to delineate the mechanism of Mcl-1 increase and Noxa increase induced by (-)-gossypol.

In conclusion, our study shows that (-)-gossypol significantly enhances the antitumor activity of docetaxel in human prostate cancer both *in vitro* and *in vivo* and may represent a promising new adjuvant therapy with a novel molecular mechanism for the treatment of hormone-refractory human prostate cancer with Bcl-2/Bcl-xL/Mcl-1 overexpression. (-)-Gossypol is now in phase II clinical trials for hormone-refractory prostate cancer. Our results provide insight into how (-)-gossypol works with chemotherapy in prostate cancer cells. Our finding contributes to the rational design of coming clinical trials and refines the selection of patients who will benefit the most from Bcl-2 molecular therapy as a promising novel strategy for overcoming chemoresistance of human prostate cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

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