Preclinical Development

Sorafenib Sensitizes (−)-Gossypol-Induced Growth Suppression in Androgen-Independent Prostate Cancer Cells via Mcl-1 Inhibition and Bak Activation

Jiqin Lian¹, Zhenhong Ni¹, Xufang Dai², Chang Su¹, Amber Rae Smith³, Liang Xu³, and Fengtian He¹

Abstract

The natural BH3-mimetic (−)-gossypol shows promising efficacy in ongoing phase II/III clinical trials for human prostate cancer. Here, we show for the first time, that treatment with (−)-gossypol and multitkinas inhibitor sorafenib synergistically suppresses the growth of androgen-independent prostate cancer cells (AI-PC) in vitro and in vivo. Our data suggest that sorafenib attenuates (−)-gossypol-induced Mcl-1 upregulation in AI-PCs. In this way, it serves as a potent chemosensitizer to affect cell death. Interestingly, (−)-gossypol and sorafenib induce cell death via two distinct pathways among different AI-PCs; DU145 cells via apoptosis and PC-3 via autophagy. The appointed death pathway may depend on the level of proapoptotic protein Bak, although the level of antiapoptotic protein Bcl-2 plays some role in it. DU145 cells with high Bak prefer apoptosis induction, whereas PC-3 cells with low Bak prefer the induction of autophagy. Furthermore, inhibiting nondominant death pathways, that is, autophagy in DU145 and apoptosis in PC-3, enhances cell killing by (−)-gossypol/sorafenib combination therapy. Ultimately, our data expose a new action for sorafenib as an enhancer of (−)-gossypol-induced cell growth suppression and reveal a novel cell death mode by Bak activation manners in AI-PCs. These new insights may facilitate the rational design of clinical trials by selecting patients most likely to benefit from the Bcl-2–targeted molecular therapy. Mol Cancer Ther; 11(2); 416–26. ©2011 AACR.

Introduction

Prostate cancer currently accounts for 28% of all new cancer diagnoses in men. It is estimated that more than 32,050 U.S. men died of this disease in 2010, and the incident rate is likely to increase as the male population ages (1). While androgen ablation is the standard initial therapy for metastatic prostate cancer, eventually all patients will develop castrate-resistant prostate cancer (2). Despite several hundred clinical studies of both experimental and approved antimtuor agents, chemotherapy has limited success with an objective response rate of less than 50% and has not shown survival benefit (3). Thus, androgen-independent disease is a major obstacle in improving the survival and quality of life in patients with advanced prostatic cancer (4). There is an urgent need for novel therapeutic strategies for the treatment of advanced prostatic cancer by specifically targeting the molecular basis of progression to androgen independence and chemotherapy resistance.

Small-molecule inhibitors of antiapoptotic Bcl-2 family members have shown promise in overcoming chemo/radioresistance in various tumor models including prostate cancer (5, 6). (−)-Gossypol (Supplementary Fig. S1; ref. 7), a natural product from cottonseed, has been identified as a small molecule pan-inhibitor of antiapoptotic Bcl-2 family members including Bcl-2, Bcl-xL, and Mcl-1 and induces apoptosis in various types of cancer (8, 9). (−)-Gossypol is now in phase II/III clinical trials for hormone refractory prostate cancer and other types of cancer at multiple centers in the United States (10). We have previously shown that (−)-gossypol sensitized androgen-independent prostate cancer cells (AI-PC) to radiation and chemotherapy both in vitro and in vivo by inhibiting antiapoptotic proteins Bcl-2 and Bcl-xL (11, 12). However, our data also showed that (−)-gossypol upregulated Mcl-1 expression level in AI-PCs, which ultimately may contribute to drug resistance (12).

Sorafenib (Nexavar, BAY 43–9006, Supplementary Fig. S2), a multiple tyrosine kinase inhibitor, has recently shown clinical benefits in patients with AI-PC (13). Several phase I, II, and III clinical trials are currently ongoing in...
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patients with prostate cancer in which sorafenib is used exclusively or in combination with additional chemotherapeutic drugs (13). Mcl-1, the antiapoptotic member of the Bcl-2 family, seems to play a key role in the proapoptotic effect of sorafenib (14). A previous study also showed that sorafenib induces apoptosis and autophagy by inhibiting mitogen-activated protein kinase (MAPK) pathway in prostate cancers in vitro (15), suggesting that the drug exerts an antiproliferative effect in this tumor histotype.

In the present study, we investigated the therapeutic potential of (−)-gossypol in combination with sorafenib in AI-PCs in vitro and in vivo. Our results should advance the rational design of clinical trials by refining the selection of patients who will benefit the most from Bcl-2 molecular therapy and will provide insight for a potential route of adjuvant therapy for patients with prostate cancer.

Materials and Methods

Reagents
(−)-Gossypol, sorafenib, 3-methyl adenine (3-MA), and Z-VA D were from Sigma-Aldrich; Annexin V–FLUOS Staining Kit was from BD Biosciences; caspase-3/CPP32 fluorometric assay kit was from Biovision; antibodies against microtubule-associated protein 1 light chain 3 (LC3), Bcl-xL, PARP, Bak, and Bax were from Cell Signaling Technology, and the antibodies against Bcl-2, Mcl-1, and Beclin1 were from Santa Cruz Biotechnology. Short interfering RNAs (siRNA) to Mcl-1, Bak, Bax, and control siRNA were obtained from Dharmacon. Bcl-2, Bak, and Bax expression vectors were purchased from OriGene. LC3 cDNA was kindly provided by Drs. N. Mizushima and T. Yoshimori (16) at Osaka University, Osaka, Japan. Lipofectamine 2000, TRIzol reagent, and Moloney murine leukemia virus reverse transcriptase were purchased from Invitrogen.

Cell lines and cell culture
Human AI-PC cell line PC-3 and DU145 were obtained from American Type Culture Collection (ATCC). These cell lines were grown in monolayer culture in Dulbecco’s Modified Eagle’s Medium (DMEM) nutrient mixture supplemented with 10% non–heat-inactivated FBS at 37°C with 5% CO₂ and 95% air. The 2 lines were originally tested by Clonetics and passaged for less than 6 months in the laboratory. Normal human prostate epithelial cell line (PrE/C) was purchased from Clonetics and maintained in prostate epithelial cell basal medium. This cell line was originally tested by Clonetics and passaged for less than 6 months in the laboratory.

Cell survival assay
Cells were cultured in 96-well culture plates and treated with the agents indicated. Viable cell number was calculated by the sulforhodamine B assay as described previously (12, 17).

Detection of apoptosis
Apoptosis was evaluated by Annexin V staining by Annexin V–FLUOS apoptosis detection kit following the manufacturer’s instructions. Caspase-3 activities and PARP cleavage were also detected by Caspase-3/CPP32 Fluorometric Assay Kit and Western blotting as additional indicator of apoptosis. For apoptosis analysis of tumors in animal studies, tumor tissues were excised and stained for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) by the ApopTag Kit (Chemicon) according to the manufacturer’s instructions.

GFP-LC3 analysis
Cells were transfected with GFP-LC3 vector (1 µg/µL) with Lipofectamine 2000 (Invitrogen). Forty-eight hours later, cells were treated with dimethyl sulfoxide (DMSO; control), (−)-gossypol (10 µmol/L), sorafenib (20 µmol/L), or their combination for 24 hours, then fixed in 4% formaldehyde for 10 minutes. Cells were then washed with PBS and examined by fluorescent microscopy.

Transmission electron microscopy
Preparation of the ultrathin sections were described previously (18). Ultrathin sections (65 nm) were examined with a TECNAI-10 transmission electron microscope.

Western blotting
Preparation of whole-cell protein lysates and Western blot analysis were described previously (12, 17).

Transfection of siRNA
siRNA oligonucleotides were from Dharmacon. Transfection of siRNA was carried out with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). siGENOME SMARTpool reagents (Dharmacon) were diluted with Opti-MEM I reduced serum medium (Invitrogen).

RNA isolation, quantitative real-time PCR
Total cellular RNA from approximately 5 × 10⁵ cells was prepared with TRIzol reagent, and reverse transcription was done by the Super-Script III First-Strand Kits (Invitrogen). The quantitative real-time PCR (qRT-PCR) was done by QuantiFast SYBR Green PCR Kit from QIAGEN. Primer sequences and conditions are available upon request.

Animal study
In vivo experiments were carried out with 5- to 6-week-old female nude mice purchased from Beijing Vital River Experimental Animals Co. Ltd. Mice were inoculated subcutaneously with 0.2 mL PC-3 or DU145 cell suspension (2 × 10⁶ cells) with a sterile 22-gauge needle. When tumors reached 100 mm³, the mice were randomized into 4 groups with 5 to 8 mice according to tumor volumes and body weights for the following treatments: vehicle control, (−)-gossypol (20 mg/kg/d orally), sorafenib (30 mg/kg/d orally), and the combination of (−)-gossypol...
and sorafenib. The tumor sizes and animal body weights were measured twice weekly and plotted as previously described (12, 17). At 1, 2, and 3 weeks, both tumors from a single mouse from each group were dissected. Tumor tissues were processed for immunoblotting as described previously (17, 19, 20). All animal experiments were carried out according to the protocol approved by Third Military Medical University Guidelines for Use and Care of Animals.

**Statistical analysis**

Two-tailed Student t test and 2-way ANOVA were used to analyze the in vitro and in vivo data, respectively, with Prism 5.0 software (GraphPad Prism). A threshold of P less than 0.05 was defined as statistically significant.

**Results**

**Sorafenib sensitizes AI-PCs to (−)-gossypol-induced growth suppression in vitro and in vivo**

Sorafenib has been reported as an enhancer of chemotherapy (such as, Bcl-2/Bcl-xL inhibitor ABT-737) in several model systems of human cancer (21, 22). To investigate whether sorafenib sensitizes AI-PCs to (−)-gossypol therapy, we examined the cytotoxic effect of the combination of sorafenib and (−)-gossypol. Seen in Fig. 1A, as the sorafenib dose increased, there was an obvious left shift of the cytotoxicity curves and the IC50 values were reduced accordingly, indicating that the DU145 and PC-3 cells were sensitized to (−)-gossypol by sorafenib. Our own studies also showed that as a single agent, sorafenib affected cell viability (Supplementary Fig. S3A) and colony formation (Supplementary Fig. S3B).

To determine whether sorafenib could enhance (−)-gossypol-induced cell death, DU145 and PC-3 cells were treated with varying concentrations of sorafenib and exposed to indicated doses of (−)-gossypol. Significant cell death occurred in both cell types when sorafenib was added to (−)-gossypol treatment (Fig. 1B), but no chemosensitization was seen when normal human PrEC underwent the same treatments (Supplementary Fig. S4). To assess whether the combined effects were synergistic or additive, the combination index (CI) values were calculated and isobolograms plotted (Fig. 1C) as described previously (11). CI less than 1 is considered synergistic of the combination treatment (11). The combination treatments of 0.5/2.0 μmol/L, 5/10 μmol/L, or 10/20 μmol/L (−)-gossypol/sorafenib resulted in synergistic effects (CIs = 0.253, 0.533, or 0.649 in DU145 cells and 0.319, 0.605, or 0.746 in PC-3 cells, respectively). The concentrations of 10 μmol/L (−)-gossypol and 20 μmol/L sorafenib are also clinically relevant and can be achieved pharmacologically (23–25).

To further examine whether sorafenib sensitized AI-PCs in vivo, we used 2 xenograft models of human prostate cancer, exposed the animals to different treatment regimens, and assessed tumor growth. The combined treatment of sorafenib and (−)-gossypol had the greatest effect on tumor growth suppression of both DU145 and PC-3 cell-derived tumors (Fig. 1D) with no significant change in body weight (Supplementary Fig. S5). These results indicate that sorafenib sensitizes AI-PCs to (−)-gossypol-induced growth suppression in vitro and in vivo and suggests that it is a good chemosensitizer candidate to enhance cell death in AI-PCs.

**Sorafenib sensitizes AI-PCs through attenuating (−)-gossypol-mediated Mcl-1 upregulation**

To further investigate the mechanisms underlying the synergistic effect of sorafenib and (−)-gossypol, we examined the expression profile of the Bcl-2 family proteins (Fig. 2A). Administration of (−)-gossypol did not affect expression of proapoptotic members Bak/Bax and antiapoptotic member Bcl-xL. Little downregulation of Bcl-2 was found in PC-3 cells upon (−)-gossypol treatment, consistent with our previous report (18). In agreement with previous research, Mcl-1 was constitutively expressed in AI-PCs. Of importance is that the levels of Mcl-1 expression were increased after (−)-gossypol treatment at either low (0.5 μmol/L) or high (10 μmol/L) concentrations in vitro and in vivo (Fig. 2A and B and Supplementary Fig. S6). Mcl-1 mRNA level did not differ between (−)-gossypol-treated cells and DMSO control (Fig. 2A), suggesting the involvement of a posttranscriptional mechanism.

Cells treated with the translocation inhibitor, cycloheximide, experienced a rapid decrease in Mcl-1 expression (Fig. 2C). When treated with (−)-gossypol and cycloheximide, Mcl-1 levels appeared unaffected, suggesting that (−)-gossypol significantly delays degradation and prolongs the stability of Mcl-1 (Fig. 2C). Similar results were found with a Bcl-2/Bcl-xL inhibitor ABT-737 in hepatoma cells (26), which protects Mcl-1 against degradation. Consistent with the Zhao study (27), both low (2.0 μmol/L) and high (10 μmol/L) concentrations of sorafenib downregulated Mcl-1 expression at the transcriptional level in DU145 and PC-3 cell lines, which may have attenuated (−)-gossypol-mediated Mcl-1 upregulation in combination treatment in vitro and in vivo (Fig. 2A and B, Supplementary Figs. S6 and S7). Downregulation of Mcl-1 by siRNA similarly sensitized DU145 and PC-3 cells to (−)-gossypol treatment in vitro (Fig. 2D). These results indicate that sorafenib sensitizes AI-PCs through attenuating (−)-gossypol-induced Mcl-1 upregulation in AI-PCs.

**Sorafenib and (−)-gossypol preferentially induce apoptosis in DU145 cells in vitro and in vivo**

To investigate whether sorafenib induces apoptosis with (−)-gossypol in AI-PCs and whether apoptosis contributes to the majority of cell death due to sorafenib/(−)-gossypol combination treatment, we compared the apoptotic response and cellular death rates among DU145 and PC-3 cells treated with sorafenib, (−)-gossypol, and a combination of the two. Individually, sorafenib and (−)-gossypol both induced programmed cell death in
The data shown are average tumor size (mm³) of each mouse. When the tumors reached 100 mm³, the mice were randomized into 5 to 8 mice per group. A total of 30 mg/kg sorafenib, 20 mg/kg (-)-gossypol, or combined sorafenib and (-)-gossypol were administrated daily via oral gavage.

**Figure 1.** Sorafenib sensitizes AI-PCs to (-)-gossypol-induced growth suppression in vivo and in vitro. A, MTT-based cell viability assay of combined sorafenib (Sora) with (-)-gossypol [(-)-G] in DU145 and PC-3 cells. Data represent the means and SD of 3 independent experiments. B, cells were treated with varying concentrations of (-)-gossypol in the presence of the indicated concentrations of (-)-gossypol. Twenty-four hours later, cells were stained with trypan blue and assessed for viability. Data represent the means and SD of 3 independent experiments. C, IC₅₀ isobologram of the combination treatments in DU145 and PC-3 cells. 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. CI < 1 is considered synergistic of the combination treatment (11). D, DU145 and PC-3 cells (2 × 10⁵) were subcutaneously injected into the flanks on both sides of each mouse. When the tumors reached 100 mm³, the mice were randomized into 5 to 8 mice per group. A total of 30 mg/kg sorafenib, 20 mg/kg (-)-gossypol, or combined sorafenib and (-)-gossypol were administrated daily via oral gavage. The data shown are average tumor size (n = 10–16), *P < 0.05 versus (-)-gossypol alone, 2-way ANOVA (n = 14–16).

DU145 and PC-3 cells, however, the ratio of apoptotic cells in PC-3 was obviously lower than in DU145 (Fig. 3A). When combining sorafenib and (-)-gossypol-treated cells, the percentage of apoptotic cells was significantly higher than (-)-gossypol treatment alone, but the absolute apoptotic cells in DU145 is much higher than that in PC-3 (Fig. 3A).

Two other differences were also evident between PC-3 and DU145 cells undergoing combination therapy. Significant caspase-3 activity and PARP cleavage could only be detected in DU145 cells in vitro (Fig. 3B). Furthermore, treating cells with the pan-caspase inhibitor, Z-VAD, inhibited cell death in DU145 cells but not in PC-3 cells (Fig. 3C). Of importance is the finding that coadministration of sorafenib and (-)-gossypol significantly enhanced apoptosis induction in DU145-derived xenograft model but not in PC-3-derived xenograft model (Fig. 3D, Supplementary Fig. S8). This suggests that DU145 and PC-3 cells die in contrasting pathways. Collectively, our data indicate that apoptosis was...
preferentially induced in DU145 cells by either sorafenib or (-)-gossypol and responsible for the majority of cell death upon combination treatment. In addition, the data strongly suggest that another mode of cell death was activated by sorafenib and (-)-gossypol treatment in AI-PCs.

**Sorafenib and (-)-gossypol preferentially induce autophagy in PC-3 cells in vitro and in vivo**

To assess whether autophagy may be responsible for cell death when PC-3 cells are treated with sorafenib and/or (-)-gossypol, we analyzed LC3 localization and processing as well as autophagosome formation. Measuring GFP-LC3 puncta formation is a well-established indicator of autophagy induction (28). Consistent with our previous results (18), autophagy was preferentially induced by either sorafenib or (-)-gossypol in PC-3 cells. Greater than 65% of PC-3 cells that received combination therapy displayed GFP-LC3 puncta versus approximately 10% of DU145 cells (Fig. 4A). Transmission electron microscopy (TEM) revealed an abundance of autophagosomes in PC-3 cells treated with sorafenib and/or (-)-gossypol (Supplementary Fig. S9). Approximately 80% of cells that received combination therapy contained autophagosomes (Supplementary Fig. S9).

Assessing the relative amount of endogenous LC3 and p62 processing between treatment groups validated the results of the fluorescent studies of GFP-LC3 puncta formation (Fig. 4B). The autophagy inhibitor 3-MA inhibited total cell death in PC-3 cells treated with (-)-gossypol or (-)-gossypol combined sorafenib, however, 3-MA had no effect on similarly treated DU145 cells (Fig. 4C). Consistent with in vitro data, sorafenib and (-)-gossypol preferentially induced autophagy in PC-3-derived xenograft model. When visualized by TEM, nearly 50% of cells from PC-3–derived tumors that received combination therapy contained autophagosomes (marked with arrows) in contrast to only 10% of DU145-derived tumors (Fig. 4D). In agreement with the TEM data, the greatest level of LC3 processing was seen in PC-3-derived tumor samples from mice that received combination therapy (Supplementary Fig. S10). Clearly, autophagic cell death is the dominant cell death pathway after treatment with sorafenib and/or (-)-gossypol in PC-3 cells. In addition, autophagy induction is significantly enhanced by sorafenib and
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Expression level of Bak plays a key role in either apoptosis or autophagy induction

To figure out the mechanisms underlying cell type-dependent apoptosis induction or autophagy induction, we examine the expression levels of Bcl-2 family proteins and BH3-only protein, Beclin1. Significant differences were found between 2 AI-PCs, that is, much higher anti-apoptotic protein Bcl-2 level in PC-3 and proapoptotic protein Bak level in DU145, at protein and mRNA levels, respectively (Fig. 5A and B). Our previous data concluded that Bcl-2 level plays an important role in apoptosis and autophagy induction in prostate cancer cells (18, 29). Overexpression of Bcl-2 in DU145 cells significantly reduced the cleavage of PARP (Fig. 5C) and the ratio of apoptotic cells (Supplementary Fig. S11A), whereas downregulation of Bcl-2 in PC-3 cells only slightly enhanced the same items (Fig. 5C, Supplementary Fig. S11B). The overexpression of Bcl-2 did not increase autophagy induction in DU145 cells whereas downregulation of Bcl-2 increased it in PC-3 cells (Fig. 5C, Supplementary Fig. S11A and S11B). These data suggest that there are

Figure 3. Sorafenib and (-)-gossypol preferentially induce apoptosis in DU145 cells in vitro and in vivo. A, Annexin V and propidium iodide staining analysis. After being treated with DMSO, 20 μmol/L sorafenib, 10 μmol/L (-)-gossypol, or sorafenib combined with (-)-gossypol for 24 hours, the cells were processed for flow cytometry. The percentage of apoptotic cells is expressed as the percentage of cells doubly positive for propidium iodide and Annexin V. Data represent the means and SDs of 3 independent experiments. *, P < 0.05, **, P < 0.01. B, cells were treated as in A, lysed, and either subjected to caspase-3 activity assay or immunoblotted to assess PARP cleavage. *, P < 0.05; NS, no significance. C, cells were treated with sorafenib (20 μmol/L) and (-)-gossypol (10 μmol/L). Z-VAD (10 μmol/L) was used alone or combined with sorafenib and (-)-gossypol. Twenty-four hours later, cells were stained with trypan blue and assessed for viability. Data represent the means and SDs of 3 independent experiments. NS, no significance; **, P < 0.01. D, xenograft tumor tissues from each treatment group were taken at the end of the first week and fixed in 10% formalin. The tumor sections were stained for TUNEL by the ApopTag Kit. The apoptotic cells have DNA fragmentation and are stained positive as brown nuclei (white arrows). Positive cells were counted under ×400 magnification at 8 different fields, and the average numbers were calculated and plotted. *, P < 0.05 compared with (-)-gossypol alone. Representative photographs and quantification of TUNEL staining positive cells are shown. FITC, fluorescein isothiocyanate; HPF, high-power field.
other key mechanisms besides Bcl-2 that regulate apoptosis and autophagy in the 2 cell lines.

Next, we investigated the involvement of potential key players in the induction of apoptosis and autophagy. As shown in Fig. 5D and Supplementary Fig. S11, down-regulation of Bak in DU145 cells dramatically reduced the cleavage of PARP and the apoptotic cell ratio while enhancing the processing of LC3 and autophagy induction. In contrast, the overexpression of Bak in PC-3 cells significantly increased the apoptosis while decreased autophagy, and downregulation of Bak in PC-3 cells obviously reduced the apoptosis while enhanced autophagy (Supplementary Fig. S12). As for Bax expression, DU145 experienced an overexpression and PC-3 cells, an underexpression (Supplementary Fig. S13). Collectively, these data strongly indicate that the expression level of Bak plays a key role in apoptosis and autophagy induction in DU145 and PC-3 cells.

Inhibiting nondominant death pathways potentiates cell killing by sorafenib/(-)-gossypol combination therapy

Because sorafenib and (-)-gossypol preferentially induce autophagy in PC-3 cells in vitro and in vivo. A, cells expressing GFP-LC3 were treated with or without 10 μmol/L (-)-gossypol together with or without 20 μmol/L sorafenib and processed for fluorescent microscopy 24 hours later. White arrows indicate the characteristic punctate pattern of GFP-LC3 that occurs upon autophagic induction. The right panel shows a statistical analysis of the percentage of cells containing 5 or more GFP-LC3 puncta. *, P < 0.05. B, cells were treated with DMSO, 20 μmol/L sorafenib, 10 μmol/L (-)-gossypol, or sorafenib combined with (-)-gossypol for 24 hours, then lysed and processed for immunoblot analysis of LC3 and p62 processing. Five millimolar 3-MA was used as an inhibitor of autophagy. C, effects of 3-MA on the combination-induced cell death in DU145 and PC-3 cells. Cells were treated with DMSO, 3-MA (5 mmol/L), (-)-gossypol (10 μmol/L), sorafenib (20 μmol/L), (-)-gossypol + 3-MA, sorafenib + 3-MA, sorafenib + (-)-gossypol, or 3-MA combined with sorafenib and (-)-gossypol for 24 hours. Viability was assessed by trypan blue staining. Results are means ± SDs of 3 independent experiments. NS, no significance; **, P < 0.01. D, representative electron micrograph images of sectioned tumor tissue showing autophagic vacuoles (arrows) with content following indicated treatment in an in vivo DU145 model and PC-3 xenograft models. The percentage of cells with autophagic vacuoles was quantified in 50 cells per group.
inhibitor or Z-VAD as an apoptosis inhibitor in PC-3. In DU145 cells, the combination of 3-MA/sorafenib/(-)-gossypol severely affected colony formation of DU145 cells versus sorafenib/(-)-gossypol whereas the effect on PC-3 cells was not significant (Fig. 6A and B). The opposite effect was true for Z-VAD/sorafenib/(-)-gossypol treatment. PC-3 colony formation was significantly lower whereas DU145 colony formation was unaffected. These observations could be tied to increased caspase-3 activation and PARP cleavage in DU145 cells (Fig. 6C) and increased GFP-LC3 puncta and processing in PC-3 cells (Fig. 6D). These data strongly suggest that suppression of the nondominant death pathways can induce more effective cell killing by sorafenib and (-)-gossypol.

**Discussion**

Mcl-1 is a very important antiapoptotic protein and it is posttranscriptionally upregulated after treatment with (-)-gossypol (12), which may result in drug resistance after long-term exposure. Our previous data showed that (-)-gossypol upregulates Noxa and Puma and binds to Mcl-1 in prostate cancers (12), these actions may block
Noxa and deubiquitinase USP9X-mediated Mcl-1 degradation and stabilize Mcl-1 (30). Because specific Mcl-1 inhibitors have not been developed and sorafenib downregulates Mcl-1 expression in multiple tumors, including prostate cancer (31), we use sorafenib to attenuate (--)-gossypol-induced Mcl-1 upregulation. Sorafenib may downregulate Mcl-1 expression at transcriptional level or posttranscriptional level (32, 33). In AI-PCs, both the mRNA and protein levels of Mcl-1 were significantly downregulated by sorafenib, but whether the translation of Mcl-1 was suppressed need to be clarified further. Combined with (--)-gossypol, sorafenib efficiently induced cell death in AI-PCs both in vitro and in vivo. These results suggest that combining sorafenib with (--)-gossypol may be an attractive strategy for clinical patients with AI-PC.

The balance between proapoptotic and antiapoptotic proteins determines the cellular decision between apoptosis or autophagy (34). According to expression profiles, high levels of Bcl-2/Bcl-xL and low levels of Bak/Bax exist in PC-3 cells, whereas low levels of Bcl-2/Bcl-xL and high levels of Bak/Bax was found in DU145 cells. This fact makes a relative high ratio of proapoptotic proteins versus antiapoptotic proteins in DU145 cells and a low ratio in PC-3 cells. While apoptosis cannot be significantly enhanced by downregulation of Bcl-2 in PC-3 cells and autophagy cannot be increased by overexpression of Bcl-2 in DU145 cells, contrasted results were found when overexpressed Bak in PC-3 cells or downregulated it in DU145 cells. In addition, Bak/Bax-induced autophagy inhibition seems to be mediated by Beclin1 cleavage (Fig. 5C and D). These results indicate that the level of proapoptotic proteins may be more important than that of antiapoptotic proteins during apoptosis or autophagy induction.

The connection between apoptotic (type I cell death) and autophagic cell death (type II cell death) in the context of cancer relevant stimuli is still unresolved, but a compensatory relationship may exist. For example, inhibition of autophagy by chloroquine has been shown to increase vorinostat-mediated apoptosis in colon cancer cells (35). Conversely, inhibiting the activity of caspase-3 enhanced autophagic cell death in lung cancer cells (36). The results of the present study provide new proof for this point. Inhibiting nondominant death pathways, that is, autophagy in DU145 and apoptosis in PC-3 potentiates cell killing by dominant death pathways, that is, apoptotic cell death in DU145 and autophagic cell death in PC-3. Long-term exposure (10–14

Figure 6. Inhibiting secondary death pathways potentiates cell killing by sorafenib and (--)-gossypol combination therapy. A and B, DU145 and PC-3 cells were passaged into 6-well plates and treated with DMSO control, 5 μmol/L sorafenib + 5 μmol/L (--)-gossypol, 10 μmol/L Z-VAD, 1 mmol/L 3-MA, sorafenib combined (--)-gossypol, and Z-VAD/3-MA. Media with drugs was changed every 7 days and colonies were stained (A) and the scored colonies were graphed (B). Each colony formation assay was conducted in triplicate and repeated for at least 3 independent experiments. * P < 0.05. C, DU145 cells were treated as indicated. Twenty-four hours later, cell lysates were prepared and assayed for caspase-3 activity analysis (top) or immunoblotted to assess PARP cleavage (bottom). * P < 0.05. D, PC-3 cells expressing GFP-LC3 were treated as indicated. After 24 hours, they were analyzed as in Fig. 4A (top). Untransfected PC-3 cells were analyzed for LC3 processing (bottom). ** P < 0.05.

![Image](image-url)
days) of 3-MA dramatically increases (−)-gossypol/sorafenib-induced cell death in DU145 cells (Fig. 6A and B), although a short-term treatment (24 hours) has no significant effect (Fig. 4C). On the other side, both long- or short-term treatments of 3-MA significantly inhibit (−)-gossypol/sorafenib-induced cell death in PC-3 cells (Figs. 6A/B and 4C). Therefore, autophagy plays a protective role in DU145 and a destructive role in PC-3. To our knowledge, this study for the first time represents the bidirectional link between cell apoptosis and autophagy verified by 2 cell models.

In conclusion, we have shown that the relative level of proapoptotic proteins versus antiapoptotic proteins is a key factor of balancing apoptosis and autophagy induction. In same cells, inhibition of secondary cell death pathway may potentiate cell killing by dominant cell death. The inhibition of Bcl-2/Bcl-xL by (−)-gossypol under sorafenib administration was effective for anti-AI-PC therapy in preclinical models. The emergence of autophagic cell death as a dominant death pathway associated with the BH3-mimetic (−)-gossypol and sorafenib presented here may provide a new direction on rational design of clinical trials as well as new strategies to exploit autophagic cell death to overcome resistance to current cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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