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Molecular mechanism of gossypol-induced cell growth inhibition and cell death of HT-29 human colon carcinoma cells

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Abstract

Gossypol, a male contraceptive drug, has been demonstrated to have antiproliferative and antimetastatic effects on many kinds of cancer cells *in vitro*. HT-29 human carcinoma cell line is one of the most susceptible cell lines to gossypol-induced cell death. Here, it is shown that treatment of HT-29 cells with gossypol not only induces cell cycle arrest on the G0/G1 phase, but also induces apoptosis. With a serial of Western blot analysis, it is revealed that gossypol-induced cell cycle arrest is involved in P21 up-regulation and cyclin D1 down-regulation; gossypol-induced apoptosis triggers down-regulation of anti-apoptosis Bcl-2 members: Bcl-X_L, Bag-1 and Mcl-1, up-regulation of pro-apoptosis Bcl-2 member Bak, activation of caspase-3, -6, -7, -8, and -9, up-regulation of Apaf-1, release of cytochrome *c* (cyto-*c*) from mitochondria, and activation of both DFF45 and PARP. Taken together, gossypol-induced cell death initiates extensive alterations of cell cycle and apoptosis proteins. Gossypol-induced apoptosis of HT-29 cells is through first the mitochondrial pathway, then the death receptor pathway, and the mitochondria pathway is, at least in part, involved in cyto-*c* release.

Keywords: HT-29; Cell cycle; Apoptosis; Cyclin D1; P21; Bcl-X_L; Caspases; DFF45; PARP; Cytochrome *c* release

1. Introduction

Gossypol is a polyphenolic compound (Fig. 1) originally isolated from cottonseed; it has been successfully used as a contraceptive drug for males for a long time [1]. It was first found by Tuszynski and Cossu to have anticancer effects against several tumor cell lines grown in tissue culture, and the most sensitive cell lines to gossypol-induced cell death were melanoma and colon carcinoma cells [2]. Since then more and more observations have been reported on its antiproliferative or antimetastatic activity, such as Ehrlich ascites tumor cells [3], P388 or L1210 murine leukemias [4], SW-13 adrenocortical carcinoma cells [5], murine erythroleukemia cells (clone 6A11A) [6], human glioma cell line HS 683, U373, U87, and U138 [7], hormone-dependent and hormone-independent breast carcinoma cells MCF-7 (drug-sensitive), MCF-7_{Ad}r (multidrug-resistant), MDA-MB-231 and T47D [8–11], melanoma cell line SK-mel-19 and SK-mel-28 [12], promyelocytic leukemia cell line HL60 [13,14], breast carcinoma cell line T47D and ovarian carcinoma cell line OVCAR-3 [15,16], colon carcinoma cell line HT-29 and LoVo [17], metastatic MAT-LyLu lung cells [18], and other kinds of human or murine normal or carcinoma cells [19–23]. Even the milk collected from gossypol-treated dairy cows was reported to have antitumor activity [24]. It is worthy to note that the *in vitro* antitumor activity of gossypol and its derivatives is prevented by serum protein [25–27]. It seems that it is possible to enhance the antitumor activity of gossypol by synthesizing certain kinds of stereo-structure or structure modification derivatives [28–30]. Besides its male antifertility activity and anticancer activity, gossypol also inhibits

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Abbreviations: Rb, retinoblastoma gene protein; PARP, poly (ADP-ribose) polymerase; DFF, DNA fragmentation factor; Smac, second mitochondria-derived activator of caspases; AIF, apoptosis inducing factor; IAP, inhibitor of apoptosis; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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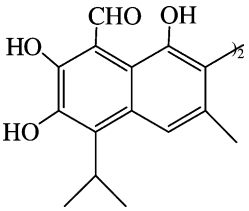

Fig. 1. Chemical structure of gossypol.

the growth of numerous parasitic organisms and shows antiviral activity against a number of enveloped virus, including HIV virus [31].

Gossypol has been used as an anticancer drug in clinical research. For metastatic adrenal cancer, the response rate is similar to the other agents currently used for adrenal cancer, but responses were seen in patients who had failed other chemotherapeutic regimes [32]. For malignant gliomas, it shows a low, but measurable response [33,34]. For refractory metastatic breast cancer, it shows a negligible antitumor activity [35]. There is one report indicating that gossypol is unlikely to be useful in patients with advanced cancer [36]. All these studies agreed that gossypol is tolerated and clinically safe.

Treatment with gossypol inhibits DNA synthesis of the cells [9], causes DNA breaks [30] or DNA fragmentation [14,37]. The other target of gossypol is mitochondria. Ultrastructurally it caused marked swelling and vacuolization of mitochondria; there was an almost complete loss of cristae but the outer membrane was retained, upon cells being treated with gossypol [15,20]. Various kinds of molecular targets have been investigated for gossypol-induced antiproliferative or antimetastatic activity. It was proposed that gossypol-induced DNA fragmentation or cell death resulted from its inhibition of protein kinase C activity [13,37,38]. Additionally, gossypol is a potent inhibitor of arachidonate 5- and 12-lipoxygenases [39]. In rat spermatocytes, gossypol-induced cell death is coupled with up-regulation of c-fos [40] and down-regulation of c-myc [41]. Most importantly, treatment of cancer cells with gossypol resulted in cell cycle arrest on G0/G1 phase [16,35,42–44]. The mechanism is that transforming growth factor-β is activated [42,43] or P53 and P21 are up-regulated, and cyclin D1 and Rb are inhibited. It has been known that these four proteins are pivotal in controlling the progress of the cell cycle [35,44].

Apoptosis is a major process for cell death. Little is known about the molecular mechanism of gossypol-induced apoptosis. There is only a brief report in HT-29 and LoVo cells indicating that gossypol-induced apoptosis may not be involved in the regulation of P53 but possibly associated with the regulation of Bcl-2 and Bax expression [17].

Since HT-29 is one of the most sensitive cell lines to gossypol-induced cytotoxicity, in the present study, the expression of molecules involved in mitosis and apoptosis pathways was examined. The results presented here demonstrate that gossypol-induced cell growth inhibition and cell death of HT-29 cells not only includes cell cycle arrest, but also includes apoptosis. HT-29 cell cycle arrest is triggered by up-regulation of P21 and down-regulation of cyclin D1; HT-29 cell apoptosis is through anti-apoptosis Bcl-2 member down-regulation, extensive caspase, DFF, and PARP activation.

2. Material and methods

2.1. Cells and reagents

Human colon cancer cell line HT-29 was obtained from National Cancer Institute (NCI), which was maintained in RPMI-1640 medium (Biofluids) supplemented with 10% (v/v) heated-inactivated bovine serum (Gibco, BRL) plus 1% glutamine (Gibco, BRL) at 37° in 5% (v/v) CO_2. The expression of apoptosis cascade components was detected by using the following antibodies: anti-Bcl-2 mAb (Oncogene) for Bcl-2, anti-Bcl-X_L mAb (Transduction Laboratories) for Bcl-X_L, anti-Bax rabbit polyclonal antibody (Upstate) for Bax, anti-Bid goat polyclonal antibody (Santa Cruz) for Bid, (Ser112)anti-phospho-Bad mAb (Cell Signaling) for phospho-Bad, anti-Apaf-1 rabbit polyclonal antibody (Santa Cruz) for Apaf-1, anti-cpp32 rabbit polyclonal antibody (Pharmingen) for caspase-3, anti-caspase-6 rabbit polyclonal antibody (Cell Signaling) for caspase-6, anti-caspase-7 rabbit polyclonal antibody (Cell signaling) for caspase-7, anti-caspase-9 rabbit polyclonal antibody (Santa Cruz) for caspase-9, anti-PARP rabbit polyclonal antibody (Roche Molecular Biochemicals) for PARP, and anti-DFF45 rabbit polyclonal antibody (Cell Signaling) for DFF45 cleavage part. Cell cycle controlling proteins were probed with the following antibodies: P53, P21 mouse monoclonal antibody (Calbiochem) for P53, P21, anti-Cyclin D rabbit polyclonal antibody (Upstate) for cyclin D1, mouse monoclonal anti-retinoblastoma (Rb) (Pharmingen) for Rb. Mouse monoclonal anti-actin antibody (Santa Cruz) for actin. Gossypol was purchased from Sigma (purity > 98%), and dissolved in absolute ethanol. A broad range caspase inhibitor Z-VAD-FMK was purchased from Enzyme Systems Products.

2.2. Detection of apoptosis by flow cytometry

Cells having undergone apoptotic events were detected by flow cytometry using a FACScan® (Becton Dickinson) with 488-nm laser line and analyzed using Cell Quest software. Phosphatidylserine exposed on the outside of the cells was determined by TACS™ Annexin V-FITC kit (Gaithersburg, MD). Briefly, cells were washed with cold PBS, pelleted and resuspended in 100 uL Annexin V-FITC diluted 1:100 in binding buffer (10 mM Hepes, 100 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) containing propidium iodide (1:10). Cells were incubated for 10–15 min on ice, then additional 400 uL binding buffer

was added before FACScan® analysis. Annexin V-FITC fluorescence was detected in FL-1, and propidium iodide was detected in FL-2.

2.3. Cell cycle assay

Cells were fixed with 2 mL of ice-cold 70% ethanol and incubated overnight at −20°. Next, the cells were washed with the ice-cold PBS once to remove the ethanol. The cell pellet was resuspended in 500 μL of the PBS, mixed with 1 mL of DNA extraction buffer (0.2 M Na_2PO_4 and 0.1 M citric acid, pH 7.8), and incubated at room temperature for 5 min. After centrifugation at 300 *g* at 4° for 8 min, the cell pellet was resuspended in 1 mL of a solution containing 0.5 mg/mL RNase and 0.08 mg/mL proteinase inhibitors for subsequent incubation in the dark at room temperature for 30 min. The PI fluorescence associated with DNA is measured on a Becton-Dickinson FACSCalibur flow cytometer with excitation at 488 nm and emission at 585 nm using the FL2 channel. The percentage of nuclei in each of the cell cycle phases (G1, S, G2/M) was calculated from the standard DNA histograms.

2.4. Immunoblot analysis

Cell lysates were prepared by the addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). Equal amounts of protein were subjected to electrophoresis with 4–20 or 8–16% gradient Tris–glycine gel (In Vitrogen), transferred onto nitrocellulose membrane (Hybond-C extra, Amersham Pharmacia Biotech), and reacted with appropriate antibodies in PBS containing 5% nonfat dry milk, 0.02% Tween 20. Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents subsequently (Amersham Pharmacia Biotech), followed by exposure to X-ray film (Kodak).

2.5. Binding assay based on fluorescence polarization

The binding affinity of gossypol to Bcl-2 and Bcl-X_L protein *in vitro* was determined by a competitive binding assay based on fluorescence polarization. For this assay, 5-carboxyfluorecein was coupled to the N terminus of a peptide, GQVGRQLAIIGDDINR, derived from the BH3 domain of Bak (Flu-BakBH3), which has been shown to bind to the surface pocket of the Bcl-X_L protein with high-affinity. Bcl-2 used in this assay was a recombinant GST-fused soluble protein (Santa Cruz Biotechnology); Bcl-X_L was soluble part of Bcl-X_L protein purified in this Lab. Flu-BakBH3 and Bcl-2 protein or Bcl-X_L were mixed in the presence or absence of gossypol under standard buffer conditions and were incubated for 30 min. The binding of Flu-BakBH3 peptide to Bcl-2 protein and Bcl-X_L was measured with a TECAN Ultra microplate reader featured with polarization function. The fluorophore was excited with vertical polarized light at 485 nm (excitation slit width 20 nm), and the polarization value of the emitted light was observed through vertical and horizontal polarizers at 530 nm (emission slit width 20 nm). The binding affinity of gossypol for Bcl-2 protein or Bcl-X_L was assessed by determining the ability of different concentrations of gossypol to inhibit Flu-BakBH3 binding to Bcl-2 or Bcl-X_L, with Bcl-2 or Bcl-X_L and Flu-BakBH3 peptide alone as 100% binding.

2.6. Clonogenic assay

3×10^4 HT-29 cells were treated with various concentrations of gossypol for 24 hr after being pretreated with desired concentration of Z-VAD-FMK for 4 hr. The survival rate of HT-29 cells after treatment was determined with a modified colorimetric clonogenic assay [45]. Briefly, HT-29 cells (2000 cells per well) were mixed with agarose (BioWhitaker Molecular Applications) in culture medium (0.3% final concentration), and added to a 96-well plate. Plates were incubated at 37°, 6.5% CO_2 for 12 days. Fifty micrograms of MTT (Sigma) in phosphate buffered saline was added to each well. After 6 hr incubation at 37°, 6.5% CO_2, 75 μL of 10% SDS (in 0.01 N HCl) was added to each well. Plates were incubated 48 hr at 37°, 6.5% CO_2 to solubilize the formazan dye. The optical density was determined by measuring the absorbance at 570 nm vs. 650 nm using TECAN Ultra microplate reader. The survival rate was illustrated as percent of control by using no treated cells as 100% growth. Data were expressed as mean ± SD of at least three experiments carried out in triplicate. Statistical analysis was performed by ANOVA followed by post-ANOVA tests (Fisher PLSD and Scheffe *F*-test). Values of $P < 0.05$ were considered statistically significant.

2.7. Measurement of caspase-3 activity

Caspase-3 activity was measured with ApoAlert® Caspase-3 Colorimetric Assay kit (Clontech). Briefly, 2×10^6 cells were collected and lyzed with 50 μL chilled lysis buffer, cell lysates were centrifuged at maximum speed for 5 min at 4°, 50 μL of supernatant was transferred to 96-well plate, then 50 μL of 2× reaction buffer/DTT mix and 5 μL of 1 mM caspase-3 substrate (DEVD-pNA) were added to each reaction, incubated at 37° for 1 hr, and read at 405 nm in a microplate reader (Molecular Device). Final caspase-3 activity was calculated by dividing the net $OD_{405\ nm}$ with the slope of a calibration curve obtained with different concentration of pNA.

2.8. Cyto-c release assay

Mitochondria and cytosol were prepared as the method reported by others [46] with some modification. Briefly, cells were harvested and washed once with ice-cold PBS

and resuspended in 1 mL ice-cold buffer C (10 mM Hepes-KOH at pH 7.4, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.5 mM sodium EDTA, 0.5 mM EGTA, 1 mM dithiothreitol) and a protease inhibitor mix (PIM). The cell suspension was homogenized on ice by passage 15 times through a 22-gauge needle. The homogenates were centrifuged twice at 750 *g* for 10 min at 4° to remove nuclei. The post-nuclear supernatant fractions were centrifuged at 10,000 *g* for 15 min at 4°, and the resulting mitochondria-enriched pellets were resuspended in 100 μL buffer C + PIM (cold). The post-mitochondrial supernatant was centrifuged at 10,000 *g* for 1 hr at 4° to remove membrane contaminants and the resulting supernatant (soluble portion) was used for cytosolic cytochrome *c* release detection; the pellet was the mitochondrial membrane (heavy membrane proteins) portion. Soluble fraction proteins and an equivalent amount of heavy membrane proteins were subjected to SDS–PAGE and analyzed by Western blot with antibody against cytochrome *c* (Pharmingen).

3. Results

3.1. Induction of cell cycle arrest and apoptosis of HT-29 cells by gossypol

To confirm gossypol-induced cell cycle arrest as reported [17], FACScan analysis was performed at only 24 hr after HT-29 cells were exposed to 5, 10 and 20 μM of gossypol. As shown in Fig. 2, 10 μM of gossypol caused more than 70% of the cells resting on G0/G1 phase of the cell cycle; at 20 μM concentration, almost all the cells died (data not shown). To quantify gossypol-induced apoptosis of HT-29 cells, Annexin V-Propidium Iodide assay was conducted. It was observed that as low as 1.0 μM of gossypol induced about 20% of the cells undergoing

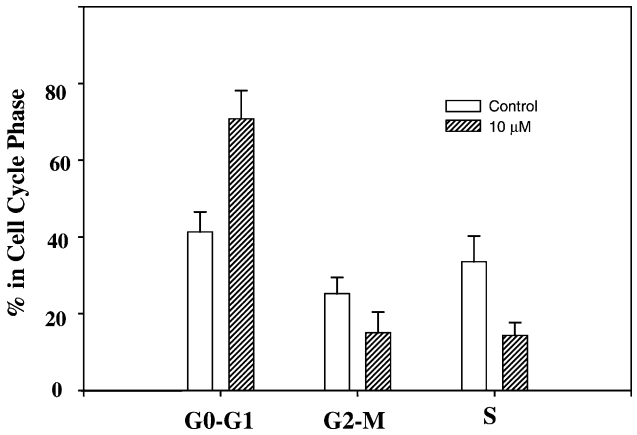

Fig. 2. Induction of cell cycle arrest of HT-29 cells by gossypol. 2×10^6 HT-29 cells were treated with different concentration of gossypol for 24 hr. DNA cell cycle analysis was performed by flow cytometry, as described in Section 2. Numbers are mean values of three independent experiments ± SD.

apoptosis; at 5.0 μM this level went up to almost 70%. Obviously gossypol-induced apoptosis is dose-dependent (Fig. 3). Taken together, gossypol not only induces cell cycle arrest, but also apoptosis of HT-29 cells.

3.2. Up-regulation of P21 and down-regulation of cyclin D1

Since P53, P21, cyclin D1, and Rb were reported to play important roles in the process of mitosis, using Western blot, the protein expression level of these molecules of HT-29 cells treated with different concentration of gossypol for various times was detected. As can be seen in Fig. 4, P21 was up-regulated at as early as 12 hr in a nice dose-dependent manner, but at 24 hr, P21 level was lower than that at 12 hr, even though it was still up-regulated with a dose-dependent manner. Cyclin D1 was down-regulated at 24 hr, also in a dose-dependent manner. The alteration of

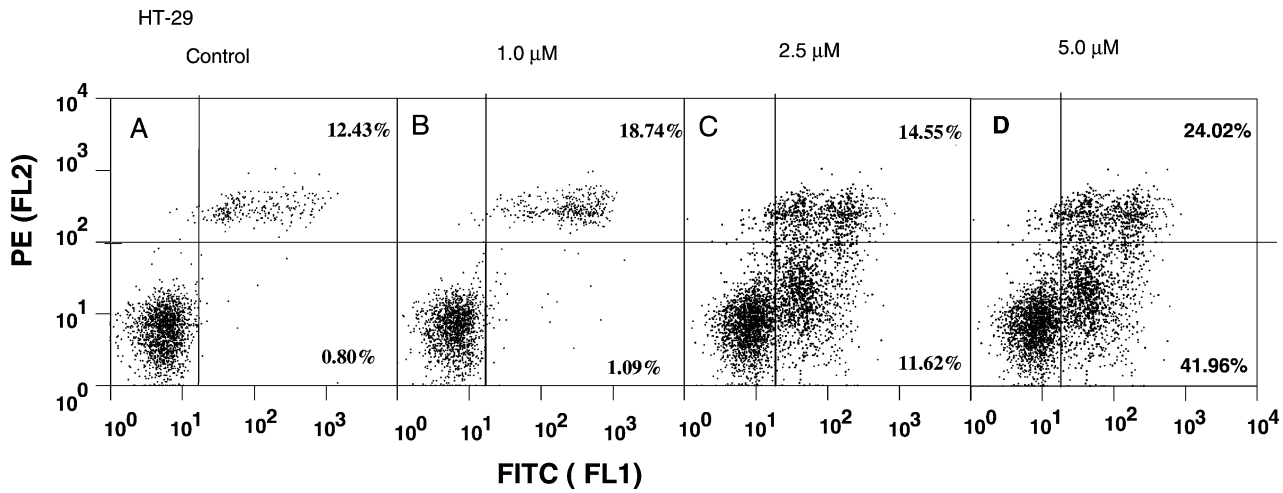

Fig. 3. Induction of apoptosis of HT-29 cells by gossypol. HT-29 cells were treated with different concentration of gossypol for 24 hr, 1×10^6 were collected to conduct Annexin V-Propidium Iodide assay, as described in Section 2. One representation of three independent experiments.

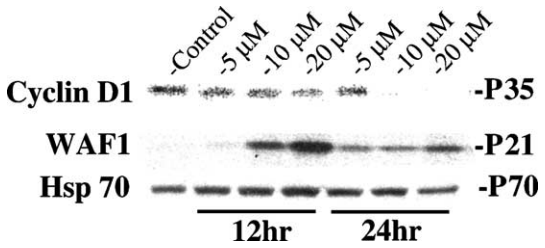

Fig. 4. P21 up-regulation and cyclin D1 down-regulation in gossypol-treated HT-29 cells. HT-29 cells were treated with different concentration of gossypol for various times. Twenty-microgram cell lysates were subject to 4–20% gradient Tris–glycine gel electrophoresis followed by immunoblot with P21 and cyclin D1-specific antibody. Hsp70 reprobing confirms equal loading of the total protein. One representation of three independent experiments.

P53 and Rb (data not shown) was not observed. These findings suggest that gossypol-induced cell cycle arrest is involved in up-regulation of P21 and down-regulation of cyclin D1.

Between two house-keeping genes actin and Hsp70, actin down-regulation was observed at 36 hr of treatment with 10 and 20 μM of gossypol (data not shown), but Hsp70 remained unaltered, so Hsp70 expression level was used as protein loading control for the Western blot analysis in this work.

3.3. Alterations of Bcl-2 family members

In order to elucidate the molecular mechanism of how gossypol works to induce apoptosis, a serial of Western blot assay was performed to trace the changes of Bcl-2 family members upon treatment with gossypol. Fig. 5 is the profile of most of the Bcl-2 family members in HT-29 cells upon treatment with different concentration of gossypol for various times. As proved by others, there was no Bcl-2 expression in HT-29 cells [17]. It was observed that Bcl-X_L was down-regulated. Also, other anti-apoptosis Bcl-2 family members like Bag-1, and Mcl-1 were all down-regulated from 24 hr of treatment. For the pro-apoptosis Bcl-2 family members: Bak was up-regulated at as early as 12 hr; (Ser112)-phospho-Bad was up-regulated at about 48 hr; Bad, Bax and Bid were down-regulated at 5 μM, but 10 μM of gossypol went back to the same level as control at 20 μM of gossypol at 24 and 36 hr. The alterations of all these molecules were dose-dependent. Collectively, gossypol treatment triggers extensive alterations of Bcl-2 family members in HT-29 cells.

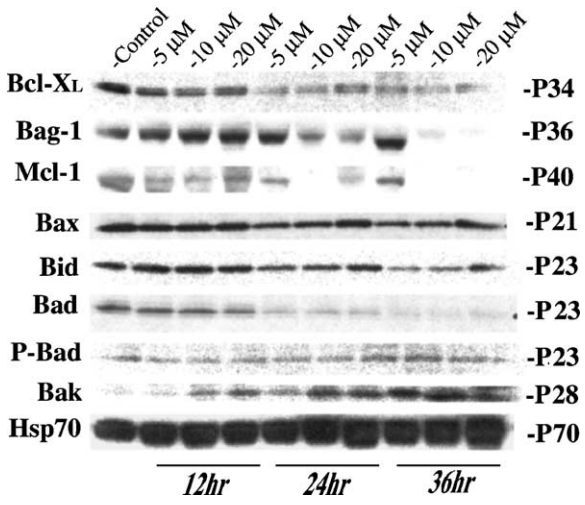

Fig. 5. Alterations of Bcl-2 family members in gossypol-treated HT-29 cells. HT-29 cells were treated with different concentration of gossypol for various times. Twenty-microgram cell lysates were subject to 4–20% gradient Tris–glycine gel electrophoresis followed by immunoblot with Bcl-X_L-, Bag-1-, Mcl-1-, Bax-, Bid-, Bad-, P(Ser112)-Bad-, and Bak-specific antibody, respectively. Hsp70 probing confirms equal loading of the total protein. One representation of three independent experiments.

3.4. Inhibition of Bcl-X_L binding to BakBH3 peptide by gossypol in vitro

It has been known that the anti-apoptosis function of Bcl-X_L is, at least in part, from its heterodimerization with pro-apoptosis Bcl-2 members, such as BAK, to further verify that gossypol can be able to inhibit the anti-apoptosis function of Bcl-X_L; binding assay based on fluorescence polarization was performed to test whether gossypol inhibits the heterodimerization of Bcl-X_L with Bak *in vitro*. As demonstrated in Fig. 6, gossypol inhibits the binding of BH3 peptide to Bcl-X_L protein, and this inhibition is dose-dependent. Also, compared with Bcl-2 protein, Bcl-X_L is more sensitive (IC_{50} = 0.4 μM) to the inhibition of gossypol than Bcl-2 (IC_{50} = 10 μM). This result is consistent with the above observation that gossypol-induced apoptosis of HT-29 cells involves Bcl-X_L down-regulation and Bak up-regulation.

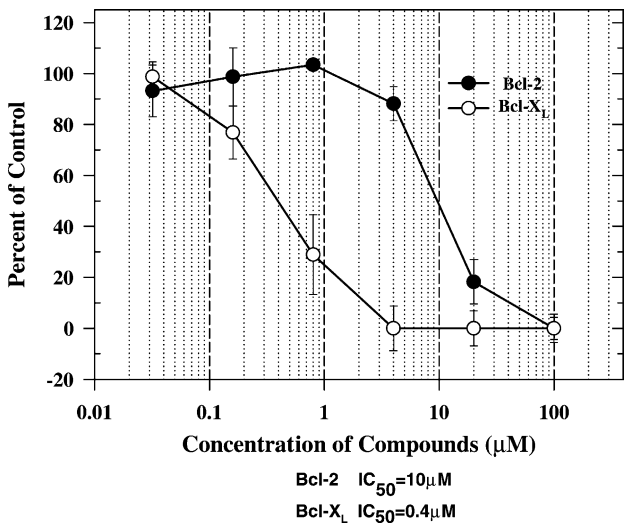

Fig. 6. Inhibition of the heterodimerization of Bcl-X_L with BakBH3 peptide by gossypol. Binding of gossypol to Bcl-X_L protein, compared with Bcl-2 protein *in vitro*, was measured by a competitive fluorescence polarization assay, as described in Section 2. Numbers are mean values of three independent experiments ± SD.

3.5. Extensive caspase activation in gossypol-induced apoptosis of HT-29 cell

Recent discoveries established that multiple distinct signaling pathways regulate apoptosis. Such pathways are activated in general by the formation of a death-inducing signaling complex (DISC). Activation of the DISC results in the early recruitment of inducer caspases (caspase-2, -8, -9, -12). These inducer caspases then amplify the apoptosis signal by cleavage and activation of effector caspases (caspase-3, -6, -7) which execute apoptosis by degrading hundreds of regulatory proteins and activation of endonucleases and other proteins [47–49]. Thus, caspases are very important in the execution of apoptosis, at least in some of the apoptosis signal transduction pathways. To investigate the roles of caspases in gossypol-induced apoptosis, the expression alteration of caspase-3, -6, -7, -8 and -9 was probed in HT-29 cells treated with different concentration of gossypol for various times with Western blot. If being activated, both P21 and P17 cleavage part of caspase-3, P12 cleavage part of caspase-6, P20 cleavage part of caspase-7, P43/P41 and P18 cleavage parts of caspase-8, both P37 and P35 cleavage parts of caspase-9 should be observed. As illustrated in Fig. 7, caspase-3 was activated from 24 hr of gossypol

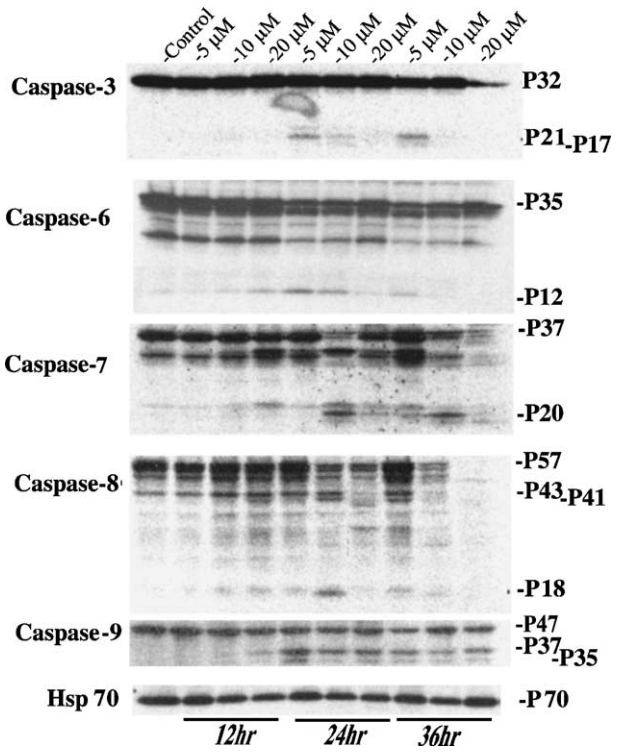

Fig. 7. Extensive caspase activation in gossypol-treated HT-29 cells. HT-29 cells were treated with different concentration of gossypol for various times. Twenty-microgram cell lysates were subject to 4–20% gradient Tris–glycine gel electrophoresis followed by immunoblot with caspase-3-, -6-, -7-, -8-, and -9-specific antibody, respectively. Hsp70 reprobing confirms equal loading of the total protein. One representation of three independent experiments.

treatment, caspase-3 cleavage part P21 was observed at 24 hr at 5 μM of concentration, and at 36 hr at 5 μM of concentration. Activation of caspase-6 presented by its cleavage part P12 appeared as early as 12 hr of gossypol treatment; at this time point, caspase-6 activation showed a nice dose-dependent manner. The peak of caspase-6 activation appeared at 24 hr at 5 μM of gossypol treatment, it was down-regulated until 36 hr of treatment. Activation of caspase-7 presented by its cleavage part P20 occurred at 24 hr at 10 μM, and also at 36 hr at 10 μM of concentration. Compared with the weak band of P21 cleavage part of caspase-3, the activation of caspase-7 was more convincing by its intensive band of P20 cleavage part. Hence, caspase-7 is the predominant executioner caspase in gossypol-induced apoptosis of HT-29 cells. P43 cleavage part of caspase-8 showed up almost all the time during the treatment; P41 cleavage part of caspase-8 appeared at 12 hr at 20 μM, 24 hr at 10 μM, and 36 hr at 5 μM of gossypol treatment; P18 cleavage part of caspase-8 was clearly observed at 24 hr at 10 μM and 36 hr of 5 μM gossypol treatment. Caspase-9 activation presented by its cleavage parts both P37 and P35 was initiated at 12 hr of 20 μM gossypol treatment and reached its peak at 24 hr at 5 μM of gossypol treatment, then went down until 36 hr of treatment. These findings demonstrate that extensive caspase activation takes part in gossypol-induced apoptosis of HT-29 cells.

To further verify that caspase activation is necessary for gossypol-induced apoptosis, HT-29 cells were treated with both gossypol and a broad range caspase inhibitor Z-VAD-FMK. Then cell survival rate and the activity of one of the important caspases, caspase-3, were measured to see whether the antiproliferative effect of gossypol was prevented, and caspase-3 activity was really inhibited. As shown in Fig. 8A, with a typical clonogenic assay with colony forming efficiency more than 90%, gossypol-induced HT-29 cell growth inhibition was blocked by Z-VAD-FMK, even at as low as 0.01 μM concentration ($P < 0.05$, compared with no Z-VAD-FMK group); caspase-3 activity was decreased as predicted, as shown in Fig. 8B. This result confirms that gossypol-induced apoptosis of HT-29 cells is caspase-dependent, and at least caspase-3 activity is necessary in this process.

3.6. Cyto c release in gossypol-induced apoptosis of HT-29 cells

One of the major apoptosis pathways is triggered by the release of mitochondrial apoptogenic protein, cyto-*c*. Cytosolic cyto-*c* binds to the CED-4 homolog Apaf-1 and induces caspase-9-dependent activation of caspase-3 [46,49]. As addressed above, casapase-9 activation takes place in gossypol-induced apoptosis, to elucidate whether cyto-*c* release pathway fits in gossypol-induced apoptosis. First the expression of Apaf-1 in gossypol-treated HT-29 cells was detected, then gossypol-treated HT-29 cells were

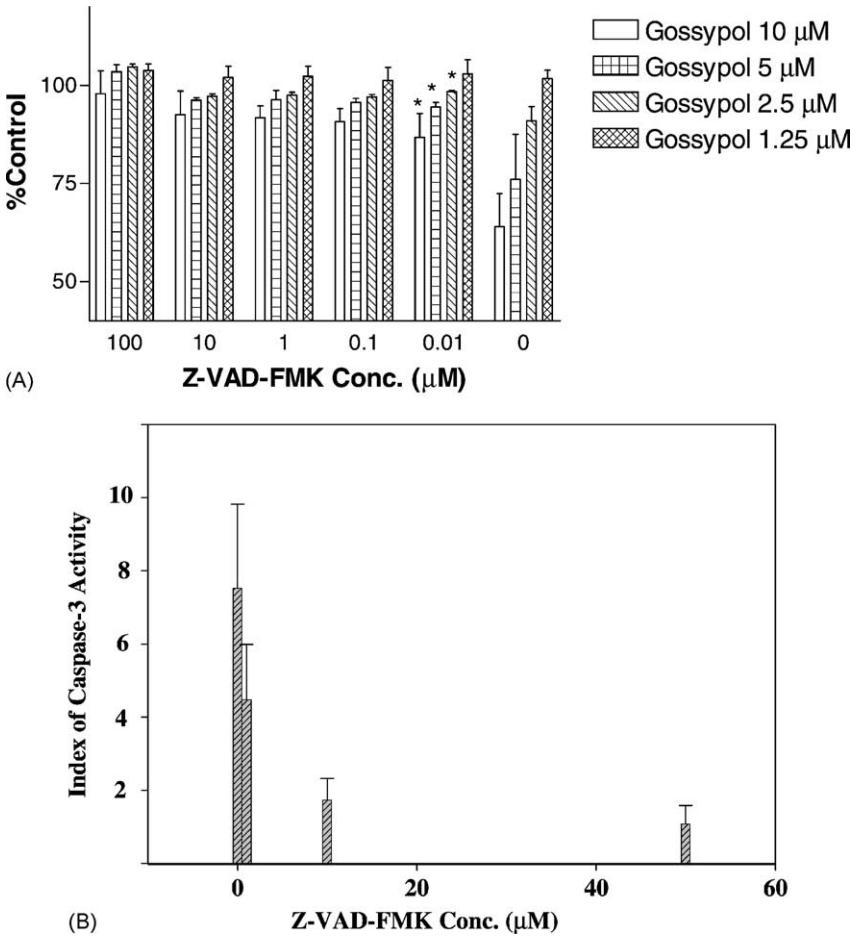

Fig. 8. (A) Caspase-dependent apoptosis of gossypol-treated HT-29 cells. 3×10^4 HT-29 cells were treated with various concentrations of gossypol for 24 hr after being pretreated with desired concentrations of Z-VAD-FMK for 4 hr. The survival rate of HT-29 cells after treatment was determined with a modified colorimetric clonogenic assay as described in Section 2 (colony forming efficiency ≥90%). Numbers are mean values of three independent experiments ± SD ($^*P < 0.05$). (B) Inhibition of caspase-3 activity in gossypol-treated HT-29 cells by Z-VAD-FMK. 2×10^6 HT-29 cells treated with the same condition were used to detect caspase-3 activity with a colorimetric method as described in Section 2. Numbers are mean values of three independent experiments ± SD.

lyzed, cytosol and mitochondrial membrane portion of the treated cells were obtained through a serial of ultra-centrifugation to probe cyto-*c* in both portions. As shown in Figs. 9 and 10, Apaf-1 up-regulation was observed at 24 hr at 20 μM and at 36 hr at 20 μM of gossypol treatment. Cyto-*c* was released to cytosol at 24 hr at as low as 5 μM gossypol treatment. Together with caspase-9 activation in gossypol-treated HT-29 cells, these results propose that cyto-*c* release to caspase-9 activation is one of the important events in gossypol-induced apoptosis of HT-29 cells.

3.7. Activation of DFF and PARP in gossypol-induced apoptosis of HT-29 cells

It has been known that DNA break or fragmentation is one of the important effects of gossypol-induced cell death.

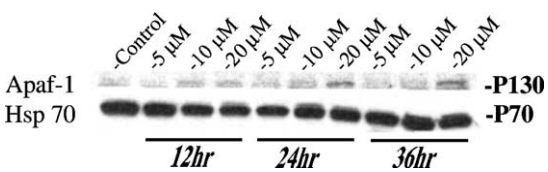

Fig. 9. Apaf-1 up-regulation in gossypol-induced apoptosis of HT-29 cells. HT-29 cells were treated with different concentration of gossypol for various times. Twenty-microgram cell lysates were subjected to 4–20% gradient Tris–glycine gel electrophoresis followed by immunoblot with Apaf-1-specific antibody. Hsp70 reprobing confirms equal loading of the total protein. One representation of three independent experiments.

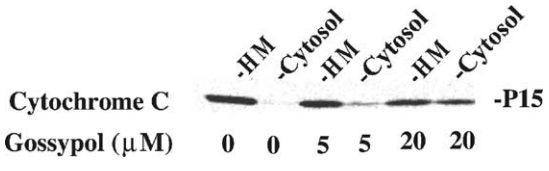

Fig. 10. Cyto-*c* release from mitochondria to cytosol in gossypol-treated HT-29 cells. 2×10^6 HT-29 cells were treated with different concentration of gossypol for 24 hr, cytosol and heavy membrane (HM) part of the treated cells were prepared as described in Section 2. Ten micrograms of cytosol and HM protein were subjected to 4–20% gradient Tris–glycine gel electrophoresis followed by immunoblot with cyto-*c*-specific antibody. One representation of three independent experiments.

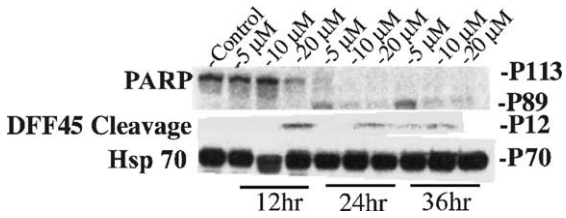

Fig. 11. Activation of both DFF45 and PARP in gossypol-treated HT-29 cells. HT-29 cells were treated with different concentration of gossypol for various times. Twenty-microgram cell lysates were subjected to 4–20% gradient Tris–glycine gel electrophoresis followed by immunoblot with DFF45 cleavage part and PARP-specific antibody, respectively. Hsp70 reprobing confirms equal loading of the total protein. One representation of three independent experiments.

It has been reported that activation of caspase-6, DFF45, or PARP results in DNA damage upon receiving the activation signal from caspase-3 or -7 [50,51]. Since caspase-6 is activated in gossypol-treated HT-29 cells, to reveal whether DFF45 and PARP are activated, the expression of DFF45 and PARP in gossypol-treated HT-29 cells was detected with Western blot. If DFF45 and PARP are activated, P21 cleavage part of DFF45, and P87 (and P24) cleavage part of PARP should be seen. As shown in Fig. 11, P12 cleavage part of DFF45 at 12 hr at 20 μM of gossypol treatment, and P87 cleavage part of PARP at 24 hr at 5 μM of gossypol treatment were observed. Combined with the observation that caspase-6 is activated, these findings suggest that the activation of caspase-6, DFF45, and PARP exists in gossypol-induced apoptosis of HT-29 cells.

4. Discussion

Van Poznak *et al.* recently proposed a model regarding gossypol-induced cell cycle arrest of breast cancer cells, which connected the relation of P53, P21, cyclin D1 and Rb to cell cycle [35]; alteration of any of these pivotal molecules would affect cell cycle. Inconsistent with previous report in HT-29 cells [17], the change of P53 was not observed in this experiment. The possible explanation is that P53 was not influenced by treatment with gossypol. Rb alterations have been observed in breast and other carcinomas [52–54], and its expression level was used as an assessment parameter for clinical breast cancer treatment [35], but the alteration of Rb was also not observed in HT-29 cells treated with gossypol. These results suggest that Poznak's model might not fit in colon cancer, especially HT-29 colon carcinoma cells, and Rb alteration cannot be used as an assessment parameter for colon cancer treatment with gossypol. In addition, unaltered P53 and up-regulated P21 indicate that P21 might be the very first target of gossypol *in vivo* to initiate cell growth inhibition of HT-29 cells.

Apoptosis is one of the major processes leading to cell death. Almost all the work related to gossypol-induced cell death has been focused on its DNA fragmentation and cell cycle arrest effects [14,30,37]. Little is known about the details on gossypol-induced apoptosis. The results of this experiment demonstrate that gossypol treatment can down-regulate all the anti-apoptosis Bcl-2 family members in HT-29 cells: Bcl-X_L, Bag-1, and Mcl-1 from 24 hr of the treatment, which have been reported to be over-expressed in many kinds of cancer cells [55–57]. As for the pro-apoptosis Bcl-2 family members: Bax, Bid, and Bad were first down-regulated at lower dose of gossypol and up-regulated at higher dose. P(Ser112)-Bad and Bak were up-regulated. Even though Bad phosphorylation leading to an anti-apoptosis consequence [58–60], and the expression pattern of Bax was not in concert with the report stating it was up-regulated in HT-29 cells [17], Bak up-regulation here is enough to antagonize the anti-apoptotic effect of this P(Ser112)-Bad, because it has been reported that Bak is more important than other pro-apoptosis Bcl-2 family members in the process of apoptosis induction [61–64]. Thus, these Bcl-2 member proteins are all the *in vivo* targets of gossypol to trigger apoptosis. As observed in *in vitro* binding assay (Fig. 6), gossypol inhibited the binding of Bcl-X_L to BakBH3 peptide (heterodimerization), with its IC_{50} value much lower than that of Bcl-2 (more than 20 times); that is probably the reason Bak expression was up-regulated. It is worthy of noting here that gossypol not only down-regulates anti-apoptosis Bcl-2 family members, but also inhibits their heterodimerization with pro-apoptosis Bcl-2 family members, at least for Bcl-X_L.

It is understandable that extensive caspase activation, from upstream caspase-9 to down stream caspase-6, takes place in gossypol-induced apoptosis of HT-29 cells. This might be the reason that dramatic alterations of apoptosis proteins happen within 24 hr. Moreover, DFF45 activation at as early as 12 hr is another reason for gossypol-induced quick apoptosis.

Mitochondria is one of the most important organelles in the cells which plays a crucial role in the mitochondrial apoptosis signal transduction pathway [65]. Mitochondria either releases cytochrome *c* to cytosol, binds to the CED-4 homolog Apaf-1 and induces caspase-9-dependent activation of caspase-3 [66–68], or releases Smac to cytosol, which functions by eliminating inhibitory effects of IAPs on caspases [69–71]. The other important factor, AIF released from mitochondria can directly cause cell apoptosis without caspase activation [72–74]. It was confirmed that both caspase-9 activation, Apaf-1 up-regulation and cytochrome *c* release happened after at least 24 hr of gossypol treatment. These results suggest that caspase activation during gossypol-induced apoptosis of HT-29 cells is, at least in part, Apaf-1-cyto-*c* dependent; that is, gossypol-induced apoptosis of HT-29 cells is through the mitochondrial pathway.

On the other hand, caspase-8 activation was observed at 24 hr at 10 μM of gossypol treatment, later than the caspase-9 activation, this means that death receptor pathway

[75–77] is another pathway involved in gossypol-induced apoptosis of HT-29 cells. That is, gossypol might bind to some death receptor(s) and trigger the death receptor signal transduction pathway. Hence, it is possible that there are at least two signal transduction pathways involved in gossypol-induced apoptosis: the one with quick response to the stimulus of gossypol is the mitochondrial pathway, the other one with slow response is the death receptor pathway.

Conclusively, gossypol-induces cell cycle arrest on G0/G1 phase as well as cell apoptosis of HT-29 cells, gossypol-induced cell death initiates extensive alterations of cell cycle and apoptosis proteins, gossypol-induced apoptosis of HT-29 cells is through first the mitochondrial pathway, then the death receptor pathway, and the mitochondria pathway is, at least in part, through cyto-*c* release.

There have been several reports regarding the structure–function relationship of gossypol [28–30]. Since this report has provided the details of gossypol-induced cell death, with no doubt, it will provide guidance to develop novel high throughput methods to screen for more potent gossypol derivatives designed by chemists.

One of the important benefits of using gossypol as an anticancer drug which should not be ignored, is that it can kill multi-drug resistant cancer cells [8–10]. One of the reasons gossypol has a low response rate on clinical trial could be the inhibition of its anticancer activity by some of the proteins from patient serum, as there have been reports stated that serum prevented the antiproliferative effect of gossypol *in vitro* [25–27]. Therefore, to reduce this inhibition *in vivo*, but not interfere with the regular physical function of these serum proteins could be one interesting subject to potentiate the pre-clinical study of gossypol. Also, as a modulator, as addressed by some authors [35], gossypol might be used to enhance the anticancer effect of other anticancer chemotherapy drugs, so combined use of some chemotherapy drugs and gossypol is another prospective area in gossypol related pre-clinical research.

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