Gossypol induces Bax/Bak-independent activation of apoptosis and cytochrome c release via a conformational change in Bcl-2

Xiaobo Lei,*,†,1 Yingyu Chen,*,‡,1 Guanhua Du,§ Wenyu Yu,‖ Xiaohui Wang,* Hong Qu,‖ Bin Xia,‖ Hongping He,¶ Jianhua Mao,* Weixing Zong,# Xudong Liao,* Maryam Mehrpour,*, Xiaojiang Hao,¶ and Quan Chen,*,2**

*The Laboratory of Apoptosis and Cancer Biology, The State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing, P.R. China; †Graduate School of the Chinese Academy of Sciences, Beijing, P.R. China; ‡Center for Human Disease Genomics, Peking University, Beijing, P.R. China; §Institute of Materia Medica, Academy Medical Science and Peking Union Medical College, Beijing, P.R. China;. ‖College of Life Sciences, Peking University, Beijing, P.R. China; ¶The State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Beijing, P.R. China; #Abramson Family Cancer Research Institute, Department of Cancer Biology, University of Pennsylvania, Philadelphia, Pennsylvania, USA; **INSERM U-487, Laboratoire de Cytokines et Immunologie des tumeurs Humaines, Institut Gustave Roussy PR1 and IFR 54, Villejuif, France

ABSTRACT **Cells without Bak and Bax are largely resistant to apoptosis (1;2), despite the presence of other key components of the apoptotic machinery. We screened 7,800 natural compounds and found several that could specifically induce caspase activation and the release of cytochrome *c* (cyto c) in the *bak*$^{-/-}$/*bax*$^{-/-}$ cells. One of these was gossypol, a polyphenolic compound naturally found in cottonseed that has been used in antifertility trials. We found that gossypol, but not other Bcl-2-interacting molecules, induced cyto c release and loss of mitochondrial membrane potential ($\Delta\psi m$) independently of mPTP and Bak/Bax activation. Furthermore, we found that gossypol induced an allosteric change in Bcl-2 in both *bak*$^{-/-}$/*bax*$^{-/-}$ cells and Bcl-2 overexpressing cells. This change in Bcl-2 conformation led to the release of cyto c in the presence of Bcl-2 and Bcl-xL in reconstituted proteoliposomes. We also observed that gossypol substantially reduced the growth of tumor xenografts from Bcl-2 overexpressing cells in nude mice. We conclude that gossypol converts the antiapoptotic molecule Bcl-2 into a proapoptotic molecule that can mediate the release of cyto c and induce apoptosis—Lei, X., Chen, Y., Du, G., Yu, W., Wang, X., Qu, H., Xia, B., He, H., Mao, J., Zong, W., Liao, X., L., Mehrpour, M., Hao, X., Chen, Q. Gossypol induces Bax/Bak-independent activation of apoptosis and cytochrome c release via a conformational change in Bcl-2. *FASEB J.* 20, E1510–E1519 (2006)**

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Studies have suggested that, when activated, Bak and Bax, proapoptotic molecules of the Bcl-2 family of proteins, create discontinuity or pores in the outer mitochondrial membrane to mediate cyto c release (3–5). More specifically, Bax translocates to mitochondria where oligomerized Bak and Bax form a megachannel or membrane pore (6, 7). Reports have shown that the mitochondrial apoptotic pathway is not activated in the absence of Bak/Bax-activating signals. Furthermore, cells lacking both Bak and Bax do not undergo apoptosis in response to death stimuli, such as DNA damaging agents, signal transduction through death receptors, growth factor deprivation, and ER stress (2, 8, 9), although they may undergo caspase-independent type II cell death (autophagic cell death) or programmed necrosis in response to high doses of DNA-damaging agents (8). Although *bak*$^{-/-}$/*bax*$^{-/-}$ mice show certain developmental abnormalities, programmed cell death appears to proceed normally *in vivo* and *in vitro* under certain experimental conditions (10). Better understanding of the mechanisms of Bak/Bax-independent cell death is important because cancer cells lacking Bak or Bax, or harboring mutations of these proteins, fail to respond to chemotherapeutic drugs and death ligands (11).

Agents that overcome drug resistance in this type of cancer are of special interest in drug development and cancer therapy. It is desirable to search for small natural compounds from a library that comprises greater structural diversity, since natural compounds

[1]These authors contributed equally to this work.

[2] Correspondence: The Laboratory of Apoptosis and Cancer Biology, The State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Bei Si Huan Xi Road 25, Haidian District, Beijing 100080, P.R. China. E-mail: chenq@ioz.ac.cn;

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have favorable pharmacological properties with minimal side effects. Given that other key components of the apoptotic machinery remain intact in these cells, we screened a library of natural compounds for small molecules that specifically induced apoptosis in *bak*$^{-/-}$/*bax*$^{-/-}$ cells. We identified a number of compounds that induced apoptosis, including gossypol, a polyphenolic compound found in cottonseed. We demonstrated that gossypol potently induced caspase-dependent apoptosis in the absence of Bak and Bax by converting Bcl-2 from an inhibitor to an activator of apoptosis.

MATERIALS AND METHODS

Cell lines and reagents

Simian virus 40 (SV40) transformed embryonic fibroblasts from *bak*$^{-/-}$/*bax*$^{-/-}$ mice were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT, USA) and penicillin/streptomycin at 37°C in a 5% CO_2 humidified atmosphere. IM-9/Bcl-2 cells were maintained as described previously (12).

Gossypol [racemic mixture of two enantiomers (–)gossypol and (+)gossypol, purity ≥ 95% (HPLC)], acridine orange, 3-MA, DPQ, and anti-Bax 6A7 monoclonal antibody (mAb) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3,3′-dihexyloxacarbocyanineiodide [DIOC$_6$ (3)] and anticyto c oxidase mAb were purchased from Molecular Probes (Eugene, OR, USA). Purified anticyto c was purchased from BD Transduction Labs (Lexington, KY, USA). Anti-Bcl-2 antibodies were purchased from BD Transduction, Abgent, and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies and enhanced chemiluminescence (ECL) reagents were purchased from Pharmingen (San Diego, CA, USA) and Pierce (Rockford, IL, USA), respectively. All other chemicals were purchased from Sigma unless otherwise specified.

Detection of cell death by Hoechst 33342 and Annexin-V-FITC

For Hoechst 33342 staining, cells were plated at a density of 5×10^4 cells/ml on glass coverslides in a six-well plate and treated with 20 μM gossypol. After 24 h, cells were stained with Hoechst 33342 in PBS for 15 min at room temperature in the dark. Cells were then washed three times with PBS and analyzed using a fluorescence microscope. At least 200 cells were counted. For quantitative analysis, apoptosis was evaluated by flow cytometry by Annexin V-FITC and propidium iodide (PI) staining following a standard protocol as described previously (13).

Cell staining with acridine orange

To detect whether gossypol induces the formation of accumulation of acidic vesicle organelles, which is characteristic of autophagy, cell staining was performed as described previously (14). After treatment with gossypol, acridine orange was added to a final concentration of 1 μg/ml for 15 min and cells were examined by fluorescence microscopy. For quantitative analysis using flow cytometry, cells were collected in PBS and stained with acridine orange for 15 min. Red fluorescence emission from 10^4 cells was measured with a FACScan using Cell Quest software.

Measurement of mitochondrial membrane potential

This assay was performed as described previously (12, 13). Briefly, after being treated with gossypol, cells were collected and $\Delta\Psi$m indicator DiOC$_6$ (3) [2 μl of 2 μM stock solution in dimethyl sulfoxide (DMSO)] was added to 0.4 ml cell suspension (4×10^5 cells/ml) in PBS (pH 7.2) and incubated at 37°C for 5 min. PI (5 μl of 500 μg/ml stock) was added before analysis. Analysis of $\Delta\psi$m was performed by flow cytometry with excitation at 488 nm. Data were obtained and analyzed with Cell Quest software from the PI-negative cell population on a BD FACScan.

Cell fractionation assay

Cells treated with gossypol were fractionated by differential centrifugation as described previously (12, 15). Briefly, cells were homogenized with a Dounce homogenizer and the homogenate was centrifuged at 800 *g* for 5 min to remove unbroken cells and nuclei. The cytosolic fractions were obtained by further centrifugation at 100,000 g for 30 min.

Immunofluorescence microscopy

For cyto c subcellular localization, cells were grown on glass coverslips, washed with PBS, and fixed in 3.7% formaldehyde-PBS (+) solution. Cells were incubated in 0.1% Triton-X100-PBS (+). Primary antibody (Ab) (mouse anticyto c mAb) was diluted 1:200 in 2% BSA-PBS (+) and incubated with the cells at 4°C for 12 h. The FITC-conjugated secondary Ab was used at a 1:50 dilution in 2% BSA-PBS (+) solution and incubated at room temperature for 2 h.

Detection of activation of casapse *in situ*

Cells were collected and washed with PBS. CaspACE™ FITC-VAD-FMK *in situ* marker was added to the cells to a final concentration of 10 μM and incubated for 20 min in the dark. Cells were then washed three times with PBS and resuspended in 400 μl PBS and analyzed with a FACScan.

Analysis for protein expression

Western blotting was performed as described previously (16). Briefly, cells were washed and lysed in buffer containing 150 mM NaCl; 25 mM HEPES, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 1 mM EGTA; 1 mM DTT; 50 μg/ml trypsin inhibitor; 1 mM PMSF; and 10 μg/mL aprotinin, leupeptin, and pepstatin. Proteins from total cell lysates were resolved on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 for 2 h at room temperature and then probed with indicated antibodies for incubation at 4°C overnight. Immune complexes were detected with HRP-conjugated secondary Ab and were visualized by ECL (Pierce).

Isolation of mouse mitochondria and measurement of mitochondrial function

Mitochondria were isolated from the liver of Balb/c mice as described previously (17, 18). Briefly, liver was minced on ice, transferred to isolated buffer, and homogenized with a glass-Teflon™ Potter homogenizer. Mitochondria were isolated by differential centrifugation in PT-1 buffer containing 250 mM sucrose; 2 mM HEPES, pH 7.4; 0.1 mM EDTA; and 0.1% fatty acid-free BSA. Mitochondria were washed twice and then resuspended in the same medium. All steps were performed on ice. Protein content of mitochondria was determined by the microbiuret method using BSA as a standard. Isolated mouse liver mitochondria were used for the following experiments: *i)* Western blotting for cyto c release. Isolated mitochondria (1 mg protein/ml) were incubated in a total vol of 50 μl PT-2 buffer (250 mM sucrose, 2 mM HEPES, 0.5 mM KH_2PO_4, and 4.2 mM potassium succinate, pH 7.4) in the presence or absence of gossypol for the indicated time at 25°C, followed by centrifugal separation of mitochondria (12,000 *g*, 10 min at 4°C). Aliquots of the supernatant (20 μl) were subjected to Western blotting. Cyto c was detected by anticyto c mAb. Equal protein loading was confirmed by immunodetection of cyto c oxidase subunit IV (COX-IV). *ii)* Determination of mitochondrial membrane potential ($\Delta\Psi$m). As described previously, $\Delta\Psi$m was measured. Briefly, isolated mitochondria (0.1 mg protein/ml) were loaded with 30 nM Rhodamine 123 and incubated at 25°C in the PT-2 medium. By measuring the $\Delta\Psi$m-dependent release of Rhodamine 123 from mitochondria using a spectrofluorimeter (Jobin-Yvon FluoroMax-2, excitation=505 nm and emission=534 nm), $\Delta\Psi$m was assessed. *iii)* Measurement of mitochondrial swelling. Mitochondrial swelling was monitored by the decrease of 90° light scatter at 520 nm in the PT-2 medium at 25°C using Jobin-Yvon FluoroMax-2 spectrofluorimeter as described (17, 19).

Immunoprecipitations for detecting Bax and Bcl-2 conformational change

Cells were lysed with 1% Chaps lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 1% Chaps] containing protease inhibitors as described (7, 20). Total protein (500 μg) was incubated with 2 μg of anti-Bax 6A7 mAb in 500 μl of Chaps lysis buffer at 4°C overnight on a rotator. Immunoprecipitates were collected by incubating with 20 μl protein G agarose for 2 h at 4°C, followed by centrifugation for 1 min. The pellets were washed three times with Chaps lysis buffer, and beads were boiled in loading buffer and analyzed by Western blotting using the anti-Bax polyclonal antibody (pAb). For detection of Bcl-2 conformational change, cells were lysed in 1% Chaps buffer (1% Chaps; 14.5 mM KCl; 5 mM $MgCl_2$; 1 mM EGTA; 1 mM EDTA; 20 mM Tris, pH 7.5) (21). Total protein (500 μg) was incubated with appropriate Ab and 20 μl protein G agarose beads overnight at 4°C. Beads were washed three times with 1% Chaps buffer before Western blotting.

Measurement of cyto c release in Bcl-xL and Bcl-2 liposomes

Liposomes were prepared by a standard method as described previously (22, 23). Briefly, 500 mg L-α-phosphatidyl choline was dissolved in 5 ml chloroform, and the solvent was then evaporated under nitrogen. A phospholipid mixture was reconstructed in 10 ml liposome buffer containing 50 mM KCl, 20 mM KH_2PO_4, 20 mM HEPES (pH 7.0), and 1 mM EDTA. After sonication, purified Bcl-xL or Bcl-2 (0.1 mg/ml, final concentration) was then mixed with liposomes and incubated for 20 min. FITC-conjugated cyto c was loaded into the proteoliposomes by three freeze-thaw cycles, and then the proteoliposomes were washed three times with the liposome buffer. Aliquots of the three types of liposomes were mixed with 50 μM gossypol and incubated for 1 h, and the reactions were terminated by centrifugation. The cyto c released in the supernatant was detected by immunoblotting assay.

Effect of gossypol on tumor growth

IM-9/Bcl-2 cells were harvested by centrifugation and suspended in HBSS. A tumor cell suspension (5×10^6 cells in 50 μl of HBSS) was injected into the back region of 6–8 wk old Balb/c mice using a 27-gauge needle. Two groups of animals (10 animals each group) were used. Animals were treated with 30 mg/kg/day of gossypol two days after injection. The mice were sacrificed 33 d after cell injection. Tumor size was monitored by measurement of the length (a) and width (b) of the tumor using a slide gauge. Tumor volumes (V) were calculated according to the formula: $V = 1/6\pi[(a+b)/2]^3$.

Statistical analysis

Statistical analysis was performed using Student's *t* test analysis, with *P* values < 0.05 considered significant.

RESULTS

Identification of gossypol inducing typical apoptosis in bak$^{-/-}$/bax$^{-/-}$ cells

Using simian virus 40 (SV40) transformed embryonic fibroblasts from Bak-Bax double knockout mice (*bak*$^{-/-}$/*bax*$^{-/-}$) (1, 8), we first screened 7800 natural compounds for their effects on cell viability by using the MTT assay. Twenty-three compounds resulted in the reduction of MTT. We then stained the cells with acridine orange and examined them using a fluorescent microscope to identify nuclear condensation (morphological hallmark of apoptosis) or accumulation of acidic vesicles in the cytoplasm (marker of autophagic cell death) of *bak*$^{-/-}$/*bax*$^{-/-}$ cells. One of these compounds, gossypol, was found to induce nuclear fragmentation in *bak*$^{-/-}$/*bax*$^{-/-}$ cells (**Fig. 1*A***). Gossypol was originally identified as a male contraceptive drug (24, 25). Although it is not fully effective as a contraceptive, there has been renewed interest in the compound for the treatment of cancer (26, 27).

To further characterize the death process, we measured phosphatidylserine exposure, a defining morphological characteristic of apoptotic cells. Gossypol induced a significant increase in the size of the Annexin V positive population (apoptotic cells) in a time-dependent manner as compared to an untreated control (Fig. 1*B*). The effect was maximal for cells exposed to 20 μM of Gossypol for 48 h (50% apoptotic cells). We next used CaspACE™ FITC-VAD-FMK *in situ* Marker to detect the intracellular caspase activity in the individual cells. As shown in Fig. 1*C*, gossypol induced caspase activation in a time-dependent manner. However, we did not detect caspase activity when *bak*$^{-/-}$/*bax*$^{-/-}$ cells were treated with VP-16, cisplatin, or other related agents. We next measured the activation of effector caspases by determining the cleavage of the chromogenic caspase tetrapeptide substrate Ac-DEVD-pNA. The DEVD cleavage activity appeared 12 h after gossypol treatment and was further elevated in a time-dependent manner (data not shown). Moreover, z-VAD-fmk (100 μM), a pan-caspase inhibitor, could potently block gossypol-induced cell death (Fig. 1*D*).

The autophagic inhibitor 3-Methylademine (3-MA) did not block cell death induced by gossypol (Fig. 1*D*).

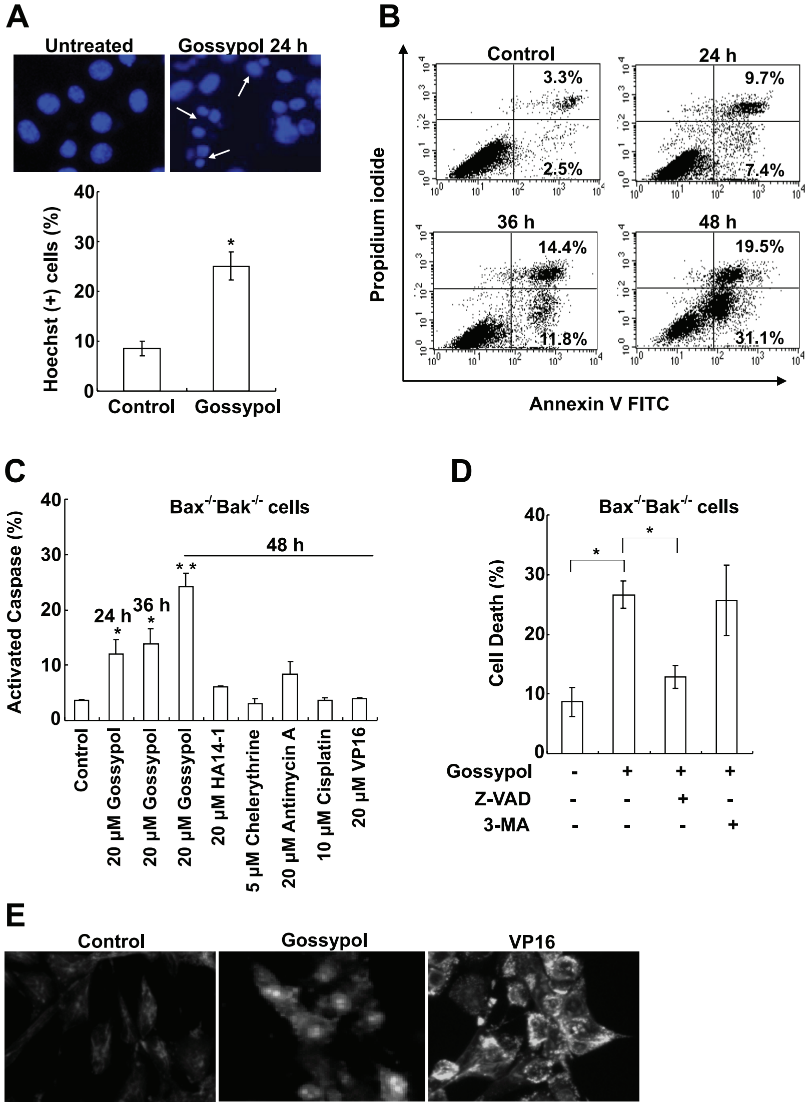

Figure 1. Apoptosis in $bak^{-/-}/bax^{-/-}$ cells induced by gossypol. *A) $bak^{-/-}/bax^{-/-}$* cells were treated with 20 μM gossypol for 24 h, followed by fixation, stained with Hoechst33342, and visualized by fluorescent microscopy. Arrows indicate the condensed, fragmented, brightly stained nuclei, which are the hallmark of apoptosis. Bar diagram showing the ratio of Hoechst positive cells. Data were the mean value of three independent experiments with each count of no less than 200 cells. $*P < 0.05$. *B)* Flow cytometric analysis of apoptosis in $bak^{-/-}/bax^{-/-}$ cells following treatment with 20 μM gossypol for the indicated times. The percentage of apoptotic cells was determined by Annexin V/PI staining. *C) $bak^{-/-}/bax^{-/-}$* cells were treated with 20 μM gossypol for the indicated time or with indicated compound for 48 h, and the percentage of cells with activated caspases were characterized as those that stained with the FITC In Situ Marker. Data represent the mean values of three independent experiments. $*P < 0.05$, $**P < 0.01$. *D) $bak^{-/-}/bax^{-/-}$* cells were exposed to 20 μM gossypol in the presence of 100 μM zVAD-fmk or 5 mM autophagic inhibitor 3-MA for 24 h. The percentage of cell death was determined by Annexin V/PI staining followed by flow cytometric analysis. Results are expressed as the mean (±SD) for three independent experiments. $*P < 0.05$. *E) $bak^{-/-}/bax^{-/-}$* cells were treated with 20 μM gossypol or 20 μM VP16 for 36 h and then stained with 1 μg/ml of acridine orange, and observed using fluorescent microscopy (×40).

The accumulation of acidic vesicle organelles was not observed after treatment with gossypol, while VP16, a positive control of autophagic cell death, induced the significant accumulation of acidic vesicle organelles in $bak^{-/-}/bax^{-/-}$ cells as examined using fluorescent microscopy (Fig. 1*E*). These results suggest that gossypol induces morphological and biochemical changes typical of apoptosis but not caspases-independent autophagic cell death.

Gossypol induced cyto c release in a mPTP-independent manner

To investigate the underlying mechanism of caspase activation in $bak^{-/-}/bax^{-/-}$ cells, we analyzed cyto c release in $bak^{-/-}/bax^{-/-}$ cells treated by gossypol. We found that gossypol induced cyto c release in a time-dependent manner. Cyto c appeared in the cytosol within 12 h of exposure to gossypol with an accompanying decrease of cyto c in the mitochondria (**Fig. 2*A***, *top*). The release of cyto c from mitochondria was concomitant with the appearance of caspase activity within cells (Fig. 1*C*). Immunostaining of cyto c revealed that, in contrast to the punctate staining in untreated cells, cyto c staining became diffuse 24 h after treatment (Fig. 2*A*, *bottom*), indicating the loss of cyto c from mitochondria and its translocation to the cytosol. To exclude the possibility that gossypol may activate intracellular pathways that induce cyto c release, we isolated mitochondria from $bak^{-/-}/bax^{-/-}$ cells and found that exposure to gossypol directly induced the release of cyto c from mitochondria in a dose-dependent manner (Fig. 2*B*) in the absence of Bak and Bax. These data clearly demonstrate that gossypol induces cyto c release from mitochondria to activate caspase and to induce apoptosis in the absence of Bak and Bax.

Currently, there are two possible pathways that may mediate the release of cyto c from mitochondria (28). One involves Bax activation, its oligomerization, and its interaction with other mitochondrial membrane proteins (7). Alternatively, the opening of mitochondrial permeability transition pores (mPTP) results in the rupture of the outer mitochondrial membrane and, thereby, the nonspecific release of cyto c. To determine

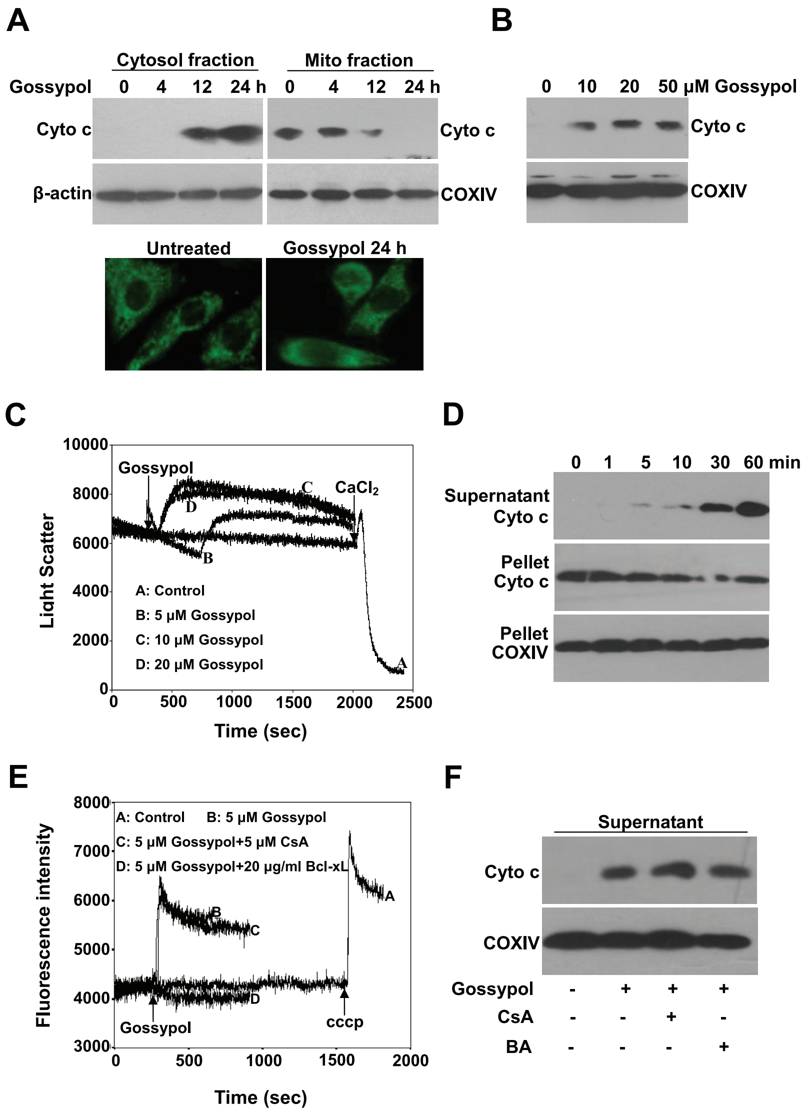

Figure 2. Gossypol induced cyto c release from mitochondria independent of the opening of mPTP. *A)* $bak^{-/-}/bax^{-/-}$ cells were exposed to 20 μM gossypol for the indicated times and subjected to subcellular fractionation. The cytosolic (cytosol) and mitochondrial (mito) fractions were analyzed by Western blotting with cyto c Ab. β-actin and COX IV were used as a loading control. Bottom images, microscopy analysis of cyto c release in $bak^{-/-}/bax^{-/-}$ cells (see Materials and Methods for detail). *B)* Isolated mitochondrial from $bak^{-/-}/bax^{-/-}$ cells were incubated with gossypol at indicated concentrations for 1 h. The presence of cyto c was detected by Western blotting. *C)* Isolated mitochondria from mouse liver cells were treated as indicated. PTP opening was monitored as described in Materials and Methods. 100 μM $CaCl_2$ was added at the end of experiments as a positive control for PTP opening. *D)* Isolated mitochondria from mouse liver were incubated in 50 μl PT-2 medium with 20 μM gossypol for indicated time at 25°C. The presence of cyto c in mitochondria supernatants and pellet was assessed by immunoblotting analysis. COX IV staining served as a loading control. *E)* $\Delta\psi m$ was assessed by measuring the fluorescence intensity of the membrane potential dependent dye rhodamin123 (30 nm) using a Jobin-Yvon Fluoromax-2. Isolated mitochondria from mouse liver were pretreated with 5 μM CsA or preincubated with 20 μg/ml Bcl-xL in 3 ml PT buffer for 10 min before gossypol (5 μM) was added. After being incubated at 25°C for 30 min, mitochondria were exposed to 1 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), a commonly used protonophore that causes rapid and complete dissipation of membrane potential. *F)* Isolated mitochondria from mouse liver were preincubated with 5 μM CsA and 50 μM BA, then treated with gossypol (20 μM) for 1 h as described previously. The presence of cyto c in mitochondria supernatants was assessed by Western blotting.

whether gossypol induces cyto c release from mitochondria via the opening of mPTP, we used isolated mitochondria from mouse liver and found that gossypol did not cause mitochondrial swelling, which is indicative of the opening of mPTP (Fig. 2*C*). Nonetheless, gossypol could induce cyto c release in a time- (Fig. 2*D*) and dose-dependent manner (data not show). The release of cyto c from mitochondria started at 10 min after treatment with 20 μM gossypol (Fig. 2*D*), whereas the mPTP opening didn't occur even 30 min after treatment with 20 μM gossypol (Fig. 2*C*). These data further support the notion that gossypol-induced cyto c release from isolated mitochondria is not the result of mPTP opening. Due to its mild uncoupling effect, gossypol could reduce mitochondrial membrane potential ($\Delta\psi m$) to a certain extent (Fig. 2*E*). Cyclosporin (CsA) and Bonkrekic acid (BA), commonly used inhibitors of mPTP opening, could not inhibit gossypol-induced cyto c release in $bak^{-/-}/bax^{-/-}$ cells (data not shown) or isolated mitochondria from mouse liver (Fig. 2*F*). These results strongly suggest that gossypol-induced cyto c release is independent of mPTP opening.

Unlike gossypol, other Bcl-2 inhibitors cannot induce Bax/Bak and mPTP opening independent cyto c release

Other compounds such as HA14-1 (reported to be a Bcl-2 inhibitor) and chelerythrine (reported to be a Bcl-xL inhibitor) induced mitochondrial swelling (**Fig. 3*A***, ***B***) and cyto c release (Fig. 3*C*) from isolated mitochondria from mouse liver in a CsA-sensitive manner, whereas CsA itself had no effect on cyto c release (Fig. 3*C*). Computer modeling suggested that gossypol, HA14–1, and chelerythrine could interact with Bcl-xL and Bcl-2 (29); we thus checked the effects of other Bcl-2 inhibitors on $bak^{-/-}/bax^{-/-}$ cells. Interestingly, HA14–1 and chelerythrine could induce apoptosis in Bcl-2 overexpressing cells (see below), although they did not induce apoptosis (data not show) and cyto c release (Fig. 3*D*) in $bak^{-/-}/bax^{-/-}$ cells. These results suggest that gossypol has the distinct property of inducing the opening of mPTP and cyto c release in $bak^{-/-}/bax^{-/-}$ cells and mouse liver.

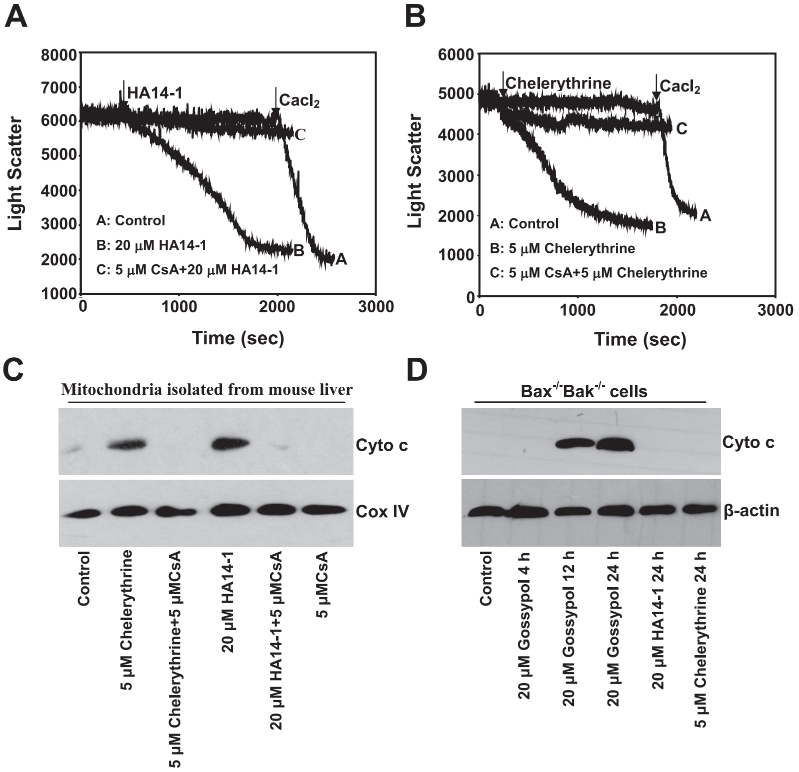

Figure 3. The effects of other Bcl-2/Bcl-xL inhibitors. *A, B)* Isolated mitochondria from mouse liver were treated as indicated and PTP opening was monitored in the presence of HA14–1 or chelerythrine as described in Materials and Methods. *C)* Isolated mitochondria from mouse liver were preincubated with or without 5 μM CsA in 50 μl PT-2 medium before being treated with chelerythrine or HA14–1 for 1 h at indicated concentrations at 25°C. The presence of cyto c in mitochondria supernatants was assessed by immunoblotting analysis. COX IV staining served as a loading control. *D)* Gossypol, but not other Bcl-2 inhibitors, induces cyto c release in *bak*$^{-/-}$/*bax*$^{-/-}$ cells. Cells were exposed to gossypol, HA14–1, and chelerythrine for the indicated times and subjected to subcellular fractionation. The cytosolic fractions were analyzed by Western blotting using cyto c Ab. β-actin was used as a loading control.

Gossypol induces allosteric Bcl-2 conformational change

In an attempt to identify the potential target(s) responsible for its apoptosis-inducing effects in the absence of Bak-Bax and opening of mPTP, we asked whether gossypol directly interacts with Bcl-2 to induce a change in Bcl-2 conformation. It has been suggested that conformational changes in Bcl-2/Bcl-xL convert them from antiapoptotic to proapoptotic (21). Using NMR chemical shift perturbation analysis, we found that gossypol, but not chelerythrine or antimycin A, interacted with Bcl-xL protein at the pocket region among its BH1, BH2, and BH3 domains (**Fig. 4*A*, *B***). Most of the residues involved in gossypol binding are located at the Bak BH3 peptide binding site (30). Careful analysis showed that this interaction also resulted in significant changes in the chemical environments of a number of amino acids outside the region. Therefore, similar to the binding of Bak and BH3I-1 (31), the binding of gossypol likely induces a conformational change in Bcl-xL to open its hydrophobic binding surface. To determine the direct effect of gossypol on Bcl-2 conformation, we used an Ab (αBcl-2/BH3-domain pAb) that recognizes the exposed epitope of BH3 in Bcl-2 molecule. Immunostaining detected by flow cytometric assay revealed that gossypol could significantly increase the fluorescent intensity of αBcl-2/BH3 domain pAb, but not mouse Ab, against the whole Bcl-2 protein (Fig. 4*C*). Immunoprecipitation analysis with a specific Ab showed that gossypol induced a conformational change in Bcl-2 in *bak*$^{-/-}$/*bax*$^{-/-}$ cells, but it did not induce a change in Bcl-2 expression levels (Fig. 4*D*). To determine whether gossypol directly induces a change in Bcl-2 conformation, we treated mitochondria isolated from *bak*$^{-/-}$/*bax*$^{-/-}$ cells with gossypol. The data depicted in Fig. 4*E* show that the exposure of gossypol directly induces a change in Bcl-2 conformation in the absence of cytosol in a dose-dependent manner. In contrast, HA14–1 and chelerythrine did not induce the Bcl-2 conformational change in *bak*$^{-/-}$/*bax*$^{-/-}$ cells (Fig. 4*F*).

To examine whether the interaction of gossypol with Bcl-2 or Bcl-xL has functional consequence for cyto c release, we reconstituted the proteoliposomes encapsulated with FITC-cyto c in the presence or absence of Bcl-2 and Bcl-xL as previously reported (22). Following treatment with gossypol, cyto c was released to supernatant in the presence of Bcl-2 and Bcl-xL (Fig. 4*G*), but there was no cyto c release in the absence of Bcl-2 or Bcl-xL or in the untreated control. This was in sharp contrast to our previous observation that Bcl-xL could prevent VDAC-mediated cyto c release in the same reconstituted proteoliposome system (22).

Gossypol induces typical apoptosis in Bcl-2 overexpressing cells in a Bcl-2 conformational change-dependent manner without activation of Bax

We next asked whether gossypol would induce Bcl-2 conformational change in other systems. We found that gossypol induced apoptosis in IM-9 Bcl-2 overexpressing cells in a time- (**Fig. 5*A***) and dose-dependent manner (Fig. 5*B*). Similar results were obtained with Bcl-xL stably overexpressed cells (data not shown). Gossypol induced the reduction of Δψm, cyto c release (Fig. 5*C*), and processing of caspase and PARP in a time-dependent manner (Fig. 5*D*). Interestingly, other

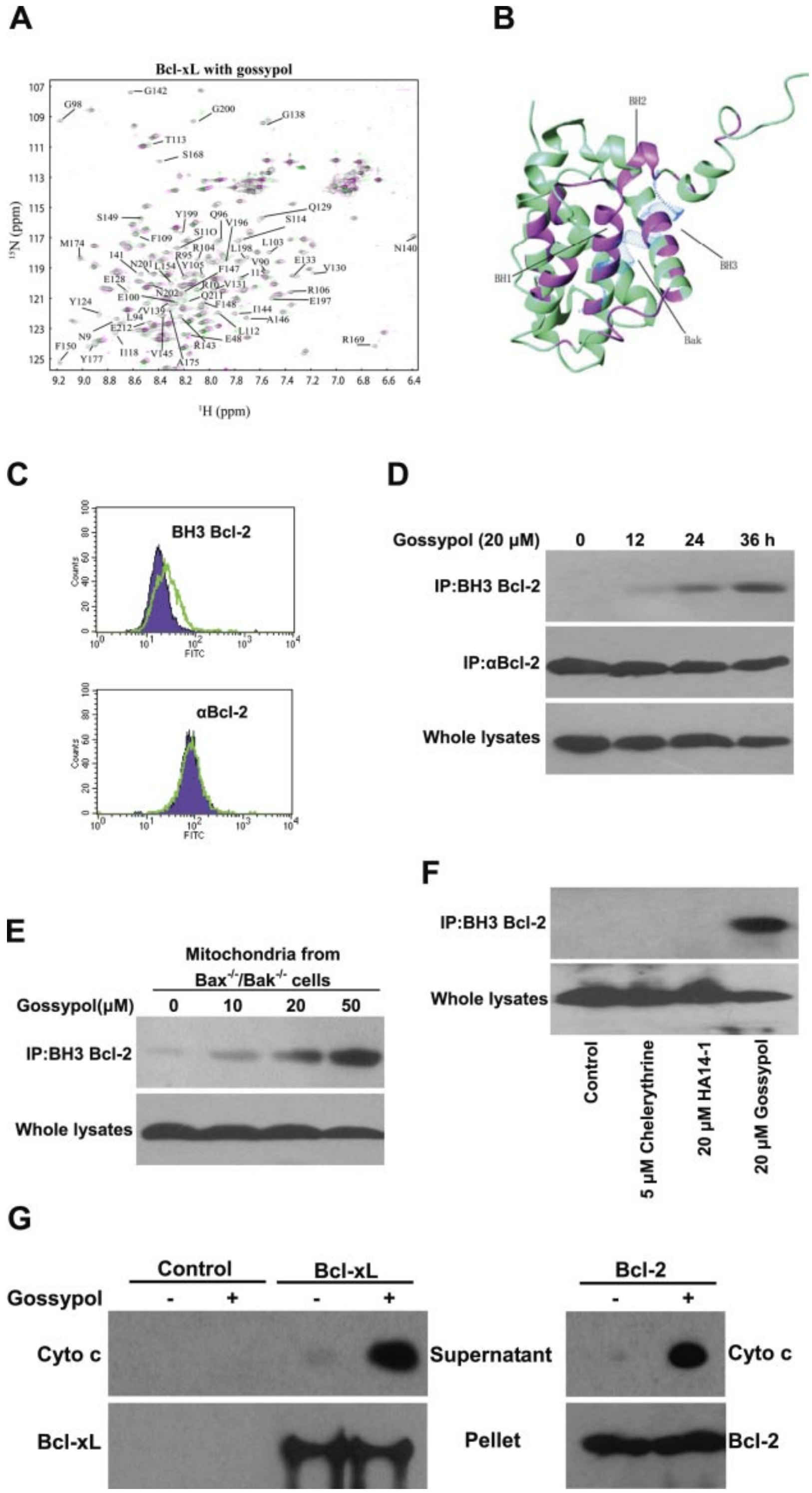

Figure 4. Gossypol induces allosteric Bcl-2 conformational change, which may be responsible for cyto c release. *A)* Superimposition of ^{1}H-^{15}N HSQC spectra recorded at different Bcl-xL/gossypol ratios: without gossypol (black peaks), 2:1(green peaks), and 1:1(magenta peaks). The residues for which the NH signals showed chemical shift or intensity changes on binding of gossypol are labeled by residue type and number. *B)* Interaction region between gossypol and Bcl-xL. The dotted blue part is the Bak peptide. All the residues whose NH chemical shifts affected by gossypol titration are indicated in pink. These residues are mostly located at BH1, BH2, and BH3 (also labeled in figure), which form the hydrophobic pocket. *C)* $bak^{-/-}/bax^{-/-}$ cells were treated with or without gossypol for 24 h and then immunostained with indicated Bcl-2 Ab, followed by FITC-conjugated secondary Ab, and identified by flow cytometry. Control (purple) and gossypol treated (green). *D)* $bak^{-/-}/bax^{-/-}$ cells were exposed to 20 μM gossypol for indicated time. Following treatment, cells were lysed in Chaps lysis buffer and immunoprecipitated with the indicated anti-Bcl-2 Ab. Immunoprecipitates were subjected to immunoblotting using anti-Bcl-2 Ab. *E)* Isolated mitochondria from $bak^{-/-}/bax^{-/-}$ cells were exposed to different concentrations of gossypol as indicated at 25°C for 60 min. After centrifugation, mitochondria were lysed in Chaps buffer and immunoprecipitation was performed as described above. *F)* $bak^{-/-}/bax^{-/-}$ cells were exposed to 5 μM chelerythrine, 20 μM HA14–1, and 20 μM gossypol for 36 h. Following treatment, cells were lysed in Chaps lysis buffer and immunoprecipitation was performed as described above. *G)* Liposomes were reconstituted as described in Materials and Methods and incubated in the presence or absence of 50 μM gossypol for 1 h at 25°C. Cyto c release was detected by immunoblotting with anticyto c Ab. Incorporation of the Bcl-2/Bcl-xL proteins in liposomes was confirmed by Western blotting with anti-Bcl-2/Bcl-xL Ab, respectively.

Bcl-2 inhibitors such as HA14–1, antimycin A, and chelerythrine induced apoptosis in Bcl-2 overexpressing cells (Fig. 5*E*), although these inhibitors had no apoptotic effect on $bak^{-/-}/bax^{-/-}$ cells (Fig. 1*C*). Gossypol did not induce Bax conformational change (Fig. 5*F*) or Bax homodimerization or oligomerization (Fig. 5*G*), but it was found to induce Bax activation in the parental cells (unpublished data). Bcl-2 conformational changes were detected 4 h after treatment in IM-9/Bcl-2 cells, concomitant with the appearance of apoptosis and cyto c release, although treatment did not induce changes in Bcl-2 expression levels (Fig. 5*H*). The compound did not kill white blood cells from healthy donors, although it induced apoptosis in IM-9 cells by activating Bax conformational change under identical experimental conditions (data not shown).

We next tested the effects of gossypol on the growth of IM-9/Bcl-2 cells after implantation in nude mice. Two days after implantation of the IM-9/Bcl-2 cells (5×10^{6}) onto the backs of the mice, we began treating

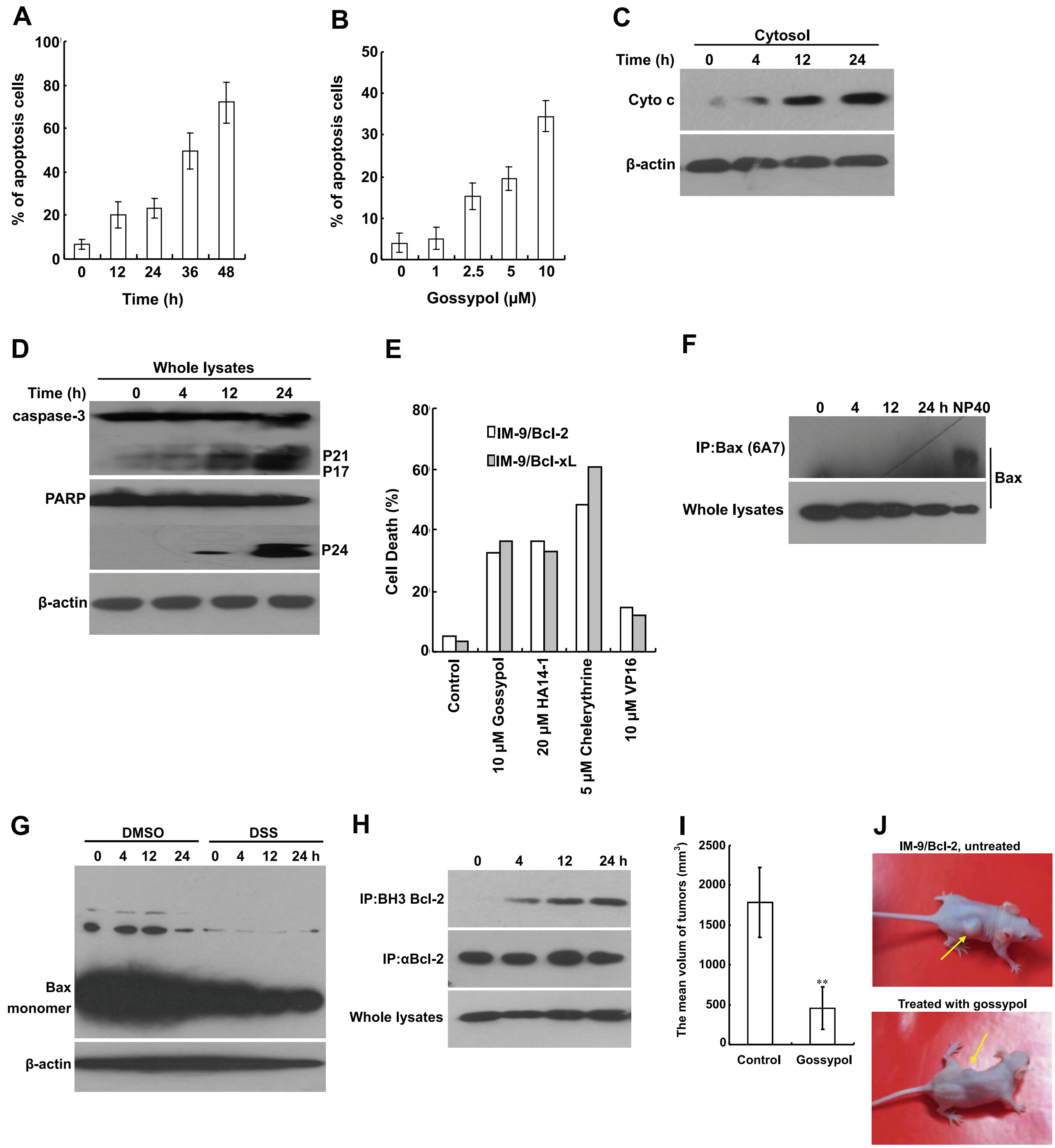

Figure 5. Gossypol induces apoptosis in Bcl-2 overexpressing cells in a Bcl-2 conformational change-dependent manner without activation of Bax. *A, B)* IM-9/Bcl-2 cells were exposed to 10 μM gossypol for indicated times (*A*) or 24 h as indicated concentration of gossypol (*B*). The percentages of cell death were determined by Annexin V/PI staining. *C)* IM-9/Bcl-2 cells were exposed to 10 μM gossypol for the indicated times and subjected to subcellular fractionation. The cytosolic fraction was analyzed by immunoblotting with Ab specific for cyto c. β-actin was used as a protein loading control. *D)* Western blotting analysis of the cleavage of Caspase-3 and PARP. IM9/Bcl-2 cells were treated with 10 μM of gossypol for 4, 12, and 24 h. Following treatment, cells were lysed and 50 μg of protein was loaded in each lane of an SDS-PAGE gel. β-actin was stained as a loading control. All data shown are representative of three separate experiments. *E)* IM-9/Bcl-2 and IM-9/Bcl-xL cells were treated with 10 μM gossypol, 20 μM HA14–1, 5 μM chelerythrine, and 10 μM VP16 for 24 h. Apoptosis was identified by Annexin V/PI staining. Data were the mean value of three independent Annexin V assays. *F)* IM-9/Bcl-2 cells were treated with 10 μM gossypol, lysed in Chaps lysis buffer, and subjected to immunoprecipitation with anti-Bax 6A7 Ab for detection of conformationally changed Bax protein. Cell lysate obtained by Nonidet P-40 lysis was used as a positive control. *G)* Cells were treated with 10 μM gossypol for 0, 4, 12, 24 h. The oligomerizatin of Bax was assessed by crosslinking with DSS followed by immunoblotting analysis. DMSO was used as the vehicle control, and actin was used as a protein loading control. *H)* IM-9/Bcl-2 cells were treated with 10 μM gossypol for indicated time. Cells were lysed in Chaps lysis buffer and subjected to immunoprecipitation with two different anti-Bcl-2 antibodies. *I)* Inhibitory effect of gossypol on implanted tumors in nude mice. IM-9/Bcl-2 cells (5×10^{6}) were injected into the backs of nude mice. Two days after the inoculation mice were treated with gossypol (30 mg/kg/day) or saline solution. Volumes of tumors in nude mice from the control groups and from the groups treated with gossypol. *J)* Pictures of nude mice. Arrows show the tumors.

them daily with gossypol or a control physiological saline solution. As shown in Fig. 5*I*, the tumor vol of the treated group [0.44 ± 0.24 cm^3] was significantly reduced compared to the control group [$1.79+0.44$ cm^3]. As shown in Figure 5*J*, gossypol inhibited tumor growth in the mice without adverse effects on body wt and activity. These results indicate that gossypol effectively inhibits the growth of IM-9/Bcl-2 cells *in vivo* without apparent adverse side effects.

DISCUSSION

In this paper, we first used $bak^{-/-}/bax^{-/-}$ cells, which are resistant to most death stimuli (2, 8), to screen for small natural molecules that can induce caspase-dependent apoptosis. Our results show that gossypol can induce cyto c release from mitochondria to activate caspase-dependent apoptosis in the absence of both Bak and Bax. We further found that gossypol induced Bcl-2 conformational change, which may convert the protective molecule of Bcl-2/Bcl-xL into a killer molecule. Recent reports show that Bcl-2 conformational change occurs during the onset of apoptosis, although it has been suggested that this conformational change may be integral to its antiapoptotic function (32). Interaction of Bcl-2 with a nuclear orphan receptor may convert Bcl-2 from a protector to a killer molecule via conformational change (21). Photodynamic therapy and ursodeoxycholi acid also cause a conformational change in Bcl-2 and promotes HA14–1 to bind to Bcl-2 (33). To the best of our knowledge, our study is the first to identify a small molecule that induces Bcl-2 conformational change and to link this type of change with cyto c release. Our results suggest that, in addition to the rheostat balancing of protective Bcl-2 protein to Bak and Bax, the conformational status of the protective molecule is also important in determining the fate of a cell. This conversion could be reminiscent of Ced-9 in *C. Elegans,* whose genome contains no Bax homologue. It has been suggested that Ced-9 performs the functions of both Bax and Bcl-2 concomitant with its conformational change (34). It is possible that the exposure of Bcl-2's BH3 domain and its insertion into the mitochondrial membrane may change the binding properties of Bcl-2 to other apoptosis-related proteins, or to lipids in the outer mitochondrial membrane, to create discontinuity or pores. Our results do not exclude other mechanisms such as hexokinase dissociation from mitochondria (35) or the conformational changes of other antiapoptotic molecules such as Bcl-xL and Mcl-1.

A wide array of Bcl-2 inhibitors are reported to interact with Bcl-2 and induce tumor cell apoptosis or regression of tumors with single-agent treatment (36). These inhibitors bind to the same hydrophobic groove, leading to the disruption of Bcl-2's heterodimerization with proapoptotic partners and inhibition of its pore forming activity. These inhibitors target mitochondria to induce cyto c release via a mechanism of CsA-inhibitable opening of PTP and in Bcl-2 overexpressing cells, but they had no killing effect on $bak^{-/-}/bax^{-/-}$ cells in our experiments. Our results suggest that gossypol is distinct from these Bcl-2 inhibitors. The discovery that gossypol changes Bcl-2 from a protector to a killer will help further elucidate Bcl-2 function and its mechanism both *in vitro* and *in vivo.* This discovery also holds promise for cancer therapy, since overexpression of Bcl-2 or Bcl-xL has been observed in 80% of B-cell lymphomas, 90% of colorectal adenocarcinomas, and many other forms of cancer (37). Recently, gossypol was shown to inhibit the growth of several tumor cell lines *in vitro* and has been suggested to be a potential antitumor drug (27, 38, 39). Given its tolerable toxicity and ability to convert Bcl-2 to a proapoptotic molecule, gossypol holds promise as a more potent and specific agent targeting Bcl-2-regulated apoptosis, both alone or in combination with other anticancer agents. Further work is underway to screen and examine novel compounds that induce Bcl-2 conformational change to change the molecule from anti to proapoptotic. FJ

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Gossypol induces Bax/Bak-independent activation of apoptosis and cytochrome c release via a conformational change in Bcl-2

Xiaobo Lei,[*,†,1] **Yingyu Chen,**[*,‡,1], **Guanhua Du,**[§] **Wenyu Yu,**[‖] **Xiaohui Wang,**[*] **Hong Qu,**[‖] **Bin Xia,**[‖] **Hongping He,**[¶] **Jianhua Mao,**[*] **Weixing Zong,**[#] **Xudong Liao,**[*] **Maryam Mehrpour,**[*,**] **Xiaojiang Hao,**[¶] **and Quan Chen,**[*,2]

*The Laboratory of Apoptosis and Cancer Biology, The State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing, P.R. China; †Graduate School of the Chinese Academy of Sciences, Beijing, P.R. China; ‡Center for Human Disease Genomics, Peking University, Beijing, P.R. China; §Institute of Materia Medica, Academy Medical Science and Peking Union Medical College, Beijing, P.R. China; ‖Beijing Nuclear Magnetic Resonance Center, Peking University, Beijing, P.R. China; ¶The State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Beijing, P.R. China; #Abramson Family Cancer Research Institute, Department of Cancer Biology, University of Pennsylvania, Philadelphia, Pennsylvania, USA; **INSERM U-487, Laboratoire de Cytokines et Immunologie des tumeurs Humaines, Institut Gustave Roussy PR1 and IFR 54, Villejuif, France

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SPECIFIC AIMS

Cells lacking both Bak and Bax are largely resistant to apoptosis induced by a number of death stimuli, including DNA-damaging agents, signal transduction through death receptors, growth factor deprivation, and ER stress. These cells may undergo caspase-independent type II cell death (autophagic cell death) or programmed necrosis in response to high doses of DNA-damaging agents. Given that the other key components of the apoptotic machinery at both pre- and postmitochondrial levels are intact in those $bak^{-/-}/bax^{-/-}$ cells, we undertook the large-scale screening of a natural compounds library for small molecules that could induce apoptosis in $bak^{-/-}/bax^{-/-}$ cells.

PRINCIPAL FINDINGS

1. We screened out compounds that can activate caspase-dependent apoptosis in Bak/Bax double knockout (bak$^{-/-}$/bax$^{-/-}$) cells

Using simian virus 40 (SV40) transformed $bak^{-/-}/bax^{-/-}$ embryonic fibroblasts, we first tested the inhibitory activity of a library of 7800 natural compounds on cell viability using the MTT assay. 23 compounds that resulted in a reduction of MTT staining, and in cells treated with those compounds, we applied acridine orange staining, Annexin V staining and an assay for intracellular caspase activity. One of these compounds, gossypol, a polyphenolic compound naturally occurring in cottonseed that has been clinically used for male contraceptive, was found to induce nuclear fragmentation and apoptosis in $bak^{-/-}/bax^{-/-}$ cells (**Fig. 1***A, B*). Gossypol-induced apoptosis is dependent on caspase activation and z-VAD-fmk (100 μM), a pan-caspase inhibitor that could potently block gossypol-induced cell death (Fig. 1*C, D*). However, other related agents did not induce caspase activation in $bak^{-/-}/bax^{-/-}$ cells (Fig. 1*C*).

2. We found that gossypol induced cyto c release in a mitochondrial permeability transition pore independent manner in bak$^{-/-}$/bax$^{-/-}$ cells

We found that gossypol induced cyto c release from mitochondria to the cytosol in $bak^{-/-}/bax^{-/-}$ cells. Therefore, we aimed to understand the mechanism of gossypol-induced apoptosis and cyto c release in the absence of Bak and Bax. Since mPTP opening is one of the pathways for cyto c release, we used isolated mito-

[1]These authors contributed equally to this work.

[2]Correspondence: The Laboratory of Apoptosis and Cancer Biology, The State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Bei Si Huan Xi Road 25, Haidian District, Beijing 100080, P.R. China. E-mail: chenq@ioz.ac.cn;
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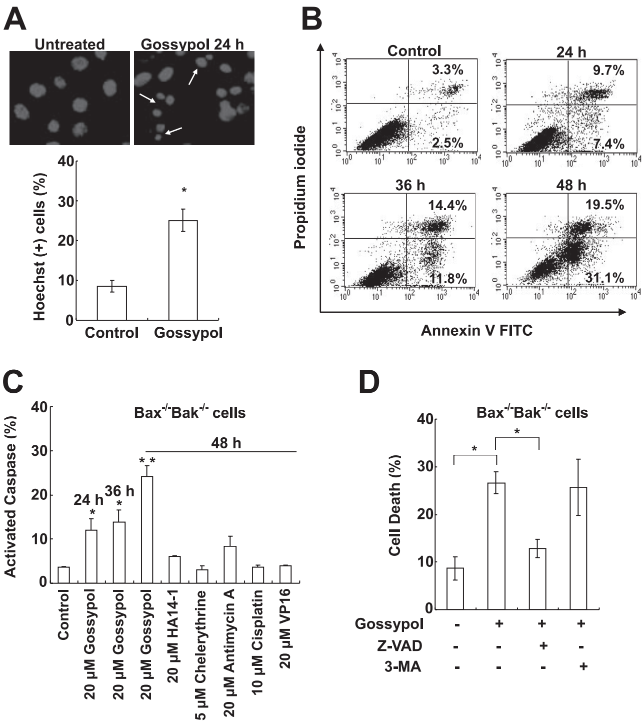

Figure 1. Apoptosis in $bak^{-/-}/bax^{-/-}$ cells induced by gossypol. *A)* $bak^{-/-}/bax^{-/-}$ cells were treated with 20 μM gossypol for 24 h, followed by fixation and stained with Hoechst33342, and visualized by fluorescent microscopy. Arrows indicate the condensed, fragmented, brightly stained nuclei, which are the hallmark of apoptosis. Bar diagram shows the ratio of Hoechst positive cells. Data were the mean value of three independent experiments with each count of no less than 200 cells. $^*P < 0.05$. *B)* Flow cytometric analysis of apoptosis in $bak^{-/-}/bax^{-/-}$ cells following treatment with 20 μM gossypol for the indicated times. The percentage of apoptotic cells was determined by Annexin V/PI staining. *C)* $bak^{-/-}/bax^{-/-}$ cells were treated with 20 μM gossypol for the indicated time or with indicated compound for 48 h, and the percentage of cells with activated caspases were characterized as those that stained with the FITC in situ marker. Data represent the mean values of three independent experiments. $^*P < 0.05$. *D)* $bak^{-/-}/bax^{-/-}$ cells were exposed to 20 μM gossypol in the presence of 100 μM zVAD-fmk or 5 mM autophagic inhibitor 3-MA for 24 h. The percentage of cell death was determined by Annexin V/PI staining followed by flow cytometric analysis. Results are expressed as the mean (±SD) for three independent experiments. $^*P < 0.05$.

chondria from mouse liver to determine whether gossypol induces cyto c release from mitochondria via the pathway, and found that gossypol did not induce the opening of mPTP. Cyclosporin (CsA), a commonly used inhibitor of mPTP, could not inhibit gossypol-induced cyto c release. These data suggest that gossypol-induced cyto c release is independent of mPTP opening.

3. We compared the apoptotic effects of gossypol with other Bcl-2 inhibitors

Gossypol was found to interact with Bcl-xL and Bcl-2. Gossypol is different from other known Bcl-2 inhibitors, such as HA14–1 and chelerythrine, which did not induce apoptosis and cyto c release in $bak^{-/-}/bax^{-/-}$ cells but did induce apoptosis in Bcl-2 overexpressing IM-9 cells.

4. We found that gossypol induced Bcl-2 conformational change and present data suggest that altered Bcl-2 is responsible for cyto c release in the absence of Bak and Bax

Using NMR chemical shift perturbation analysis (**Fig. 2*A***) and a specific antibody (Ab) that recognizes the exposed epitope of BH3 in the altered Bcl-2 molecule (**Fig. 2**), we showed that gossypol induced conformational change in Bcl-2/Bcl-xL, thus converting their antiapoptotic function into a proapoptotic one. Using reconstituted proteoliposomes encapsulated with FITC-cyto c, in the presence or absence of Bcl-2 and Bcl-xL, we showed that the interaction of gossypol with Bcl-2 or Bcl-xL has functional consequence for cyto c release.

5. Gossypol could be useful for fighting cancers with dysregulated Bcl-2 family proteins

We found that gossypol overcame Bcl-2-conferred drug resistance in lymphoblast cells by inducing a conformational change in Bcl-2 but not activating Bax. *In vivo* study showed that gossypol inhibited tumor growth in nude mice without adverse effects on body wt and activity.

CONCLUSIONS AND SIGNIFICANCE

In this study, we used $bak^{-/-}/bax^{-/-}$ cells, which are resistant to most death stimuli, to specifically screen for

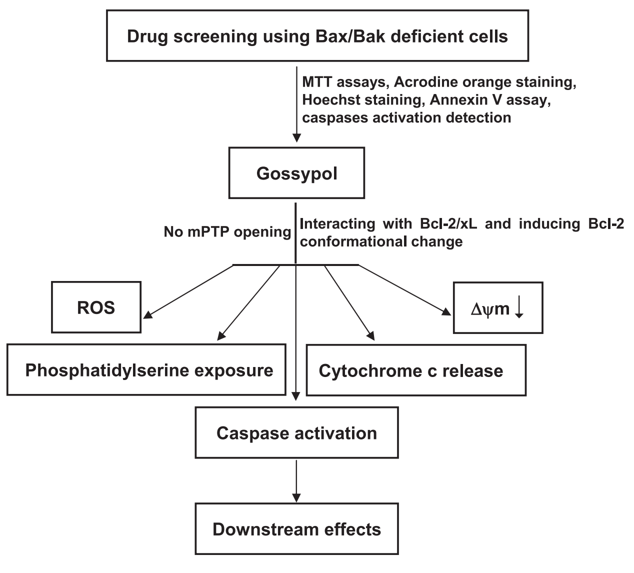

Figure 3. Schematic diagram of apoptotic mechanism induced by gossypol. We found that gossypol induces Bax/Bak independent apoptosis. It can directly impact on mitochondria to induce the reduction of Δψm and cytochrome c release without affecting mPTP opening. Our results showed that the interaction between gossypol with Bcl-2 and the subsequent Bcl-2 conformational change play critical roles for the apoptotic effects.

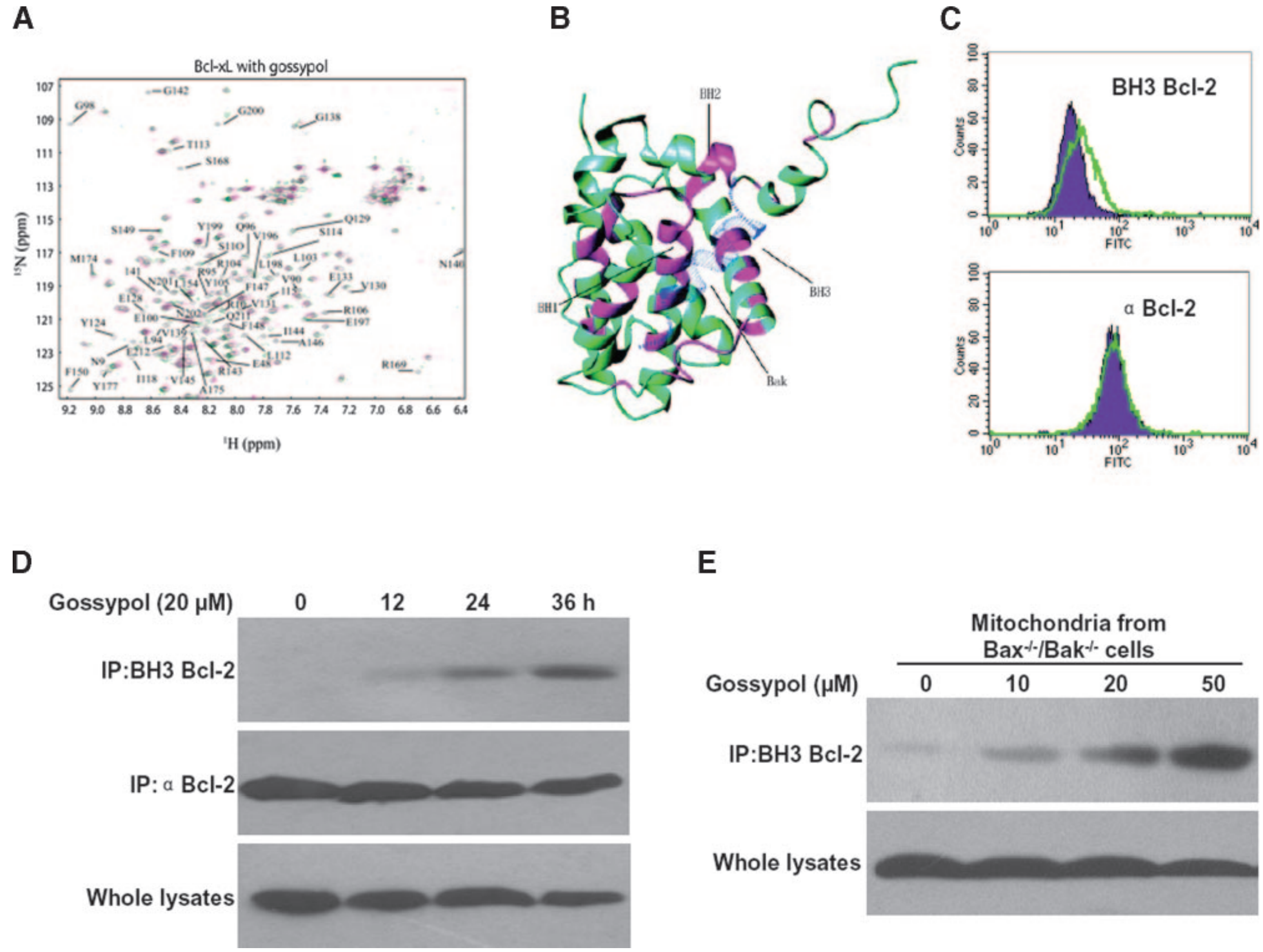

Figure 2. Gossypol induces allosteric Bcl-2 conformational change, which may be responsible for cyto c release. *A)* Superimposition of ^{1}H-^{15}N HSQC spectra recorded at different Bcl-xL/gossypol ratios: without gossypol (black peaks), 2:1(green peaks), and 1:1(magenta peaks). The residues for which the NH signals showed chemical shift or intensity changes on binding of gossypol are labeled by residue type and number. *B)* Interaction region between gossypol and Bcl-xL. The dotted blue part is the Bak peptide. All the residues whose NH chemical shifts affected by gossypol titration are indicated in pink. These residues are mostly located at BH1, BH2, and BH3 (also labeled in figure), which form the hydrophobic pocket. *C)* $bak^{-/-}/bax^{-/-}$ cells were treated with or without gossypol for 24 h, and then immunostained with indicated Bcl-2 Ab, followed by FITC-conjugated secondary Ab, and identified by flow cytometry. Control (purple) and gossypol treated (green). *D)* $bak^{-/-}/bax^{-/-}$ cells were exposed to 20 μM gossypol for indicated time. Following treatment, cells were lysed in Chaps lysis buffer and immunoprecipitated with the indicated anti-Bcl-2 Ab. Immunoprecipitates were subjected to immunoblotting using anti-Bcl-2 Ab. *E)* Isolated mitochondria from $bak^{-/-}/bax^{-/-}$ cells were exposed to different concentrations of gossypol as indicated at 25°C for 60 min. After centrifugation, mitochondria were lysed in Chaps buffer and immunoprecipitation was performed as described above.

small natural molecules that can induce caspase-dependent apoptosis. Our results reveal that gossypol can induce cyto c release from mitochondria to activate caspase-dependent apoptosis in the absence of both Bak and Bax. We found that gossypol induced Bcl-2 conformational change, which may convert it from a protective to a killer molecule. To our best knowledge, this is the first study describing a small molecule that can induce Bcl-2 conformational change and links this change to cyto c release. Our results suggest that, in addition of the rheostat balancing of protective Bcl-2 protein to proapoptotic Bcl-2 family proteins, the conformational status of Bcl-2 is also important in determining the fate of the cell. This conversion could be reminiscent of Ced-9 in *C. Elegans*, in which the genome contains no Bax homologue and Ced-9 may perform functions similar to that of both Bax and Bcl-2 by conformational change. The discovery that gossypol can change Bcl-2 from a protector to a killer will be help further elucidate Bcl-2 function. This discovery also holds promise for cancer therapy, as overexpression of Bcl-2 or Bcl-xL has been observed in 80% B-cell lymphoma, 90% of colorectal adenocarcinomas, and many other forms of cancer. Given its tolerable toxicity, gossypol represents a promising lead for the development of more potent and specific agents targeting Bcl-2-regulated apoptosis both alone or in combination with other anticancer agents. FJ