ApoG2 inhibits antiapoptotic Bcl-2 family proteins and induces mitochondria-dependent apoptosis in human lymphoma U937 cells

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Lymphoma is one of the most common types of hematological malignancies and proteins from the Bcl-2 family are highly expressed in human lymphomas. Apogossypolone (ApoG2), the most potent gossypol derivative, has been classified as a novel small-molecule inhibitor of antiapoptotic Bcl-2 family proteins. Here, we assessed the in-vitro cytotoxicity of ApoG2 on human U937 lymphoma cells, and explored the underlying intracellular molecular mechanisms of ApoG2. Using the WST-8 assay, we found that ApoG2 inhibited growth of U937 cells in a dose-dependent and time-dependent manner, and the IC\textsubscript{50} values were 30.08, 14.81, and 9.26 \textmu mol/l for 24, 48, and 72 h treatments, respectively. ApoG2 also induced apoptosis in U937 cells, as noted through changes in morphological characteristics, including cellular internucleosomal DNA fragmentation and the appearance of a sub-G1 apoptotic peak. Treatment with ApoG2 downregulated Bcl-xL and Mcl-1 protein expression and blocked the binding of Bcl-2 with Bax protein. Furthermore, ApoG2 led to an abundant release of cytochrome c from mitochondria and a five-fold increase in the activity of caspase-3 and caspase-9. Taken together, our results suggest that ApoG2 could effectively suppress the growth of human lymphoma cell line U937 through the inhibition of the antiapoptotic Bcl-2 family proteins and the induction of mitochondria-dependent apoptotic cell death.

Keywords: apogossypolone, apoptosis, Bcl-2, caspase, cytochrome c release, mitochondria, U937

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Introduction

Apoptosis is one of the major processes that leads to cell death, and overexpression of the antiapoptotic Bcl-2 family proteins is commonly observed in a variety of human cancers. Among hematological malignancies, Bcl-2 or Bcl-xL overexpression directly correlates with resistance to chemotherapeutic drugs [1–7], indicating poor prognosis [8–12]. Thus, there is an urgent need to develop agents that directly target antiapoptotic Bcl-2 family proteins for treating chemotherapy-resistant malignancies. Several research groups have been developing strategies to block the activity of antiapoptotic Bcl-2 family proteins and trigger apoptosis. For example, peptides or small molecules that act on the Bcl-2 binding pocket have been developed to prevent Bcl-2 heterodimerization with and sequestration of proapoptotic molecules. Furthermore, there has been some promise in treating certain types of lymphomas, specifically those with Bcl-2/Bcl-xL/Mcl-1 overexpression, with nonpeptidic, drug-like, cell-permeable, and potent small-molecule inhibitors that interact with the BH3 binding groove in Bcl-2/Bcl-xL heterodimers and block their antiapoptotic function [13–16].

Gossypol, a natural product isolated from cottonseeds and roots, has been used as a contraceptive drug in men for a long time [17]. Currently, gossypol is regarded as a potential antitumor drug because it can inhibit growth of several types of tumor cells, both \textit{in vitro} [18–21] and \textit{in vivo} [22,23]. Recently, studies have suggested that gossypol can effectively inhibit growth of non-Hodgkin’s lymphoma cells, and the combination of gossypol with certain secondary chemotherapeutic agents has synergistic cytotoxicity [24]. More importantly, gossypol has been successfully used to treat metastatic adrenal cancer [25]. The antitumor properties of gossypol are associated with its interaction with members of the Bcl-2 family, which, in turn, regulates Bcl-2 and Bax expression [26]. However, gossypol contains two reactive aldehyde groups that may cause potential nonspecific toxicity [27]. Apogossypolone (ApoG2), a novel derivative without the two reactive aldehyde groups, has been synthesized (Fig. 1). Compared with gossypol, ApoG2 has a more potent binding affinity to antiapoptotic Bcl-2 proteins Mcl-1 and Bcl-2, with K\textsubscript{i} values of 25 and 35 \textmu mol/l, respectively [28]. Nevertheless, it is still unknown whether ApoG2 has antitumor activity toward lymphoma.
cells. In this study, we investigated the in-vitro antitumor effects of ApoG2 in human leukemic monocyte lymphoma cell line U937 and examined the ApoG2-induced cell death pathway. The results demonstrate that ApoG2 inhibited cell growth and induced apoptosis in this cell line. Moreover, ApoG2 triggered apoptosis through down-regulating Bcl-xL and Mcl-1 and blocking the interaction between Bcl-2 and Bax. ApoG2 also induced mitochondria-dependent apoptotic cell death in U937 cells through cytochrome c (cyto-c) release and activation of caspases-3/9.

Materials and methods

Cells and reagents

U937 cells (human leukemic monocyte lymphoma cell line) were maintained in RPMI 1640 (Invitrogen Corporation, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (Gibco Corporation, Carlsbad, California, USA), 1 unit/ml penicillin G, and 1 µg/ml streptomycin at 37°C and 5% CO2. ApoG2 was dissolved in dimethylsulfoxide (DMSO) to make a stock solution of 20 mmol/l. Z-VAD-fmk was obtained from Promega Corporation (Cat. G7231; San Luis Obispo, California, USA). The primary antibodies used for immunoblots and immunoprecipitations were anti-Bcl-2 (sc-509; Santa Cruz, California, USA), anti-cyto-c (sc-13156; Santa Cruz), anti-Mcl-1 (sc-819; Santa Cruz), anti-Bax (Cat.2772; Cell Signaling, Danvers, Massachusetts, USA), and anti-Bcl-xL (Cat. 2762; Cell Signaling). Cell lysis buffer was purchased from Beyotime Biotechnology, Haimen, Jiangsu, China.

WST-8 assay

The in-vitro cytotoxic effect of ApoG2 toward U937 cells was determined using the Cell Counting kit (Dojin Laboratories, Kumamoto, Japan) [29]. Briefly, U937 cells were plated in 96-well tissue culture plates (Costar, Cambridge, Massachusetts, USA) at a density of 8 x 10^4 cells/ml. Serial dilutions were made from a stock solution of ApoG2 to the desired concentrations. All experimental concentrations were tested in triplicate. Four hours before the desired time points, 10 µl of WST-8 was added. Then, after 4 h of incubation, optical density (OD) values were measured (Thermo Multiskan MK3; Thermo Labsystems, Helsinki, Finland). Percentage absorbance relative to control was plotted as a linear function of drug concentration. The 50% inhibitory concentration (IC50) was identified as the concentration of drug required to achieve 50% growth inhibition relative to control populations. Inhibition of cell growth was measured by the percentage of cells that were viable compared with the control (% = (ODC –ODT)/ODC x 100%. OD_T is the OD values of treated samples, and OD_C is the OD values of control samples.

Flow cytometry analysis of apoptosis

U937 cells were incubated with either 0.1% DMSO or 5, 10 or 20 µmol/l of ApoG2 for 12, 24, or 48 h. Then cells were harvested, washed with phosphate-buffered saline (PBS), and fixed in 70% ice-cold ethanol overnight. The fixed cells were incubated with 5 µg/ml RNase I and 1 µg/ml propidium iodide. The cellular DNA content was determined by a flow cytometer (Beckman Coulter, Fullerton, California, USA). Apoptotic cells were identified by the sub-G1 phase in the cell-cycle distribution.

Detection of cells undergoing apoptosis

ApoG2-induced apoptosis in U937 cells was assessed by 4,6-diamidine-2-phenylindole (DAPI) staining. After treatment with 0.1% DMSO or 10, 20, or 40 µmol/l ApoG2 for 48 h, U937 cells were harvested and smeared on slides. The slides were air dried, fixed in methanol-acetone (3:1, v/v), and stained with DAPI (2 µg/ml) at 37°C for 15 min. Nuclear morphology was examined to determine cells undergoing apoptosis (DFC480; Leica Microsystems, Wetzlar, Germany).

DNA ladder formation assay

Isolation and analysis of apoptotic DNA fragments were performed as described [30]. After treatment with 0.1% DMSO or 5, 10 or 20 µM ApoG2 for 48 h, U937 cells were harvested and washed with PBS. Cells were then pelleted and lysed with lysis buffer (1% NP-40, 20 mmol/l EDTA and 50 mmol/l Tris–HCl, pH 7.5; 10 µl/10^6 cells) for 10 s. After centrifugation at 2000 rpm for 5 min, the supernatant was collected, and the extraction was dissolved...
in the same amount of lysis buffer. The supernatant was brought to 1% SDS and treated with RNase A (final concentration of 5 μg/μl) for at least 2 h at 37°C. After adding 0.5 volumes of 10 mol/l ammonium acetate and 2.5 volumes ethanol, the DNA was precipitated, dissolved in loading buffer, and separated by electrophoresis in a 1.5% agarose gel. The ladder bands were visualized under an ultraviolet transilluminator (Bio-Rad, Hercules, California, USA).

**Immunoblot and immunoprecipitation analysis**

U937 cells were treated with 10 μmol/l of ApoG2 for 0, 12, 24, or 48 h. Whole cell lysates were prepared by adding 2 × SDS sample buffer (125 mmol/l Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.02 mg/ml bromophenol blue, and 5% mercaptoethanol). Equal amounts of protein (50 μg/sample as determined by UV spectrometry) were electrophoresed on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were then blocked for 60 min at room temperature with 5% nonfat dry milk/TBS–Tween 20 and treated with appropriate antibodies for Bcl-2, Bcl-xL, Bax, or Mcl-1 (1:1000 dilution in blocking buffer) overnight at 4°C with gentle rocking. Following incubation with the primary antibody, membranes were washed in TBS–TWEEN 20 and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution in blocking buffer) for 1 h at room temperature. Proteins were visualized by incubation with enhanced chemiluminescence detection reagents subsequently (7072; Cell Signaling), followed by exposure to radiograph film (Kodak, Rochester, New York, USA). Immunoblot data were quantitated with Quant analysis software (Quantity One; Bio-Rad).

For immunoprecipitation U937 cells were treated with 20 μmol/l of ApoG2 for 48 h. Cells were collected, washed with ice-cold PBS, and lysed in ice-cold lysis buffer (20 mmol/l Tris, pH7.4, 150 mmol/l NaCl, 1% Triton X-100, 2 μg/ml aprotinin, 2 μg/ml leuprin, 2 μg/ml pepstatin, and 1 mmol/l phenylmethylsulfonyl fluoride). The cell lysates were centrifuged at 12 000 × g for 15 min, and the resulting supernatant was incubated overnight at 4°C with anti-Bax antibody (or normal rabbit IgG) or anti-Bcl-2 antibody (or normal mouse IgG) (0.5 μg/ml). The antibody-protein complex was pelleted using protein A-agarose (sc-2001; Santa Cruz). Precipitates were harvested with a short centrifugation (2000 rpm, 10 s, 4°C) and washed five times with ice-cold lysis buffer. Immunoprecipitated proteins were eluted with 2 × SDS sample buffer and further analyzed by SDS-PAGE and immunoblotting with either an anti-Bcl-2 antibody or an anti-Bax antibody to determine the effect of ApoG2 on the interactions of Bax and Bcl-2.

**Measurement of caspase-3 and caspase-9 activity**

Caspase-3 and caspase-9 activity was evaluated using a caspase colorimetric protease assay kit (Keygen Biotech. Co. Ltd., Nanjing, China) according to the manufacturer’s instructions. Briefly, log-phase cell cultures were treated with 0.1% DMSO or 5, 10, 20, or 40 μmol/l ApoG2 for 12 h and then harvested by centrifugation. The cell pellets were washed with PBS and resuspended in 50 μl ice-cold cell lysis buffer for 20 min. Then, the same amount of 2 × reaction buffer and 5 μl caspase-3 or caspase-9 substrate were added to an equal amount of cell lysates (100 μg proteins/50 μl). After incubating in the dark at 37°C for 4 h, enzymatic activity was measured in a microplate reader at 405 nm. Caspase-3 and caspase-9 activity levels were calculated by comparing OD values with those of the control.

**Effect of caspase inhibitor on apoptosis and expression of Bcl-xL and Mcl-1**

U937 cells were preincubated with 20 μmol/l z-VAD-fmk for 30 min before incubation with different concentrations of ApoG2 (0.1% DMSO or 5, 10, or 20 μmol/l) for 24 h. Following the 24 h treatment, the cells were analyzed for apoptosis with flow cytometry as indicated above. Cells were preincubated with 20 μmol/l z-VAD-fmk for 30 min before being treated with 10 μmol/l ApoG2 for 48 h. The expression levels of Bcl-xL and Mcl-1 expression were then detected with immunoblotting.

**Cytochrome c release assay**

U937 cells were treated with either 0.1% DMSO or 5, 10 or 20 μmol/l ApoG2 for 48 h, harvested by centrifugation, washed once with PBS, and resuspended in resuspension buffer (30 mmol/l sucrose, 75 mmol/l KCl, 3 mmol/l KH₂PO₄, 0.5 mmol/l MgCl₂, 10 mmol/l HEPES pH 7.4, 1.5 mmol/l PMSF, 3 μg/ml leupeptin, and 20 μg/ml aprotnin). The resuspended cells were then homogenized using an ultrasonic homogenizer. The resultant homogenates were centrifuged at 3000 rpm for 5 min to clear nuclear fragments. Cytoplasmic and mitochondria fractions were further isolated by centrifugation at 15 000 rpm for 20 min at 4°C. Proteins of both fractions were separated by SDS-PAGE followed with immunoblot analysis using an anti-cyto-c antibody.

**Statistical analysis**

All assays were performed in triplicate. Data are expressed as the mean ± SD. Statistical analyses were performed using analysis of variance by SPSS 14.0 software. A value of *P* < 0.05 was considered statistically significant.

**Results**

**Effect of apogossypolone on the growth of U937 cells**

To investigate whether ApoG2 has a cytotoxic effect on human leukemic monocyte lymphoma cells, WST-8 assays were performed on U937 cells after 24, 48 or 72 h of treatment with ApoG2. ApoG2 strongly inhibited the proliferation of U937 cells in a dose-dependent and
time-dependent manner, with IC\textsubscript{50} values of 30.08, 14.81, or 9.26 \(\mu\)mol/l for 24, 48, or 72 h of treatment, respectively (Fig. 2a).

**Apogossypolone-induced apoptosis in U937 cells**

Propidium iodide staining and flow cytometry were used to investigate whether ApoG2 inhibits the growth of U937 cells by initiating the apoptotic pathway. As shown in Fig. 2b, U937 cells underwent apoptosis after being exposed to ApoG2 for 12, 24, or 48 h at a range of concentrations (from 5 to 20 \(\mu\)mol/l). The percentage of apoptotic cells in the sub-G1 phase of the cell cycle increased in a time-dependent and dose-dependent manner (\(P < 0.05\)).

Apoptotic nuclear morphology was observed with DAPI staining using fluorescence microscopy. After being exposed to 10 \(\mu\)mol/l ApoG2 for 48 h, U937 cells began to exhibit obvious apoptotic characteristics, such as cell shrinkage and nuclear condensation/fragmentation (Fig. 3a).

**Apogossypolone-induced DNA fragmentation**

To further study whether the morphological changes associated with ApoG2 treatment were due to activation of the apoptotic pathway, we examined internucleosomal DNA fragmentation, one of the hallmark characteristics of a cell undergoing apoptosis. U937 cells were treated with three doses of ApoG2, 5, 10, or 20 \(\mu\)mol/l, which, respectively, correlated with low, medium, or high cytotoxicity toward U937 cells according to the growth inhibition assays. The appearance of DNA laddering was observed in cells treated with all three doses of ApoG2 for 48 h (Fig. 3b). The DNA laddering pattern became stronger as the concentrations of ApoG2 increased. In contrast, low molecular weight DNA did not appear in untreated cells.

**Effect of apogossypolone on the protein expression level of Bcl-2 family members**

To explore the molecular mechanisms by which ApoG2 induces apoptosis, western blot analysis was performed to observe the changes in expression levels of Bcl-2 family members upon treatment with ApoG2. Figure 4 shows the profiles of most Bcl-2 family members in U937 cells after treatment with 10 \(\mu\)mol/l ApoG2 for various times. As shown in Fig. 4a, ApoG2-treated U937 cells showed detectable levels of Bcl-2 family members, including Bcl-xL, Bcl-2, Bax, and Mcl-1 proteins. There were no significant changes in the expression levels of Bcl-2 and Bax at different treatment times. However, Bcl-xL and Mcl-1 proteins were downregulated by about 66 and 81\%, respectively (\(P < 0.05\)), and these alterations were dependent on the duration of ApoG2 (Fig. 4b).

**Inhibition of the binding between Bax and Bcl-2**

As ApoG2 was a new derivative from gossypol, which is now considered a potent small-molecule inhibitor of Bcl-2 and Bcl-xL proteins, we speculated that ApoG2 might trigger apoptosis of U937 cells by influencing the expression and interaction of antiapoptotic proteins (Bcl-2) with proapoptotic proteins (Bax). To verify this hypothesis, whole lysates from treated and untreated U937 cells were collected, immunoprecipitated with an anti-Bax (or anti-Bcl-2) specific antibody, and then probed with an anti-Bcl-2 (or anti-Bax) specific antibody. As illustrated in Fig. 5a, Bax and Bcl-2 binding became significantly decreased after ApoG2 treatment. These
observations demonstrate that ApoG2 inhibited the heterodimerization of Bcl-2 with Bax in U937 cells.

ApoGossypolone promotes cytochrome c release and caspase-3 and caspase-9 activation in U937 cells

To investigate the roles of caspases in ApoG2-induced apoptosis, we detected activation of the major cellular executioner caspases, caspase-3 and caspase-9, in U937 cells after a 12-h treatment with different concentrations of ApoG2. As shown in Fig. 5b, the activity of caspase-3 and caspase-9 increased significantly, up to five-fold, in U937 cells treated with different concentrations of ApoG2, when compared with untreated cells ($P < 0.05$).

We also detected cyto-c release in U937 cells after ApoG2 treatment. Subcellular fractionation was then performed with immunoblotting for cyto-c to show that ApoG2 specifically promoted cyto-c release from the mitochondria into the cytosol of U937 cells (Fig. 6a).

Caspase inhibitor affects apoptosis and expression of Bcl-xL and Mcl-1 in U937 cells

After treating U937 cells with both ApoG2 and the caspase inhibitor z-VAD-fmk, the rate of apoptosis was measured to see whether apoptosis had been prevented. As shown with flow cytometry (Fig. 2b), ApoG2 induced apoptosis of U937 cells, and this was blocked by z-VAD-fmk ($P$ compared with the no z-VAD-fmk group).

As the expression of Bcl-xL and Mcl-1 in U937 cells was downregulated by ApoG2 treatment, we further investigated whether the changes were because of caspase cleavage. After treatment with both ApoG2 and z-VAD-fmk, there were no significant changes in the expression of Bcl-xL and Mcl-1 compared with the treatment group receiving only ApoG2 (Fig. 6b).

Discussion

In this study, we investigated the effect of ApoG2 on the human leukemia monocyte lymphoma cell line U937, which expresses Bcl-2, Bax, Bcl-xL, and Mcl-1 proteins. We found that ApoG2 effectively inhibited the growth of U937 cells in a dose-dependent and time-dependent manner. Our data suggest that ApoG2 may have great potential as an antitumor agent.

Earlier studies have shown that gossypol induces cell death through its effects on DNA damage and cell cycle arrest [27,31,32]. However, little is known about whether ApoG2 can induce apoptosis. Here, we found that treating U937 cells with 5 $\mu$mol/l ApoG2 was sufficient to induce significant apoptosis. Morphologically, ApoG2-treated U937 cells presented typical features of apoptosis, such as nuclear condensation/fragmentation and internucleosomal DNA fragmentation. Therefore, we believe that the antitumor effect of ApoG2 on U937 cells was owing to the induction of cellular apoptosis.
In the current study, we were able to make some very interesting and encouraging observations related to ApoG2. First, we found that ApoG2 could not only directly inhibit the antiapoptotic function of Bcl-2 by downregulating Bcl-2 and Bax binding, but it also downregulates expression of Bcl-xL and Mcl-1 in U937 cells. Although gossypol can regulate the expression level of Bcl-2 family proteins in many other types of cancer cells [33–35], ApoG2 did not induce any changes in Bcl-2 and Bax proteins in our system, indicating that Bcl-2 family proteins such as Bcl-xL and Mcl-1 may be the specific targets of ApoG2-induced apoptosis in U937 cells. The heterodimerization of Bcl-2 family proteins is believed to be pivotal to the antiapoptotic function of these proteins [36,37]. Moreover, given that ApoG2 could downregulate the binding of Bcl-2 and Bax, we speculate that ApoG2 may be able to block subsequent hetero-

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**Fig. 4**

(a) Apogossypolone (ApoG2) alters the expression of Bcl-2 family members. U937 cells were treated with 10 µmol/l ApoG2 for 0–48 h. The effect of ApoG2 on Bcl-2, Bcl-xL, Bax, and Mcl-1 proteins expression level was evaluated by immunoblotting (a). Immunoblotting data were quantitated from at least three experiments. The relative amount of each protein was calculated compared with GAPDH. Results are presented as mean ± SD. *P<0.05 was obtained using analysis of variance by comparing the relative amounts of each protein in cells treated with ApoG2 with those of untreated control cells (b).

(b) ApoGossypolone (ApoG2) (10 µmol/l) alters the expression of Bcl-2 family members. U937 cells were treated with 10 µmol/l ApoG2 for 0–48 h. The effect of ApoG2 on Bcl-2, Bcl-xL, Bax, and Mcl-1 proteins expression level was evaluated by immunoblotting (a). Immunoblotting data were quantitated from at least three experiments. The relative amount of each protein was calculated compared with GAPDH. Results are presented as mean ± SD. *P<0.05 was obtained using analysis of variance by comparing the relative amounts of each protein in cells treated with ApoG2 with those of untreated control cells (b).

**Fig. 5**

(a) Apogossypolone (ApoG2) interrupted the binding of Bcl-2 with Bax and induced caspase activation. (a) The binding between Bax and Bcl-2 was examined by immunoprecipitation. Equivalent lysates harvested from U937 cells were immunoprecipitated with primary antibodies to the Bax (or normal rabbit IgG) or Bcl-2 (or normal mouse IgG). Bcl-2 and Bax were preferentially bound to each other. (b) Caspase-3 and caspase-9 activities were measured in U937 cells after treatment with 0.1% dimethylsulfoxide or 5–40 µmol/l ApoG2 for 12 h. Bars represent the means for three independent experiments (bars, ± SD). *P<0.05 was obtained by comparing the optical density (OD) values for ApoG2 treated and untreated cells, using analysis of variance.
ApoG2-induced cell apoptosis in human lymphoma U937 cells

Fig. 6

(a) Cytosolic fraction
Mitochondrial fraction

(b) ApoG2 z-VAD-fmk

Apoposopholine (ApoG2) promoted cytochrome c (cyto-c) release from mitochondria into cytosol and z-VAD-fmk affected Bcl-2 and Mcl-1 expression in ApoG2-treated U937 cells. (a) U937 cells were treated with 0.1% dimethylsulfoxide or 5, 10, or 20 μmol/l ApoG2 for 48 h. After cytosolic fractions were prepared, cyto-c release was assessed by immunoblotting. (b) U937 cells were preincubated with 20 μmol/l z-VAD-fmk for 30 min before adding 10 μmol/l ApoG2 for 48 h. The ApoG2 treatment was followed by immunoblotting to examine Bcl-xL and Mcl-1 expression. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

dimerization of Bcl-2 with Bax, which would further initiate downstream apoptotic events in U937 cells.

Caspase-3 activation occurs following mitochondrial cyto-c release during drug-induced apoptosis. Moreover, Bcl-2 and Bcl-xL prevent caspase activation and subsequent apoptotic cell death by preventing the release of cyto-c into the cytosol. Therefore, agents that inhibit Bcl-2 or Bcl-xL might promote cyto-c release. We also noticed that ApoG2 induced the release of cyto-c from the mitochondria into the cytosol and caused a five-fold increase in the activity of caspase-3 and caspase-9 in U937 cells. Mitochondria play a crucial role in the apoptotic signal transduction pathway [38]. It is well known that the release of cyto-c from mitochondria into the cytosol is an important upstream event of mitochondrial-dependent apoptosis, which activates both caspase-9 and caspase-3 [39,40]. Therefore, our data suggest that caspase activation during ApoG2-induced U937 cell apoptosis is, at least in part, cyto-c dependent. In other words, ApoG2-induced apoptosis of U937 cells occurs through the mitochondrial pathway.

To further verify that caspase activation is necessary for ApoG2-induced apoptosis, the caspase inhibitor z-VAD-fmk was used during ApoG2 treatment. Given that ApoG2-induced apoptosis can be blocked by z-VAD-fmk, we confirmed that ApoG2-induced death of U937 cells is caspase dependent. We further investigated whether the downregulation of Bcl-xL and Mcl-1 were because of caspase cleavage after ApoG2 treatment. Results showed that the downregulation of Bcl-xL and Mcl-1 could not be inhibited by z-VAD-fmk. These data provide strong evidence that the loss of Bcl-xL/Mcl-1 in ApoG2-induced apoptosis of U937 cells was caspase independent. Whether ApoG2 exerts a direct effect on the downregulation of Bcl-xL or Mcl-1 still needs to be thoroughly examined in future studies.

In summary, this study reports that ApoG2 can inhibit the growth of tumor cells and induced mitochondria-dependent apoptosis in the lymphoma cell line U937, which expresses proteins from the Bel-2 family. Therefore, ApoG2, as a small-molecule inhibitor of Bcl-2, Bcl-xL, and Mcl-1, has therapeutic potential in lymphoma. Additionally, our study may provide some useful information for the development of more potent gossypol-derivatives.

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