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Gossypol inhibits phosphorylation of Bcl-2 in human leukemia HL-60 cells

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ABSTRACT

Gossypol is an attractive therapeutic anti-tumor agent as an apoptosis inducer and is being evaluated in preclinical tests. However, the molecular mechanisms underlying apoptosis induction by gossypol in malignant cells have not been completely enunciated. Here we investigate the alterations of Bcl-2/Bcl-xL/Mcl-1 protein levels and Bcl-2 phosphorylation in gossypol-induced apoptosis in human leukemia HL-60 cells. We found that gossypol treatment inhibited cell growth and induced apoptosis in HL-60 cells. Bcl-2/Bcl-xL/Mcl-1 protein levels were slightly reduced and phosphorylation of Bcl-2 at threonine 56 (phospho T56) was not altered. However, phosphorylation of Bcl-2 at serine 70 (phospho S70) was strikingly down-regulated in gossypol-exposed cells. This reduction was found to be not only in both dose- and time-dependent fashion but also obviated by phorbol l2,13-dibutyrate (PDBu), an activator of protein kinase C (PKC). In addition, pre-treatment of PDBu partially prevented gossypol-induced apoptosis in HL-60 cells. Collectively, gossypol treatment can reduce phosphorylation of Bcl-2 at serine 70 in leukemia HL-60 cells and gossypol may be a promising therapeutical candidate for leukemia patients especially expressing phosphorylated Bcl-2 at Ser70.

1. Introduction

Aberrant regulation of apoptosis in cells, especially insufficient apoptosis, has been proven to play a pivotal role in the pathogenesis of cancer (Reed, 2008). From a therapeutic point of view, selective induction of apoptosis in cancer cells is now considered an efficacious strategy and has been widely used to treat a variety of cancers (Adams and Cory, 2007; Marzo and Naval, 2008). The dysregulation of apoptosis in cancer cells often involves Bcl-2 family proteins, which include anti- and pro-apoptotic members and are central to cell apoptosis (Cory and Adams, 2002, Certo et al., 2006). For instance, anti-apoptotic Bcl-2 proteins are found to be over-expressed in a variety of human cancers (Simonian et al., 1997) and dysregulation of Bcl-2 phosphorylation triggered by abnormal upstream signaling pathways is present in certain cancer cells (Ruvolo et al., 2001). Consequently, therapeutic approaches to boost apoptosis in cancer cells often target the Bcl-2 family members. It has been suggested that the apoptosis-inducing effect of some anti-cancer agents is associated with inducing reduced expressions of Bcl-2 anti-apoptotic members, modulating their phosphorylation, or binding directly to them and thus inhibiting their functions (Letai, 2008).

One potential anti-cancer agent currently under clinical evaluation is gossypol (2,2-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene,$C_{30}H_{30}O_8$) (Tuszynski and Cossu, 1984; Mohammad et al., 2005a,b). This compound is a polyphenol extracted from cottonseeds and roots. It is initially investigated in China as a male contraceptive candidate (Wang et al., 1987; Wu, 1989). Previous reports have indicated that the anti-cancer effect of gossypol is connected to its ability to interfere with the functions of Bcl-2/Bcl-xL/Mcl-1 proteins (Mohammad et al., 2005a,b; Meng et al., 2008; Etxebarria et al., 2008), the anti-apoptotic Bcl-2 family members. Each of these proteins functions as a binding protein for pro-apoptotic Bcl-2 proteins containing BH3 domain and gossypol is recently noticed to be a natural BH3 mimetic that can bind to the BH3 pocket of Bcl-2/Bcl-xL/Mcl-1 proteins (Kitada et al., 2003; Kang and Reynolds, 2009).On the other hand, gossypol treatment is reported to reduce expression of anti-apoptotic Bcl-2 family proteins such as Bcl-2, Bcl-xL and Mcl-1 in a variety of tumor cells (Wang et al., 2000; Zhang et al., 2003; Huang et al., 2006, 2009; Balakrishnan et al., 2008).

Gossypol has been reported to be a nonspecific protein kinase C (PKC) inhibitor (Jarvis et al., 1994) and PKC was initially identified as a Bcl-2 kinase (May et al., 1994; Ito et al., 1997). However, no report in the literature has been found regarding the effect of gossypol on Bcl-2 phosphorylation. Here we focus on whether gossypol can induce dose- and time-dependent changes at the levels of Bcl-2 phospho S70 and phospho T56 in leukemia HL-60 cells, which display robust Bcl-2 phosphorylation. Meantime, gossypol-induced apoptosis in the same cells was monitored using FACS assay, DNA fragmentation, and cleavage of Poly ADP Ribose Polymerase (PARP). Finally, the PKC activator phorbol l2,13-dibutyrate (PDBu) was used to investigate the possible roles of PKC in the observed effects of gossypol in HL-60 cells.

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2. Materials and methods

2.1. Reagents

Gossypol, phorbol l2,13-dibutyrate (PDBu) and 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium (MTT) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All reagents were prepared and used as recommended by their suppliers.

2.2. Cell line and cell culture

HL-60 cell line was kindly provided by Dr. Jun Yin (Shantou University, Shantou, China). Cells were cultured in RPMI 1640 supplemented with penicillin, streptomycin, and 10% FBS. Cells were collected at a concentration of 1×10^5 cells/ml, to which were added the designated agents, and maintained in a 37 °C, 5% CO_2, fully humidified incubator for the indicated time.

2.3. MTT assay

Cells were treated with different concentrations of gossypol for 24 h and 48 h. Cell viability was estimated by the modified MTT assay described previously (Mosmann, 1983; Liu et al., 2009). Briefly, 10 μl of MTT solution (5 mg/ml in ddH_2O) was added to each well already containing 100 μl of cell suspension. Plates were then incubated for 4 h at 37 °C. Intracellular formazan crystals were dissolved by addition of 100 μl of isopropanol with 0.04 N HCl to each well, until the solution turned purple and absorbance analyzed in an enzyme-linked immunosorbent assay (ELISA) plate reader at 490 nm. Rate of inhibition was calculated by using the equation: rate of inhibition = (Ac − At) / Ac × 100, where At and Ac represent the absorbance in treated and control cultures, respectively.

2.4. Annexin V–FITC/propidinium iodide (PI) fluorescence-activated cell sorting (FACS) analysis

Apoptosis of cells exposed to gossypol for 24 h was determined by flow cytometry using a commercially available Annexin V–FITC/propidinium iodide apoptosis detection kit (KeyGen Biotech Co., Ltd., NanJing, China). After drug treatment, cells were collected and washed twice in ice cold PBS and resuspended in 500 μl of binding buffer at 1×10^5 cells/ml and incubated with 1 μl of Annexin V/FITC and 5 μl of propidinium iodide in the dark for 15 min at room temperature. Finally, samples were analyzed by flow cytometry and evaluated based on the percentage of early apoptotic cells for Annexin V positive and PI negative.

2.5. DNA fragmentation assay

DNA fragmentation was analyzed after the extraction of DNA from cells exposed to the indicated doses of gossypol for 24 h using Apoptotic DNA-ladder Kit (Applygen Technologies Inc., Beijing, China). The DNA was separated on a 1.5% agarose gel and visualized under UV light by ethidium bromide staining.

2.6. Western blot analysis

A modified method as previously described was used (Dorsey et al., 2000; Liu et al., 2009). Briefly, collected cells were lysed immediately in buffer [1% Triton X-100, 150 mM NaCl, 25 mM Tris–HCl (PH 7.2), 0.5 mM EDTA, 0.5 μM Na_3VO_4] supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Protein concentration was determined using Micro BCA kit (Beyotime Biotechnology, Haimen, China). Equal amounts of protein (60 μg) were boiled for 5 min, separated by SDS-PAGE, and electroblotted to nitrocellulose membrane. After blocking, the blots were incubated with an appropriate dilution of specific antisera or monoclonal antibodies [PARP, Bcl-2, Bcl-xL, Mcl-1, phosphorylation of Bcl-2 at serine 70 (phospho S70) and at threonine 56 (phospho Thr 56), Cell Signaling Technology, Beverly, MA, USA] for 1 h at room temperature. Blots were washed three times and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were again washed three times and then developed using a chemiluminescence assay. Blots were stripped and reprobed for β-actin (Cell Signaling Technology, Beverly, MA, USA) to be used as a loading control.

3. Results

3.1. Gossypol inhibited growth of HL-60 cells

The effect of gossypol on growth of HL-60 cells was examined by MTT assay. Fig. 1 shows that the rate of inhibition of gossypol treatment at 24 h with 10 μM was (29.9 ± 0.04) % and increased to (68.6 ± 0.01) % after cells incubated with 20 μM. The IC_{50} of gossypol at 24 h is 15.3 μM. The rate of inhibition was not significantly elevated after prolonged treatment to 48 h.

3.2. Gossypol-induced apoptosis in HL-60 cells

Gossypol-induced apoptosis in HL-60 cells was estimated using different approaches. With Annexin V–FITC assay, we detected that gossypol treatment for 24 h caused the increase of early apoptotic population by 21.4% with 10 μM and by 30.9% with 30 μM (Fig. 2A). No significant DNA fragmentation was observed after gossypol treatment with 10 μM but a remarkable enhancement appeared with 30 μM (Fig. 2B). Consistently, the results of western blot analysis showed that gossypol treatment with 30 μM induced significant marked PARP cleavage yielding a characteristic 89KD fragment (Fig. 2C).

3.3. Gossypol decreased phosphorylation of Bcl-2 in HL-60 cells

Protein levels of Bcl-2/Bcl-xL/Mcl-1 and phosphorylation of Bcl-2 in gossypol-treated HL-60 cells were examined using western blot analysis. As shown in Fig. 3A, slight down-regulations of Bcl-2/Bcl-xL/Mcl-1 protein levels were noticed by gossypol treatment with 10 μM at 24 h but the reduction was not enhanced with 30 μM. Gossypol treatment did not alter phospho Thr56 of Bcl-2. Phospho S70 of Bcl-2, however, was strikingly reduced at a dose of 10 μM and completely abolished at 30 μM. The time-dependent test showed that phospho

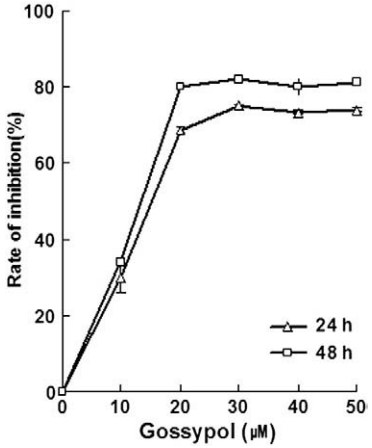

Fig. 1. Growth-inhibiting effects of gossypol on HL-60 cells. Cells were incubated with different dosages of gossypol for the indicated time and rate of inhibition was determined by MTT assay. Data mean plus or minus the standard deviation (S.D.) of three independent experiments.

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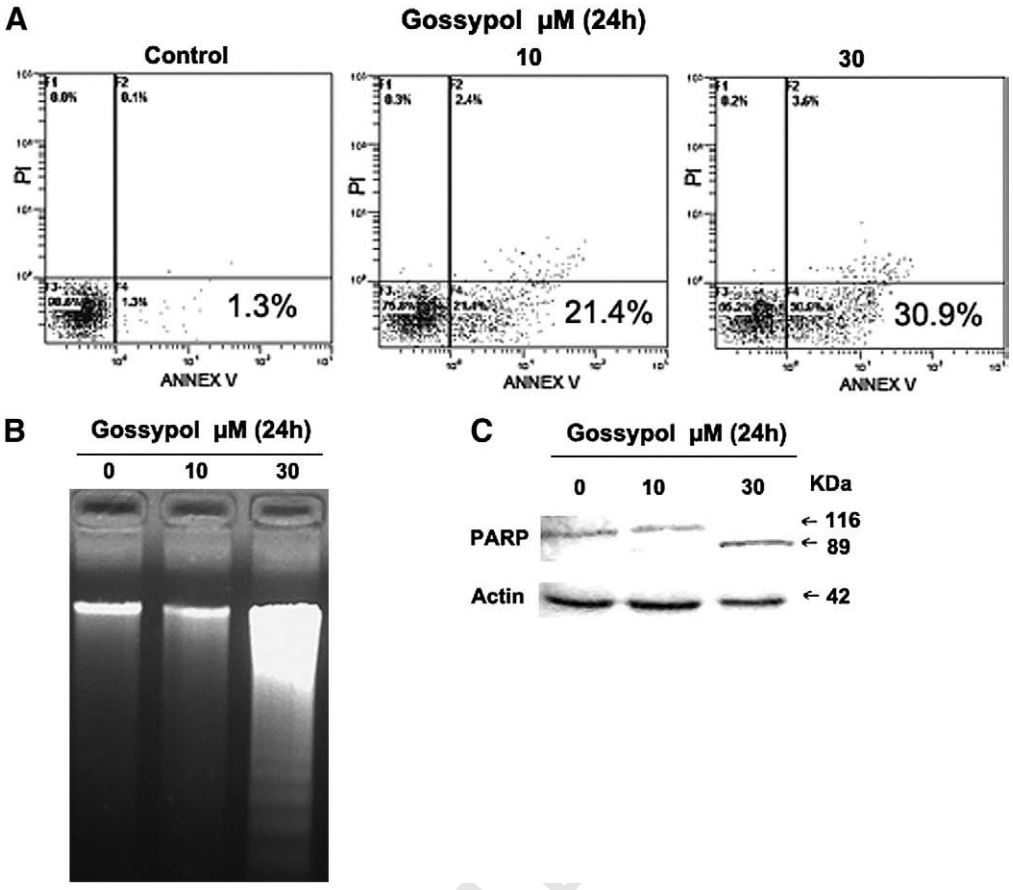

Q3 **Fig. 2.** Apoptosis induction of gossypol in HL-60 cells. After cells incubated with gossypol for 24 h, (A) the percentage of early apoptotic cells was detected using Annexin V/PI FACS assay; (B) DNA fragmentation was analyzed with agarose gel electrophoresis of DNA; (C) PARP cleavage was examined by western blot analysis.

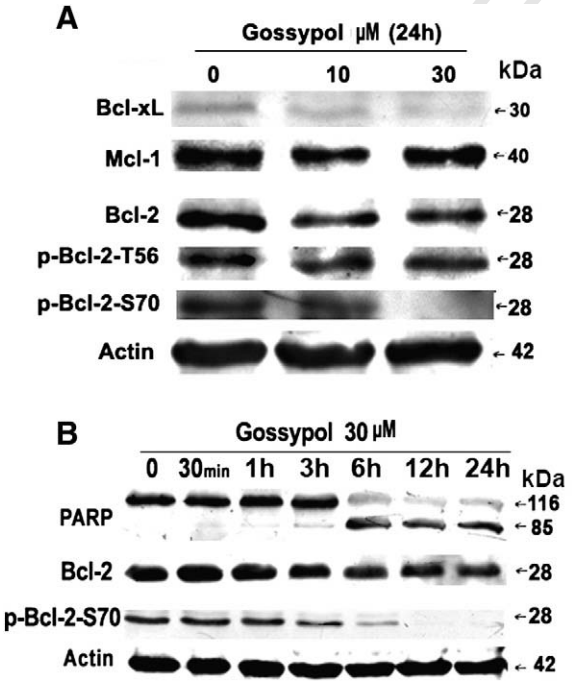

Fig. 3. Decrease of Bcl-2 phosphorylation in gossypol-treated HL-60 cells. (A) Protein levels of Bcl-2/Bcl-xL/Mcl-1, phospho T56 of Bcl-2 (p-Bcl-2-T56), phospho S70 of Bcl-2 (p-Bcl-2-S70), and β-actin were measured by western blot analysis. (B) Time-dependent changes of phospho S70 of Bcl-2, Bcl-2, and PARP cleavage were examined by western blot analysis. Each experiment was performed three times and similar results were obtained.

S70 of Bcl-2 began to decrease as early as at 3 h and was fully abrogated at 12 h after cells incubated with 30 μM of gossypol. In parallel with the decrease of Bcl-2 phospho S70, PARP was cleaved into fragmentation of 89 KD (Fig. 3B).

3.4. PDBu prevented gossypol-induced apoptosis in HL-60 cells

As PKC is reported to be one of the Bcl-2 kinases which can phosphorylate Bcl-2 at phospho S70, the effect of a PKC activator PDBu on gossypol-induced decrease of Bcl-2 phospho S70 and apoptosis was investigated. As shown in Fig. 4A, pre-treatment with 150 nM of PDBu for 1 h obviated the reduction of Bcl-2 phospho S70 and partially prevented the cleavage of PARP in HL-60 cells exposed to 30 μM of gossypol for 24 h. FACS assay showed that the early apoptotic population of gossypol-treated cells with and without pre-incubation of PDBu for 1 h was 21.44% and 30.56%, respectively (Fig. 4B).

4. Discussion

In this study, we demonstrate that gossypol treatment slightly reduced protein levels of Bcl-2/Bcl-xL/Mcl-1 in HL-60 cells. Phospho S70 of Bcl-2 was decreased by gossypol treatment in both dose- and time-dependent fashion and the reduction was obviated by the PKC activator PDBu. Pre-treatment of PDBu was found to partially prevent gossypol-induced apoptosis in HL-60 cells.

Previous studies show that gossypol is a potent apoptotic inducer and gossypol treatment down-regulates expression levels of Bcl-2/Bcl-xL/Mcl-1 proteins in multiple tumor cell lines. For examples, gossypol treatment completely abolished Bcl-2 protein expression at 24 h in LoVo cells (Wang et al., 2000) and down-regulated Bcl-2 and

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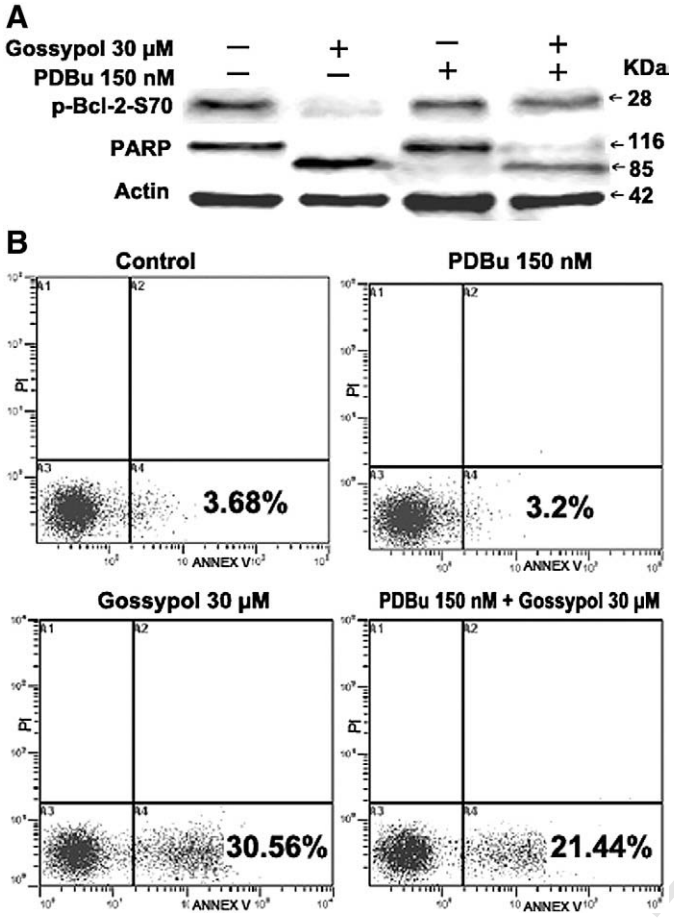

Fig. 4. Gossypol-induced apoptosis was partially prevented by PDBu in HL-60 cells. (A) Protein levels of phospho S70 of Bcl-2 (p-Bcl-2-S70), β-actin, and PARP cleavage were measured by western blot analysis. (B) Early apoptotic population of gossypol-treated cells with or without pre-incubation of PDBu for 1 h was examined using Annexin V/PI FACS assay. Each experiment was performed twice and similar results were obtained.

Bcl-xL at the protein levels in DU-145 (Huang et al., 2006), MAT-LyLu and MLL cell lines (Huang et al., 2009). Gossypol has also been reported to induce a reduction of Mcl-1 protein level in B-CLL cells (Balakrishnan et al., 2008) and down-regulations of both Bcl-xL and Mcl-1 in HT-29 cells which have absent Bcl-2 protein expression (Zhang et al., 2003). Consistent with a previous report (Jarvis et al., 1994), we found that gossypol treatment only slightly reduced protein levels of Bcl-2/Bcl-xL/Mcl-1 in leukemia HL-60 cells, which suggest that gossypol-induced apoptosis in HL-60 cells is less related with regulations of Bcl-2/Bcl-xL/Mcl-1 protein expression levels.

The results presented here indicate that gossypol could be a promising therapeutical candidate for AML patients especially expressing phosphorylated Bcl-2 at Ser70. The Bcl-2 protein is well known to undergo phosphorylation including mono- and multi-site phosphorylation at Thr56, Thr69, Ser70, Thr74 and Ser87 in response to diverse types of stimuli (Puthalakath and Strasser, 2002; Perez-Galan et al., 2008). Although it remains controversial whether these post-translational modifications can positively regulate the anti-apoptotic function of Bcl-2 (Deng et al., 2004; Reed, 2008), Bcl-2 phosphorylation at Ser70 (phospho S70) has been found necessary for its full and potent anti-apoptotic function, and has also been reported to be associated with poor survival in acute myeloid leukemia (AML) (May et al., 1994; Ruvolo et al., 2001; Ito et al., 1997). For example, leukemia cells from patients expressing phosphorylated Bcl-2 at Ser70 are shown to exhibit greater resistance to chemotherapy (Kurinna et al., 2006) and the decrease in Bcl-2 phosphorylation can improve the sensitivity of leukemia cells to anti-tumor agents (Perez-Galan et al., 2008). Thus Bcl-2 phosphorylation has become a therapeutic target for AML (Ruvolo et al., 2001). HL-60 cells, which was derived from a patient with acute promyelocytic leukemia, display robust Bcl-2 phosphorylation (Breitman et al., 1980; Ruvolo et al., 1998). Although gossypol treatment is reported to induce apoptosis in HL-60 cells (Jarvis et al., 1994; Hou et al., 2004), the exact mechanism is still not clear. In this study, we found that gossypol treatment decreased Bcl-2 phospho S70 in a time- and dose-dependent manner in these cells but did not alter phospho Thr 56 of Bcl-2. The decrease in Bcl-2 phophorylation was shown to be in parallel with PARP cleavage, a hallmark of apoptosis. Moreover, we found that HL-60 cells were more sensitive to gossypol-induced apoptosis than leukemia K562 cells lacking expression of BcL-2 protein (data not shown). This is likely that gossypol-induced apoptosis is partially correlated with the inhibition of Bcl-2 phophorylation. Further studies are warranted to determine the efficacy of gossypol or its combination with other anti-leukemia treatment regimens in AML especially expressing phosphorylated Bcl-2 at Ser70.

Our findings suggest that gossypol-induced decrease in Bcl-2 phosphorylation is likely to be associated with its inhibition of PKC. Previous studies show that Bcl-2 is always phosphorylated with activated PKC and mitogen-activated protein kinases (MAPK) Erk1/2 in AML blast cells (Kurinna et al., 2006). It has been reported that Erk1/2 is a Bcl-2 kinase which can phosphorylate Bcl-2 at Ser70 (Ruvolo et al., 2001). In this study, we did not find that gossypol treatment altered phosphorylation of Erk1/2 in HL-60 cells (data not shown). As gossypol is a nonspecific PKC inhibitor, we hypothesized that gossypol-induced reduction of phospho S70 of Bcl-2 in HL-60 cells was mediated by PKC inhibition. Thus PDBu, a PKC activator which is reported to protect gossypol-induced inhibition of PKC activity in spermocytes (Teng, 1995), was used to test the hypothesis. As expected, we found that the presence of PDBu obviated gossypol-induced reduction of phospho S70 of Bcl-2 and partially prevented gossypol-induced apoptosis with the evidence of alleviated PARP cleavage and apoptotic population.

In conclusion, gossypol-induced apoptosis in leukemia HL-60 cells is partially mediated by reduction of Bcl-2 phospho S70 through inhibition of PKC pathway. Therefore, gossypol may be a promising therapeutical candidate for AML patients especially expressing phosphorylated Bcl-2 at Ser70.

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