Induction of apoptosis and antitumor effects of a small molecule inhibitor of Bcl-2 and Bcl-xl, gossypol acetate, in multiple myeloma *in vitro* and *in vivo*

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Abstract. Gossypol is a naturally occurring polyphenolic compound extracted from cotton plants. Recent studies revealed that gossypol is a non-peptidic small molecule inhibitor of Bcl-2/Bcl-xl. The aim of the present study was to investigate the induction of apoptosis and antitumor effects of gossypol acetate in multiple myeloma and the possible mechanism(s) of action. Our results showed that gossypol acetate resulted in a dose- and time-dependent inhibition of multiple myeloma cell proliferation, with an IC_{50} value to both U266 and Wus1 cells at 2.4, 2.2 μM at 48 h after treatment. Gossypol acetate effectively induced the apoptosis of multiple myeloma cells as demonstrated by typical morphological changes, DNA ladder formation and increase in the percentage of cells in subdiploid peak. Furthermore, colorimetric assays showed activation of both caspase-3 and caspase-9. Bcl-2 and Bcl-xl expression was decreased by 86.5±1.2% and 35.9±3.6%, respectively, after treatment with gossypol acetate at 25 μmol/l for 24 h. Preliminary studies *in vivo* showed that a growth inhibition (T/C) of 30.9% (gossypol acetate 40 mg/kg) was obtained in Balb/C mice bearing Wus1 cells. In addition, there was no body weight loss for the treated group in comparison with the vehicle mice. Our results demonstrated that the potent inhibitor of Bcl-2 and Bcl-xl gossypol acetate had significant antiproliferative and antiapoptotic effects on multiple myeloma cells *in vitro* and *in vivo*. Gossypol acetate may represent a promising new anticancer agent with a novel molecular mechanism and warrants further investigation as a single agent, or in combination with other chemotherapeutics, for human multiple myeloma with Bcl-2 overexpression.

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Introduction

Multiple myeloma (MM) is a clonal plasma cell malignant disease characterized by anemia, bone disease, M-component and impaired renal function (1). The pathogenesis has yet to be fully elucidated. Although new therapeutic modalities such as thalidomide (2), bortezomib (3,4), and lenalidomide (5,6) have been developed in recent years, MM remains incurable.

The Bcl-2 family includes both death antagonists, such as Bcl-2 and Bcl-xl, and death agonists such as Bax, Bak, Bid (7-10). It is now recognized that Bcl-2 is overexpressed in most MMs and it plays a major role in drug resistance in myeloma cell lines and in freshly isolated myeloma cells (11). Thus, inhibition of the antiapoptotic function of Bcl-2 family member protein targeting represents an attractive new strategy for the treatment of MM. Antisense Bcl-2 and Bcl-xl studies have provided important proof-of-the-concept that inhibition of Bcl-2 and Bcl-xl may be effective for the treatment of MM (11).

Gossypol acetate is a naturally occurring polyphenolic compound extracted from cotton plants. Since the 1960s, gossypol had been shown to possess antineoplastic activity against a variety of malignant cell types. Some preclinical studies have demonstrated that gossypol acetate has antitumoral activity and induces apoptosis in several types of cancer, including lung cancer (12), melanoma (13), colon carcinoma (14), breast cancer (15), and central nervous system tumor (16). Gossypol has also been demonstrated to be well-tolerated and has achieved few effects in some clinical trials, but the mechanisms are not well understood (17). Previous studies revealed that gossypol was a nonpeptidic small-molecule inhibitor of Bcl-2/Bcl-xl. It can bind to the BH3 (Bcl-2 homology domain 3) binding site in Bcl-2/Bcl-xl, and then block the heterodimerization of Bcl-2/Bcl-xl with proapoptotic members in the Bcl-2 protein family such as Bad, Bak, Bid (18,19).

Nevertheless, whether gossypol acetate has antitumor activity on MM cells remains unclear. In this study, we investigated the effect of gossypol acetate on MM cells *in vitro* and *in vivo*, and discussed the possible molecular mechanisms of action.

Materials and methods

Reagents. Gossypol acetate (provided by the University of Michigan Comprehensive Cancer Center) was dissolved in dimethyl sulfoxide (DMSO) to make a 50 mM stock solution and was preserved at -20˚C (Fig. 1). Treatment solutions were prepared by the dilution of stock solution in RPMI-1640.

Cell culture. Human MM cell lines U266 and Wus1 were obtained from the hematology laboratory of Peking Union Medical College Hospital (PUMCH). Both cell lines were cultured in RPMI-1640 medium containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine and 10% fetal bovine serum in a humidified incubator (37˚C, 5% CO_2 and 95% air). Culture medium was changed every 48-72 h.

Proliferation assay. MM cells were plated in 24-cell culture clusters at a density of $1x10^5$ viable cells/l per well. Triplicate wells were treated with 1, 5, 10, 25 and 50 μmol/l gossypol acetate, and the negative control group was supplemented with 0.1% DMSO. Then, cell numbers at different treatment time points (0, 24, 48 and 72 h) were determined by using a hemocytometer and the trypan blue dye-exclusion method. The trypan blue dye-exclusion method was used to evaluate the cell viability. The cells were examined in a counting chamber under a light microscope. Only viable cells were recorded.

Apoptosis assay

Morphological observation. Cells were exposed to 25 μmol/l gossypol acetate for 24 h. Three methods were employed to confirm the morphological changes of myeloma cells following exposure to gossypol acetate: i) light microscopic examination: 10^6 cells were collected and stained with Wright-Giemsa. Slides were analyzed under light microscopy (Nikon); ii) fluorescence microscopic examination: 10^6 cells were collected and fixed in 4% formaldehyde at 4˚C for 10 min. Then, Hoechst 33258 (100 μl) was added and stained for 10 min. Cells were observed under fluorescence microscopy (Olympus BX51) using 340 nm excitation wavelength; iii) transmission electron microscopic examination: 10^6 cells were collected and fixed in 2.5% glutaraldehyde and preserved at -4˚C. The work was completed by the Electron Microscopy Center, Peking Union Medical College.

DNA fragmentation analysis. Cells ($5x10^6$) were collected after exposure to gossypol acetate at a concentration of 25 μmol/l for 24 h. The cell pellet was washed twice with PBS and resuspended in 200 μl of 6 mol/l NaI solution, 400 μl chloroform and isoamyl alcohol (24:1). Following centrifugation at 10,000 x g for 10 min, supernatant was transferred to another centrifuge tube. Isopropanol (400 μl) (100%) was added and the mixture was mixed vigorously and incubated at room temperature for 10 min. DNA was pelleted by centrifugation at 10,000 x g for 10 min and washed twice with 1.0 ml of 70% isopropanol. After centrifugation, DNA was air dried, redissolved in 50 μl of TE, and preserved at -20˚C. The control group was supplemented with 0.1% DMSO.

DNA (10 μl) was mixed with 2 μl 6X loading buffer, and was separated on a 1.5% agarose gel (including 0.5 μg/ml EB) by electrophoresis at 80 V for 90 min in electrophoresis buffer (0.5X TBE). DNA bands were photographed on a gel imaging system (UVI GAS7001B).

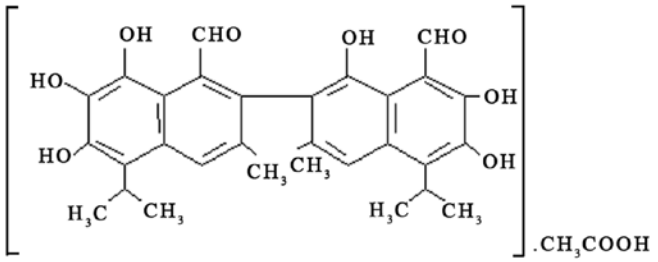

Figure 1. Chemical structure of gossypol acetate.

DNA contents analysis by flow cytometry. Cell apoptotic rate was quantitatively determined by flow cytometry. The percentage of cells with a sub-G_1 DNA content was taken as the fraction of apoptotic cell population. Cells ($1x10^6$) were collected after exposure to gossypol acetate at different concentrations for different times. The control group was supplemented with 0.1% DMSO. Cells were fixed with 2 ml of ice-cold 70% ethanol and incubated overnight at -20˚C. Prior to analysis, cells were washed with ice-cold PBS and resuspended in PBS, then incubated with 25 μg/ml RNase for 30 min and 50 μg/ml propidium iodide (PI) for 30 min in the dark. The PI fluorescence associated with DNA was measured on a flow cytometer (Coulter EPICS XL). At least 10,000 cells were detected in each sample.

Caspase fluorometric activity assay. Caspase-3, -9 activity was measured with caspase-3 colorimetric assay kit and caspase-9 colorimetric assay kit (R&D). Briefly, after incubating with 25 μmol/l gossypol acetate for 0, 6, 12, 18 and 24 h, $2x10^6$ cells were collected and washed twice with PBS and then lysed at 4˚C with 50 μl cell lysis buffer for 10 min. Following centrifugation at 2,000 x g at 4˚C, 50 μl of supernatant was transferred to a 96-well plate, 50 μl 2X reaction buffer containing 10 mmol/l DTT was added to each sample. Then, 5 μl caspase-3 substrates (DEVD-pNA) or caspase-9 (LEHD-pNA) was added to each sample and incubated for 120 min at 37˚C. pNA fluorescence, released by caspase activity, was measured on a fluorescence plate reader (Bio-Rad 450) set at 405-nm excitation filter. The results are expressed as fold increase in caspase activity of apoptotic cells over that of non-induced cells.

Analysis of Bcl-2 and Bcl-xl protein expression by flow cytometry. Cells ($2x10^6$) were collected after exposure to 25 μmol/l gossypol acetate for 24 h. Cells was fixed with 4% paraformaldehyde and incubated at room temperature for 40 min. Following centrifugation, 0.2% triton-X100 and PBS (including 5% FBS) 1 ml was added and cells were incubated on ice for 10-20 min. FITC-mouse anti-human Bcl-2 monoclonal antibody (40 μl) was added and incubated for 40 min on ice. Cells were washed twice with cold PBS and analyzed on a flow cytometer.

When analyzing Bcl-xl expression, primary antibody rabbit anti human Bcl-xl was added first and, following incubation, second antibody FITC-goat anti rabbit IgG was added. The other steps were same as those of Bcl-2 analysis.

Table I. Effect of gossypol acetate on MM cell growth.

Cells	Gossypol concentration (μM)	24 h	48 h	72 h
U266	0	33.3±9.0	141.1±16.0	201.1±46.0
	1	31.2±9.1	107.4±44.1	110.4±56
	5	26.7±8.7	37.8±10.5[a]	42.7±9.3[a]
	10	15.5±3.4[a]	19.5±5.4[a]	14.1±6.4[a]
	25	9.5±3.9[a]	2.9±0.4[a]	0.6±1.0[a]
	50	0.3±0.3[a]	0.1±0.2[a]	0.0[a]
Wus1	0	42.7±9.5	172.3±43.7	178.9±13.9
	1	42.2±3.3	139.7±11.8	143.8±2.9
	5	27.7±2.5[a]	28.9±3.3[a]	30.4±5.3[a]
	10	14.7±0.6[a]	15.0±5.0[a]	11.0±3.6[a]
	25	2±0.4[a]	0.2±0.3[a]	0.0[a]
	50	0.6±0.2[a]	0.0[a]	0.0[a]

Values are expressed as means ± SD; n=3; unit, x10^4 cells/ml. [a]P<0.05, significantly different from DMSO group.

Table II. IC_{50} of gossypol acetate on myeloma cells (unit, μmol/l).

	U266			Wus1		
	24 h	48 h	72 h	24 h	48 h	72 h
IC_{50}	9.0	2.4	0.9	6.5	2.2	2.2

Human myeloma cell line Wus1 xenografts. Female Balb/c nude mice (4-6 weeks old) were obtained from Charles River Laboratories Inc. Wus1 cells (1x10^6) (in serum-free RPMI-1640) were injected subcutaneously into the flanks of the mice. On the 4th day after Wus1 cells were injected into mice, each mouse received gossypol acetate i.p. Mice were randomized to 3 groups (4 mice per group) including the blank group (normal mice not administered gossypol), the control group and the gossypol acetate 40 mg/kg group (20 mg/kg, day 4 and 6).

Tumors were measured at their greatest length and width, and the weight was calculated as tumor weight (mg) = (AxB^2)/2, where A and B are the tumor length and width (millimeters), respectively. Tumor growth inhibition (T/C) was calculated by using the median tumor weight in the treated group (T) when the median tumor weight in the control group (C) reached ~1,000 mg. At day 8, liver and spleen were isolated to determine the size and weight.

Statistical analysis. Data are expressed as the means ± standard deviation (SD). SPSS12.0 was used for the statistical analysis. Statistical differences between means were evaluated using the t-test and LSD test followed by normality test and homogeneity test of variances. A P-value of <0.05 was considered to indicate a statistically significant difference.

Table III. Apoptotic rate of myeloma cells after induction with gossypol acetate at different concentrations for 24 and 48 h.

Time	Gossypol acetate concentration (μM)	U266 (%)	Wus1 (%)
24 h	0	1.0±0.5	1.2±1.4
	5	4.2±2.2	2.4±1.1
	10	4.7±2.1	3.0±0.3
	25	16.6±9.6[a]	39.4±8.8[a]
48 h	0	1.0±0.8	1.7±2.7
	5	8.3±1.9[a]	12.5±3.4[a]
	10	28.9±3.9[a]	44.3±6.9[a]
	25	80.1±5.3[a]	82.4±8.4[a]

Values are expressed as means ± SD; n=3. [a]P<0.05, significantly different from DMSO group.

Results

Gossypol acetate inhibits myeloma cell proliferation in vitro. Cell viability was determined by trypan blue exclusion assay. Gossypol acetate resulted in a dose- and time-dependent inhibition of cell proliferation (Table I). Gossypol acetate inhibited the growth of myeloma cell lines at concentrations >10 μmol/l. Gossypol acetate (1 μmol/l) had no effect on the myeloma cell lines. The IC_{50} of gossypol acetate on the myeloma cell lines is listed in Table II.

Induction of apoptosis in myeloma cell line by gossypol acetate. Next we investigated whether gossypol acetate was able to induce apoptosis in myeloma cell lines. Apoptosis is distinguished from necrosis by characteristic morphological and biochemical changes, including compaction and fragmentation of the chromatin, the activation of certain proteases and nucleases and the appearance of cells at sub-G_0/G_1 (the subdiploid peak). In our study, evidence of apoptosis was assessed by morphology, DNA ladder and analysis of cell DNA content by flow cytometry.

Gossypol acetate causes morphological changes in myeloma cells. After exposure to 25 μmol/l gossypol acetate for 24 h, typical apoptotic changes of U266 and Wus1 cells were found under light microscopy, fluorescence microscopy and transmission electron microscopy (Figs. 2-4).

Gossypol acetate induces DNA fragmentation. The results indicated that DNA from the control group showed no degradation (Fig. 5, lane 4 and 5). By contrast, DNA ladder can be observed after incubation with gossypol acetate for 24 h (Fig. 5, lane 2 and 3). This typical pattern of DNA degradation with oligonucleosome-sized fragments of 180-200 bp and multiples thereof is the biochemical hallmark of apoptotic cell death, and it suggested that gossypol acetate induced myeloma cell apoptosis.

Gossypol acetate induces appearance of subdiploid DNA of myeloma cells. Flow cytometry analysis revealed the hallmark

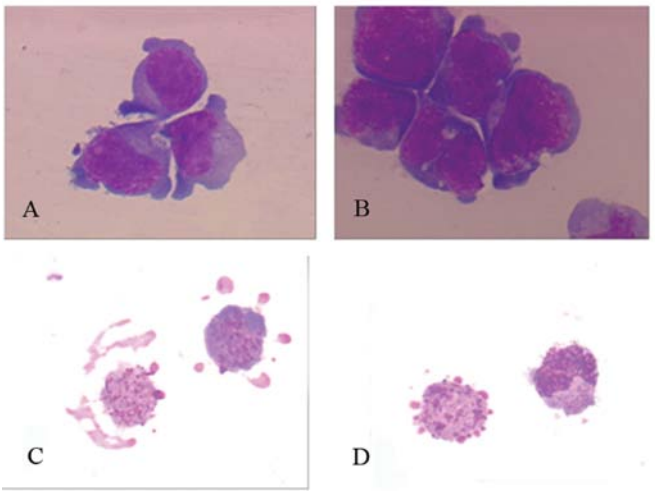

Figure 2. Morphological characteristics of apoptotic cells under light microscopy in MM cells after treatment with gossypol acetate 25 μmol/l for 24 h. Untreated MM cells showed large, round and irregular cell body, pseudopodium was seen in partial cells, neucleo is large, mostly round, oval or kidney shape, nucleoli are observed in some cells. Characteristics of apoptotic MM included diminished size, integrated cytomembrane, enhanced acidophilia, condensed chromatin, formation of apoptotic body. (A) Untreated U266 cells; (B) untreated Wus1 cells; (C) apoptotic U266 cells; (D) apoptotic Wus1 cells (magnification, x1,000).

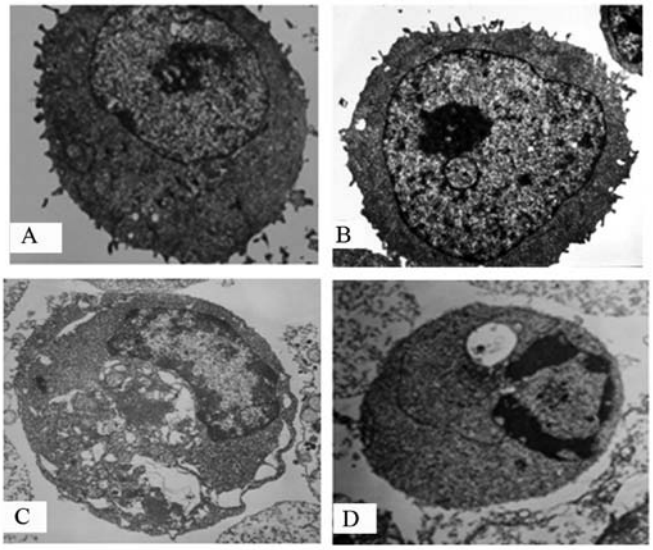

Figure 4. The apoptosis-inducing effect of gossypol acetate on myeloma cells confirmed by electron microscopy. Apoptotic cells showed diminished microvilli, cell shrinkage, nuclear membrane intact, nuclear chromatin condensation and peripheral shift of condensed chromatin to nuclear membrane or nuclear collapsing into patches, vacuole formation. (A) Untreated U266 cells; (B) untreated Wus1 cells; (C) apoptotic U266 cells; (D) apoptotic Wus1 cells (magnification, x6,000).

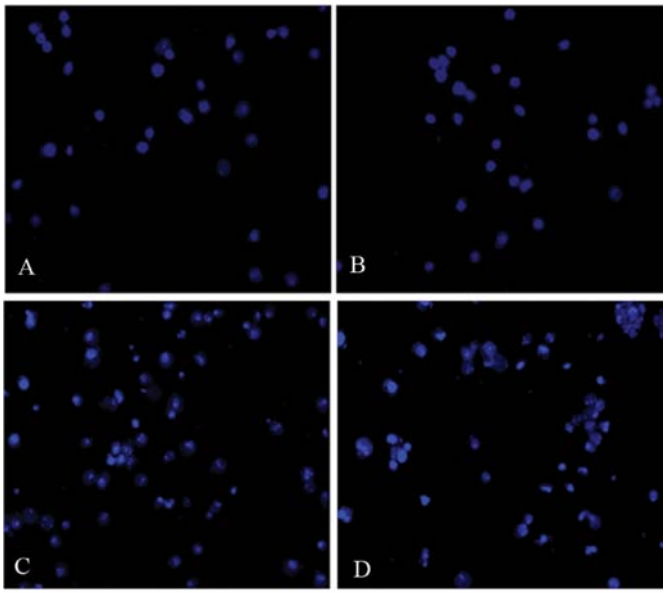

Figure 3. Gossypol acetate induces apoptotic morphological changes of MM cells by Hoechst 33258 staining under fluorescence microscope. Apoptotic cells showed chromatin condensation, fragmented apoptotic nucleus. (A) Untreated U266 cells; (B) untreated Wus1 cells; (C) apoptotic U266 cells; (D) apoptotic Wus1 cells (magnification, x200).

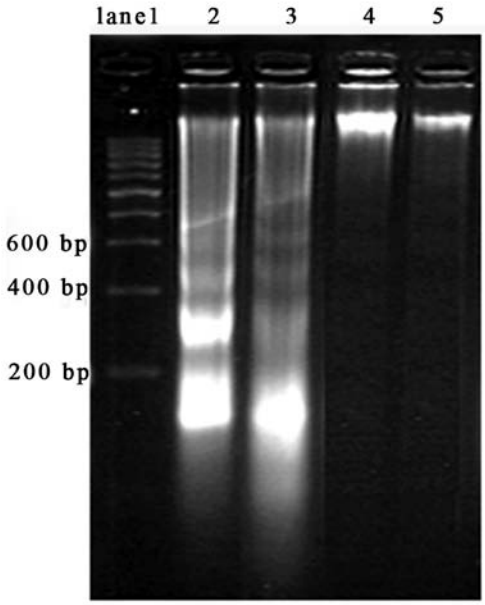

Figure 5. Gossypol acetate induces DNA fragmentation of myeloma cells. Lane 1, DNA marker; lane 2, 25 μmol/l gossypol acetate on U266 for 24 h; lane 3, 25 μmol/l gossypol acetate on Wus1 for 24 h; lane 4, U266 cells in control group; lane 5, Wus1 cells in control group.

features of apoptosis, with the appearance of subdiploid DNA. Quantitative analysis revealed that gossypol acetate induced the apoptosis of myeloma cells in a time- and dose-dependent manner (Table III). The subdiploid peak began to appear in the DNA content distribution after treatment with gossypol acetate at the concentration of >25 μmol/l for 24 h or 5 μmol/l for 48 h (Figs. 6 and 7).

Activation of caspase-3 and -9 by gossypol acetate. Apoptosis is associated with the activation of specific cysteine proteases referred to as caspases. We assessed whether gossypol acetate activated specific caspases during apoptosis of myeloma cells. Treatment of U266 and Wus1 with 25 μmol/l gossypol acetate for 0,6,12,18,24 h resulted in the activation of caspase-9 and -3 as early as 6 h (Fig. 8). The maximum increase in caspase-9

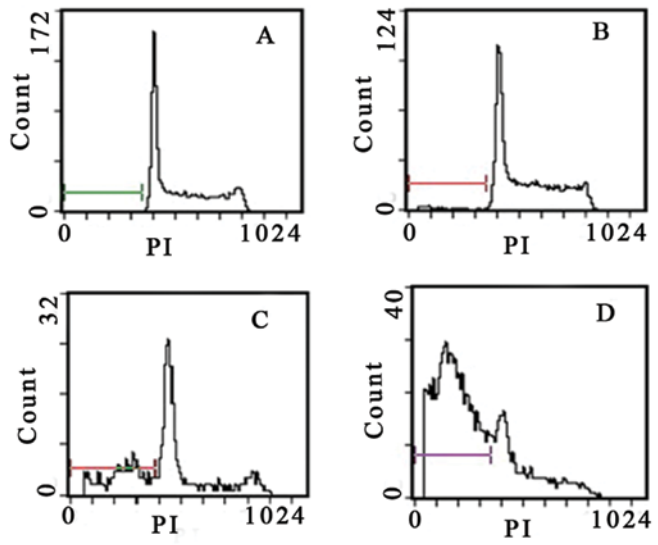

Figure 6. DNA content analysis of U266 cells after incubation with gossypol acetate. (A-D) Results of flow cytometry for U266 cells after induction with DMSO, 5, 10, 25 μM of gossypol acetate for 48 h.

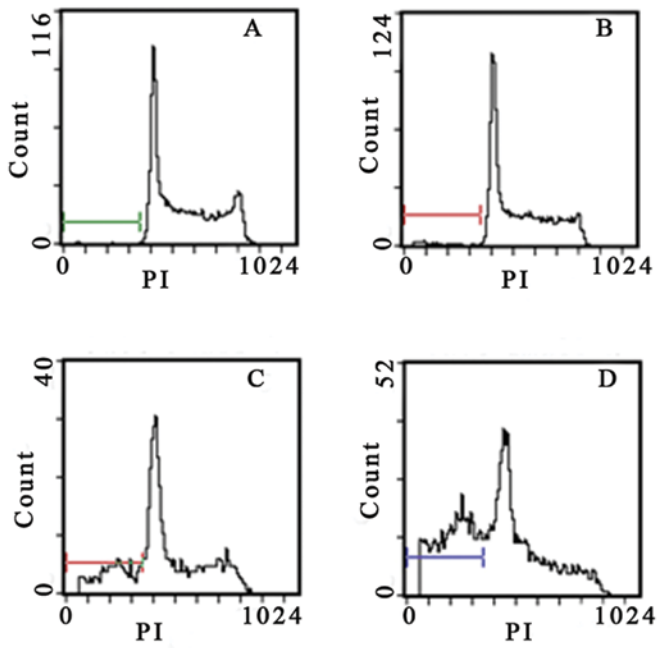

Figure 7. DNA content analysis of Wus1 cells after incubation with gossypol acetate. (A-D) Results of flow cytometry for Wus1 cells after induction with DMSO, 5, 10, 25 μM of gossypol acetate for 48 h.

Table IV. Changes of liver and spleen weight in Wus1 Balb/c mice at day 8 after gossypol acetate treatment.

	Normal mice	DMSO group	Gossypol acetate 40 mg/kg group
Liver weight (g)	0.53±0.03	0.70±0.16	0.55±0.08[a]
Spleen weight (g)	0.03±0.01	0.07±0.03	0.03±0.01[a]

[a]P<0.05, significantly different from DMSO group.

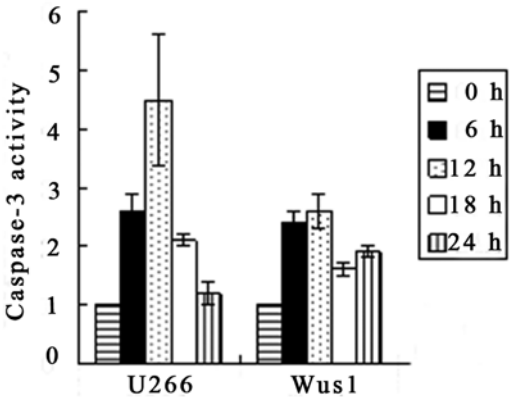

Figure 8. Caspase-3 fluorometric activity assay on Wus1 and U266 cell exposure to 25 μmol/l gossypol acetate for 0, 6, 12, 18 and 24 h.

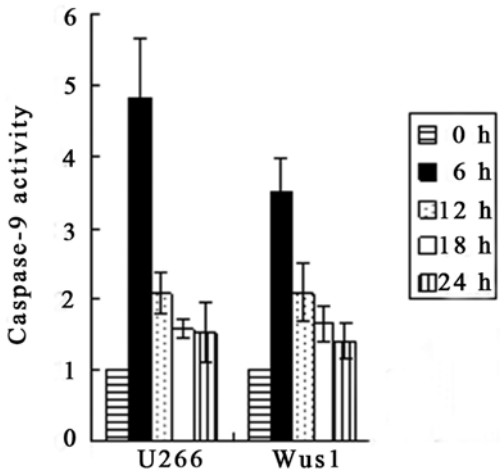

Figure 9. Caspase-9 fluorometric activity assay on Wus1 and U266 cells exposed to 25 μmol/l gossypol acetate for 0, 6, 12, 18 and 24 h.

activity was observed at 6 h, whereas the maximum caspase-3 activity was seen at 12 h with treatment of 25 μmol/l gossypol acetate (Fig. 9). The time to maximum caspase-3 activity is longer than that of caspase-9. The results indicated that gossypol acetate activated caspase-3 and -9 of myeloma cell lines.

Gossypol acetate downregulates Bcl-xl and Bcl-2 expression of myeloma cells. In our study, we found that Bcl-2 was not expressed in normal peripheral blood mononuclear cells and the U266 cell line, but was highly expressed in the Wus1 cell line. Bcl-xl was not expressed in normal peripheral blood mononuclear cells, but was highly expressed in the U266 and the Wus1 cell line. Thus, we only analyzed Bcl-2 and Bcl-xl changes in Wus1 cells after treating with gossypol acetate.

The Bcl-2 level of Wus1 was decreased by 86.5±1.2% after treating with 25 μmol/l gossypol acetate for 24 h (Fig. 10). Gossypol acetate at a concentration of 25 μmol/l on Wus1 for 24 h downregulated expression of the Bcl-xl level by 35.9±3.6% (Fig. 11).

The results suggested that gossypol acetate downregulates Bcl-2 and Bcl-xl expression of MM cells, and the effect of Bcl-2 downregulation was more evident than that of Bcl-xl.

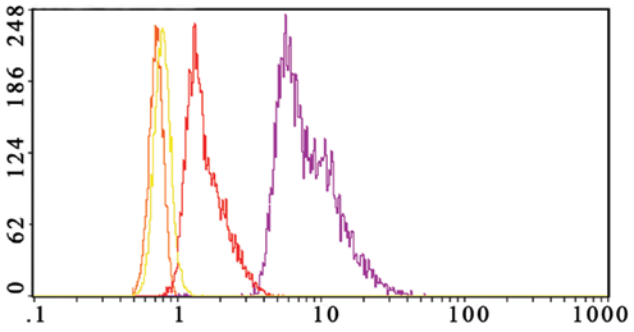

Figure 10. Bcl-2 expression of Wus1 after treating with 25 μmol/l gossypol acetate for 24 h. Purple, BCL-2 expression in untreated Wus1 cells; red, BCL-2 expression in Wus1 cells treated with gossypol acetate; orange, IgG homotype control in untreated Wus1 cells; yellow, IgG homotype control in Wus1 cells treated with gossypol acetate.

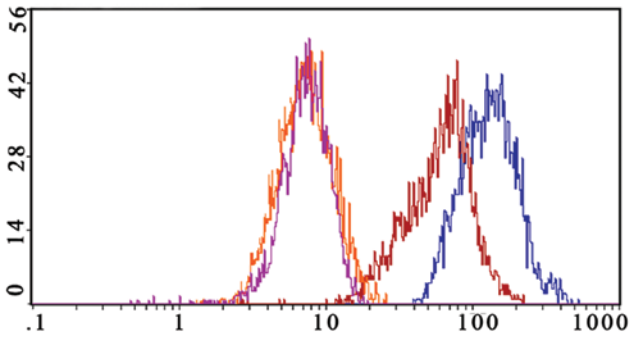

Figure 11. Bcl-xl expression of Wus1 after treating with 25 μmol/l gossypol acetate for 24 h. Blue, Bcl-xl expression in untreated Wus1 cells; brown, Bcl-xl expression in Wus1 cells treated with gossypol acetate; orange and yellow, IgG homotype control in untreated Wus1 cells; purple, IgG homotype control in Wus1 cells treated with gossypol acetate.

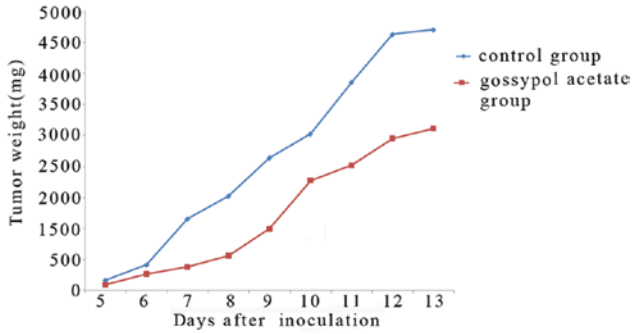

Figure 12. Effect of gossypol acetate on tumor weight in Balb/c mice bearing Wus1 cells.

Gossypol acetate has antitumor effects in Balb/c mice bearing Wus1 cells. We next tested the toxicity and antitumor activity of gossypol acetate in Wus1 Balb/c mice model. Antitumor activity of gossypol acetate against Wus1-bearing mice was observed (Fig. 12). T/C was 30.9% (gossypol acetate 40 mg/kg). Enlarged liver and spleen contributed to myeloma can be diminished after gossypol acetate treatment (Table IV). In addition, there was no body weight loss for the treated group in comparison with the vehicle treated mice. Of note, mouse survival was not prolonged.

Discussion

MM is a malignant plasma cell disease. The clinical manifestation includes hyperglobulinemia, renal dysfunction, bone damage and cytopenia. Advances in the therapy have been made in recent years, but MM remains incurable and the overall survival has not improved.

Bcl-2, frequently expressed in follicular lymphomas bearing the t(14;18) chromosomal translocation, is also widely expressed in several other B- and T-cell lymphomas such as lymphoma, chronic lymphocytic leukemia, MM (20). The study from Pettersson *et al* showed that Bcl-2 was expressed in most MM cell lines such as U266-1970, U266-1984, U1958, U1996, L363, Karpas 707, OPM1, OPM2 (21). Our results revealed that the human MM Wus1 cell line which was established in our laboratory expressed a high level of Bcl-2. It was controversial whether U266 expressed Bcl-2 highly. Some studies found that during the continuous cultivations, U266 had undergone cytogenetic changes and Bcl-2 expression changed (21-23). Overexpression of Bcl-xl has been observed in 30% MM (24). Our studies found that human MM cell lines U266 and Wus1 expressed a high level of Bcl-xl.

There is increasing evidence that high expression of Bcl-2, Bcl-xl, or both, may play a critical role in MM progression and resistance to chemotherapeutic agents (25). Therefore, therapy targeting Bcl-2 or Bcl-xl has become a promising strategy. There are several methods that could inhibit Bcl-2 such as antisense nucleotide or monoclonal antibody of Bcl-2. Antisense Bcl-2 and Bcl-xl studies showed that inhibition of Bcl-2 and Bcl-xl may be an effective treatment of MM (11). Previous studies revealed that gossypol was a nonpeptidic small-molecule inhibitor of Bcl-2/Bcl-xl. It can bind to the BH3 binding site in Bcl-2/Bcl-xl, and then block the heterodimerization of Bcl-2/Bcl-xl with proapoptotic members in the Bcl-2 protein family such as Bad, Bak, Bid and Bas, and can initiate downstream apoptosis pathways (18).

Gossypol has antiproliferative and antimetastatic effects on several tumors. Its mechanism and molecular targets may be variable, for example inhibition of protein kinase C, regulation of cell cyclin-related protein Rb and cyclin D1, anti-angiogenesis, impacting signal transduction pathway (26,27). Gossypol has now been found to have inhibitory effects on proliferation or to induce apoptosis in ovarian cancer, endometrial cancer, adrenal cortical tumor, thyroid cancer, lung cancer, colon carcinoma, leukemia, pancreatic cancer, melanoma and lymphoma. In addition, gossypol can increase the sensitivity of drug-resistant tumor cells to chemotherapy and radiotherapy (12,13,15,26,28-36). Some clinical trials showed gossypol was well-tolerated, and partial responses were observed in some patients (17,37-39). We found gossypol acetate was able to induce dose- and time-dependent apoptosis of MM cells, as evidenced by typical morphological changes, DNA ladder and increase in the percentage of cells in subdiploid peak.

The mitochondrial pathway is a key pathway in cell apoptosis. DNA-damaging agents signal cell death by altering the mitochondrial transmembrane protein, activating Bcl-2 family members with subsequent cyto *c* release, and activating the caspase family of proteins. Caspase-3 is a central component in cell apoptosis caused by exogenous or

endogenous apoptotic signal. We revealed that caspase-3 and caspase-9 were activated, and Bcl-2/Bcl-xl expression was downregulated in the process of MM cell apoptosis induced by gossypol acetate. Therefore, induction of MM cell apoptosis by gossypol acetate may be through the mitochondrial pathway, inhibiting Bcl-2/Bcl-xl expression and activating caspase-9, caspase-3. Our results are consistent with previous findings (14,36,40,41).

As a new antitumor agent, the effect of gossypol acetate should be confirmed *in vivo*. Our *in vivo* experiments proved that gossypol acetate could inhibit tumor growth in Wus1-bearing mice, but the survival of mice was not prolonged, and tumor grew rapidly after short inhibition. Some studies reported that the effect may improve if gossypol is combined with the other chemotherapeutics. Thus, whether these effects may be improved when gossypol acetate is combined with other antimyeloma drugs, such as dexamethasone, thalidomide, requires further investigation.

In the present study, we demonstrated that gossypol acetate had an antiproliferative effect and induced apoptosis of MM cells for the first time. The mechanisms might involve downregulation of Bcl-2/Bcl-xl expression and affecting the cell cycle. Gossypol acetate was also able to inhibit tumor growth in Wus1-bearing mice. Our findings provide evidence that gossypol acetate warrants further preclinical and clinical research in the treatment of MM.

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