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A Steroid Fraction of Chloroform Extract from Bee Pollen of *Brassica campestris* Induces Apoptosis in Human Prostate Cancer PC-3 Cells

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Bee pollen of *Brassica campestris* L. is widely used in China as a natural food supplement and an herbal medicine in strengthening the body's resistance against diseases including cancer. The present study was carried out to investigate the effect of a steroid fraction of chloroform extract from bee pollen of *Brassica campestris* L. on human cancer cell viability. Our studies show that among nine cancer cell lines of different origin (PC-3, LNCaP, MCF-7, Hela, BEL-7402, BCG-823, KB, A549 and HO8910), this steroid fraction displayed the strongest cytotoxicity in human prostate cancer PC-3 cells. The mode of cell death appeared to be apoptosis in PC-3 cells, as shown by flow-cytometric analysis and fluorescence microscopes. Caspase-3 activity was obviously enhanced after the cells were treated with the fraction. A time-dependent decrease in the expression of anti-apoptotic protein Bcl-2 was also observed by Western blot analysis. It is suggested that the steroid fraction could induce cytotoxicity in prostate cancer PC-3 cells by triggering apoptosis. The studies indicate that the steroid fraction of chloroform extract from bee pollen of *Brassica campestris* L. may be a promising candidate for the treatment of advanced prostate cancer. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Brassica campestris L.; prostate cancer; apoptosis.

INTRODUCTION

Bee-collected pollen is an apicultural product that is composed of nutritionally valuable substances and contains considerable amounts of biological active substances that may possibly be used to prevent or treat cancer (Aliyazicioglu *et al.*, 2005). *Brassica campestris* L. is planted in most regions of the world. In China, the bee pollen of this plant is widely used as a natural supplement to everyday meals and as an herbal medicine to strengthen the body's resistance to diseases including cancer (Ren and Liu, 2001). In cancer treatment, traditional Chinese medicine believes that increasing the body's defense to disease is the principal method, and as a natural food, bee pollen of *Brassica campestris* L. serves this purpose (Gong, 1991). It was reported that the polysaccharide from bee pollen of this plant can inhibit the growth of tumors by regulating the immune system (Yang and Wu, 2006). However, whether the biological active components from *Brassica campestris* L. could be used to induce cytotoxicity in human cancer cells remains unknown. Therefore, the present study was carried out to evaluate the effect of its chloroform extract on human cancer cell viability, and to explore the underlying mechanism.

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MATERIALS AND METHODS

Materials. Trypan blue, RNase, aprotinin, leupeptin and propidium iodide were purchased from Sigma (USA); acridine orange (AO) and ethidium bromide (EB) were from Fluka (USA). F-12 (Ham), DMEM (Dulbecco's modified Eagle's medium) and RPMI-1640 from Life Technologies (USA). Caspase-3 colorimetric assay kits and caspase-3 inhibitor (z-DEVD-fmk) were obtained from Biovision (USA).

Preparation and analysis of bee pollen of *Brassica campestris* L. extract. Bee pollen of *Brassica campestris* L. was collected in Xinjiang (China) in June 2002 and identified by the College of Pharmaceutical Sciences, Zhejiang University (Hangzhou, China), where the voucher specimen of this plant (NO.777) is deposited. Air-dried bee pollen of *Brassica campestris L* (1000 g) was extracted with 100% EtOH, and the solvent was concentrated in vacuum and extracted with chloroform, which was then applied to silica gel column chromatography, and eluted with petroleum ether-acetone (10:1 w/w), 1.95 g fraction (chloroform extract from bee pollen *Brassica campestris L*, CPBC) was obtained.

CPBC gave positive response to Liebermann-Burchard's reaction and Carr-Price's reaction, indicating that CPBC was a steroid fraction.

CPBC solution (50 mg/mL) was prepared in dimethyl sulfoxide (DMSO) and stored at –20 °C. Final CPBC at different concentrations used for different experiments were prepared by diluting the stock with culture medium, and control cultures received the carrier

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solvent (0.2% DMSO). In control experiments, exposure to 0.2% DMSO in the culture medium showed no effect on cell growth (data not shown).

Cell culture. Nine different human cancer cell lines were used: Androgen-independent prostate cancer PC-3 and estrogen-responsive breast adenocarcinoma MCF-7, androgen-sensitive prostate cancer LNCaP, human cervix carcinoma Hela, gastric adenocarcinoma BCG-823, hepatocarcinoma BEL-7402, squamous carcinoma KB, lung carcinoma A549 and ovarian carcinoma HO8910 cell lines were obtained from Institute of Biochemistry and cell Biology, CAS (Shanghai, China). PC-3 and LNCaP were maintained in F-12 (Ham) media containing 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, supplemented with 10% heat-inactivated fetal bovine serum. MCF-7 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Hela, HO8910, BEL-7402, BCG-823, A549 and KB cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum and antibiotics as above. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell viability assay. The cell viability was determined by the trypan blue exclusion assay. Briefly, the logarithmically growing cells were planted to 24/96-well culture plates and cultured overnight. CPBC was added to the wells with final concentration at 12.5, 25, 50 and 100 μg/mL. After 24 h treatment with CPBC, the cells were counted with hemocytometer by exposed to 0.4% (w/v) trypan blue at 37 °C for 1 min.

Flow cytometry analysis of apoptosis. PC-3 cells were seeded into 50 mL culture flask and after being treated with 0, 12.5, 25 and 50 μg/mL CPBC for 24 h; cellular DNA content was detected by flow cytometry via determination of propidium iodode. Briefly, the cells were trypsinized, washed twice with ice-cold PBS and fixed with 70% ethanol. After overnight refrigeration at 4 °C and subsequent rehydration in PBS for 30 min at 4 °C, cells were stained at 37 °C for 20 min with 50 μg/mL propidium iodide and 100 units/mL RNase. Analysis was performed on a FAC Scan (Becton Dickinson, San Jose, CA). Data acquisition and analysis was performed with CellQuest 3.1 software and ModFit LT 3.0 software (Variety Software House, Inc. Topsham, ME), respectively.

Fluorescent analysis of apoptosis. PC-3 cells were seeded into 50 mL culture flask and treated with 50 μg/mL CPBC for 24 h, and then the cells were collected and stained with AO and EB. Following staining, the cells were observed by fluorescence microscope (Leica Germany).

Caspase-3 activity assay. After the treatment with indicated agents, PC-3 cells were harvested and washed with PBS by centrifugation at 750 × **g** for 5 min at 4 °C. The activity of caspase-3 was assayed using a caspase-3/CPP32 colorimetric assay kit (BioVision Research Products) according to the manufacturer's protocol. Quantitative detection of apoptotic cells were performed by flow cytometry.

Western blot analysis. PC-3 cells were treated with 50 μg/mL CPBC for 0, 6, 12, 24 and 48 h. Both adherent and floating cells were collected and frozen at −80 °C. The expression of Bcl-2 in PC-3 cells was detected by Western blot analysis. Briefly, the cell pellets were resuspended in lysis buffer, including Hepes 50 mmol/L pH 7.4, Triton-X 100 1%, sodium orthovanada 2 mmol/L, sodium fluoride 100 mmol/L, edetic acid 1 mmol/L, egtazic acid 1 mmol/L, PMSF 1 mol/L, aprotinin 0.1 g/L, leupeptin 0.01 g/L, then lysed in 4 °C for 1 h. After 13 000 × **g** centrifugation for 10 min, the protein content of supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad, USA). The protein lysates were separated by electrophoresis in 12% SDS polyacrylamide gel and blotted onto nitrocellulose membrane. Protein expression was detected by rabbit polyclonal anti Bcl-2 antibody (Santa Cruz Biotechnology, USA) and secondary antibody conjugated with peroxidase (goat anti-rabbit IgG) (Santa Cruz Biotechnology, USA).

Statistical analysis. Results are reported as mean ± standard deviation, and all statistical comparisons were made by means of *t*-test and values of $p \leq 0.05$ were considered significant.

RESULTS

Cell viability assay

All cell lines used in this study were treated with various concentrations of CPBC (12.5–100 μg/mL) for 24 h. The results showed that CPBC remarkably induced concentration-dependent cytotoxicity in PC-3 and LNCaP cells. But only 100 μg/mL CPBC could obviously induce cytotoxicity in MCF-7, Hela, BEL-7402, BCG-823, KB, A549 and HO8910 cells (Fig. 1).

Flow cytometry analysis of apoptosis

Quantitative detection of apoptotic cells and analysis of cell cycle kinetics were performed by flow cytometry.

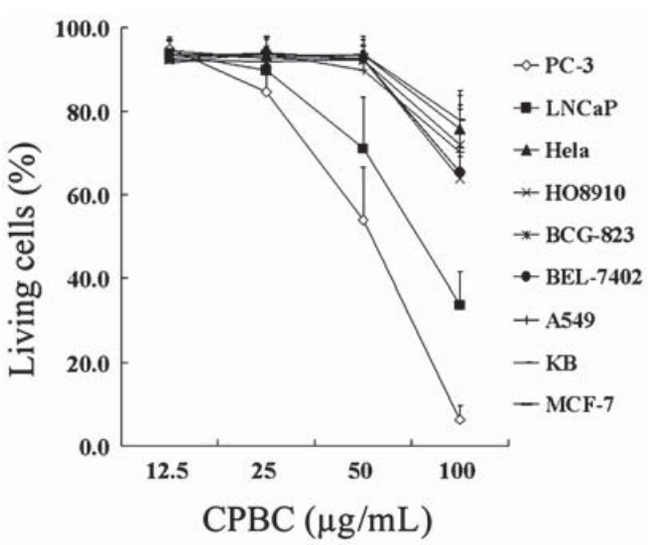

Figure 1. Assessment of cell viability in nine human cancer cell lines treated with CPBC (12.5, 25, 50 and 100 μg/mL) for 24 h. The viable cells were counted with a hemocytometer by trypan blue staining, $n = 3$, mean ± SD.

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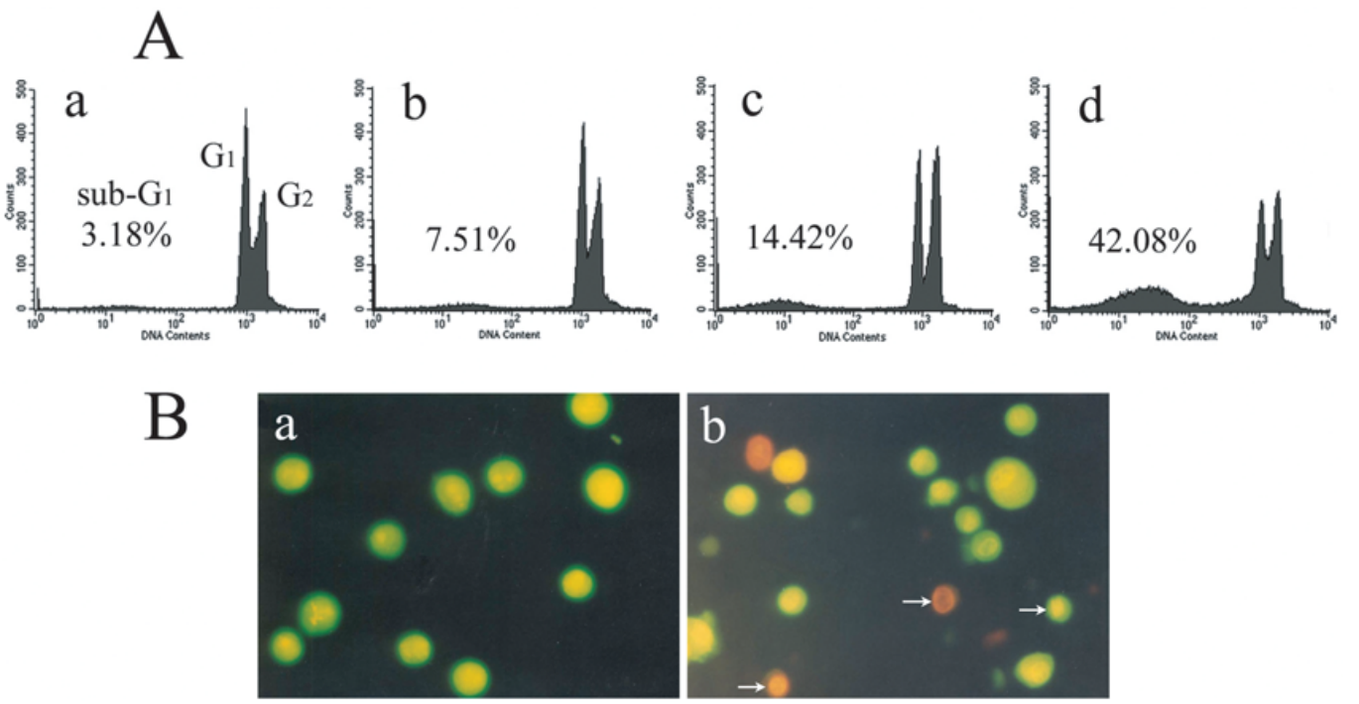

Figure 2. (A) Flow cytometry analysed the DNA contents of PC-3 treated with CPBC. (a, b, c, d) cells treated with 0, 12.5, 25 and 50 μg/mL CPBC for 24 h, respectively. The sub-G1 peak formed by cells with reduced DNA content represented the presence of apoptotic cells. The two major peaks represented G0/G1 and G2/M phase of cell cycle, $n = 3$. Figure data from a representative experiment. (B) Morphological changes of PC-3 induced by CPBC under fluorescence microscope (stained by AO and EB). (a) untreated normal cells showed uniform chromatin stained with AO. (b) cells treated with CPBC (50 μg/mL) 24 h. Apoptotic cells displayed well defined condensed chromatin clearly stained with AO; these cells were further classified as either viable (EB–, right arrow) or nonviable (EB+, left arrow), depending on the integrity of the cytoplasmic membrane. In addition, there were some late apoptotic cells undergoing degradation; these cells were invariably EB (middle arrow), with obviously dispersed nuclear chromatin (× 400).

The sub-G_1 peak formed with reduced DNA content represented the presence of apoptotic cells. The two major peaks represented the G_0/G_1 and G_2/M phase of cell cycle. The results showed that PC-3 cells treated with CPBC (0, 12.5, 25 and 50 μg/mL) for 24 h induced a concentration-dependent increase in the apoptotic rate and marked accumulation in G_2/M phase of cell cycle (Fig. 2A).

Fluorescent analysis of apoptosis

After staining with AO/EB, the fluorescent assay revealed that PC-3 untreated cells showed uniform chromatin stained with AO. In contrast, after 24 h exposure to 50 μg/mL CPBC, PC-3 cells showed characteristic apoptotic alterations: condensed chromatin clearly stained with AO; these cells were further classified as either viable (EB–) or nonviable (EB+), depending on the integrity of the cytoplasmic membrane. In addition, there were some late apoptotic cells undergoing degradation; these cells were invariably nonviable (EB+), with obviously dispersed nuclear chromatin (Fig. 2B).

Caspase-3 activity assay

The present study examined whether caspase-3 activation is involved in the apoptotic process triggered by CPBC. PC-3 cells were pretreated with 50 mM caspase 3 inhibitors (z-DEVD-fmk) for 2 h, and then induced to undergo apoptosis by treatment with 50 μg/mL CPBC for 24 h. The results clearly showed that administration of caspase 3 inhibitor alone did not affect the cell viability and caspase-3 activity. However, z-DEVD-fmk (specific caspase-3 inhibitor) significantly inhibited CPBC-induced cell apoptosis and caspase-3 activation in PC-3 cells (Fig. 3).

Western blot analysis

PC-3 cells were treated with 50 μg/mL CPBC for 0, 6, 12, 24 and 48 h. After 6 h, Bcl-2 expression began to decrease and was almost undetectable after 24 h (Fig. 4).

DISCUSSION

The results show that CPBC, a steroid fraction of chloroform extract from bee pollen of *Brassica campestris* L., displayed a differing extent of cytotoxic activity on human cancer cells. Since it is possible that different cell lines exhibit different sensitivities towards the plant extracts, nine cell lines of different origins and with different biological characteristics were chosen to evaluate the effects of CPBC. Among these cell lines, the cells derived from human prostate (PC-3, LNCaP) were more sensitive than those of non-prostate origin (Fig. 1). It is therefore speculated that CPBC may be a selective cytotoxic agent of cancer cell lines derived from human prostate.

The PC-3 cell line is a much-studied prototype of cells from a highly malignant human prostate cancer that is hormone-refractory and resistant to further

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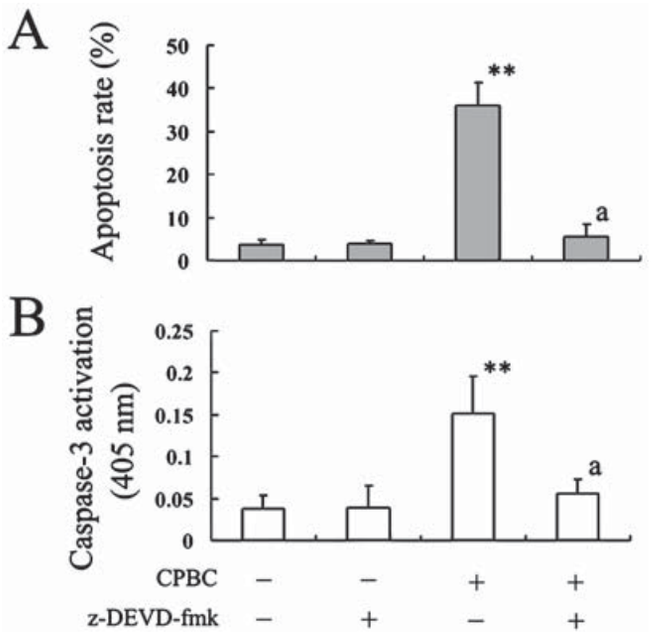

Figure 3. Inhibition of caspase-3 activity and attenuation of CPBC-induced cell apoptosis by caspase-3 inhibitor (z-DEVD-fmk). PC-3 cells were treated with 50 mM specific caspase-3 inhibitor (z-DEVD-fmk) 2 h prior to 24 h of 50 μg/mL CPBC treatment. After incubation, the cell apoptosis rate (a) and caspase-3 activity (b) were examined as described in Methods section. Mean ± SD, $n = 5$. ** $p < 0.01$ compared with the respective CPBC and z-DEVD-fmk free control and [a] $p < 0.01$ comparison between the absence and presence of z-DEVD-fmk in the same CPBC treatment group.

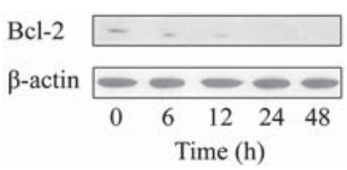

Figure 4. CPBC effect on the expression of Bcl-2 in PC-3 cells. PC-3 cells were treated with 50 μg/mL CPBC for 0, 6, 12, 24 and 48 h, repectively. Equal amounts of proteins (40 μg/lane) were loaded and membranes were sequentially probed, stripped and reprobed with rabbit polyclonal antibody to Bcl-2. The expression of β-actin was the loading control.

treatment. Numerous studies have shown them to be highly tumorigenic and metastatic, hormone unresponsive and resistant to normal rates of apoptosis (Kaighn *et al.*, 1978; van Bokhoven *et al.*, 2003). Considering that PC-3 cells are the most sensitive to CPBC, they were selected to explore the underlying mechanism. Different methods were applied to determine the cell death model of PC-3 cells exposed to CPBC. As shown by fluorescence microscopes, apoptosis was the dominant model of cell death induced by CPBC. Further studies using flow-cytometric analysis confirmed a concentration-dependent increase of apoptotic cell, together with the accumulation of G_2/M phase cells. Caspases are believed to serve as the central executioners of the apoptotic pathway. Morphological changes observed in apoptotic cells, such as cell membrane blebbing and chromatin condensation, are known to be closely associated with the activation of caspases in apoptotic cells (Kothakota *et al.*, 1997). The results show that caspase-3 activity was obviously increased after CPBC treatment, it is suggested that CPBC are capable of inducing apoptosis through activation of caspases in PC-3 cells.

To develop more effective treatments of prostate cancer, it is important to understand the molecular mechanisms regulating the apoptotic pathway in this disease. Bcl-2 protein has been correlated with aggressive histology and high rates of recurrence in prostate cancers (Krajewska *et al.*, 1996) and contributes to the phenotype of androgen-resistant prostate cancers (McDonnell *et al.*, 1992). In addition, it has been shown that Bcl-2 overexpression makes prostate cancer cells resistant to various treatments, including radiation, hormonal ablation and chemotherapy (Raffo *et al.*, 1995; Huang *et al.*, 1998; Petrylak, 2005). These findings prompted us to test the effect of CPBC on the expression of Bcl-2 in PC-3 cells. The results show that CPBC could decrease the expression of Bcl-2 in a time-dependent manner. This suggests that CPBC-induced apoptosis in PC-3 cells is associated with the down-regulated Bcl-2 expression.

Since apoptosis plays an important role in the regulation of tumor response to various forms of cancer therapies, and regulating apoptosis is an effective way to improve tumor therapy, it is speculated that CPBC may be used in the treatment of prostate cancer. Specifically, in the treatment of prostate carcinoma, therapeutic efficacy is achieved by hormonal or androgen-ablative therapy through increasing the apoptotic response of androgen-dependent prostate cancer cells. However, current therapy for prostate cancer is hindered because of the disease progression from the androgen-dependent to the androgen-independent state. In the latter case, hormonal ablation is always ineffective, thus promoting apoptosis of androgen-independent prostate cancer cells becomes a formidable challenge. The results showed CPBC could induce the apoptosis of androgen-independent prostate cancer cell line PC-3, and therefore it is suggested that CPBC could possibly be used to prevent or treat advanced prostate cancer.

In conclusion, it was found that CPBC could induce cytotoxicity in PC-3 cells by triggering apoptosis. These results indicate that the steroid fraction of chloroform extract from bee pollen of *Brassica campestris* L. may be a promising candidate for the treatment of advanced prostate cancer.

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