Department of Pharmacology and Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China

Brassinolide, a plant sterol from pollen of *Brassica napus* L., induces apoptosis in human prostate cancer PC-3 cells

YAO-DONG WU, YI-JIA LOU

Received July 27, 2006, accepted September 1, 2006

Yi-Jia Lou, Department of Pharmacology and Toxicology, College of Pharmaceutical Sciences, Zhejiang University, 310031 Hangzhou, China
yijialou@zju.edu.cn

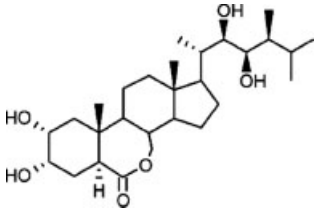
Pharmazie 62: 392–395 (2007) *doi: 10.1691/ph.2007.5.6660*

Brassinolide is a plant sterol first isolated from pollen of rape (*Brassica napus* L.). The present study was carried out to investigate the effect of brassinolide on androgen-independent human prostate cancer PC-3 cell viability. Results showed that brassinolide could induce a time and concentration-dependent cytotoxicity in PC-3 cells. The mode of cell death appeared to be predominately apoptosis, as shown by flow-cytometric analysis, fluorescence and transmission electron microscopes. Caspase-3 activity was obviously increased after brassinolide treatment. Western blot studies indicated that treatment with brassinolide triggered a time-dependent decrease in the expression of anti-apoptotic protein Bcl-2. We suggest that brassinolide could induce cytotoxicity in PC-3 cells by triggering apoptosis. Brassinolide might therefore be a promising candidate for the treatment of prostate cancer.

1. Introduction

Epidemiological studies suggest that dietary plant sterols may offer protection for some types of cancer including prostate cancer. Plant sterols are found in significant amounts in various parts of plants including seeds, nuts, fruits and vegetable oils. Thus, eating habits and availability of the source of plant sterols govern human intake of plant sterols (Schaller 2003). It has been shown that dietary consumption of plant sterols is lower in Western countries (80 mg/day) than in Asia (400 mg/day) (Messina and Barnes 1991; Rao and Janezic 1992), where the incidence rate of prostate cancer is at a minimum level (Crawford 2003). In addition, it was demonstrated that some plant sterols could inhibit the proliferation of human prostate cancer cells both *in vitro* and *in vivo* (Awad et al. 2001; von Holtz et al. 1998). These data suggest that plant sterols may possibly be used to prevent or treat prostate cancer.

In 1979 a plant sterol, termed brassinolide, was first isolated from pollen of rape (*Brassica napus* L.) (Grove et al. 1979). It is a naturally occurring plant hormone that promotes growth, increases yields for grain and fruit crops, and makes plants more resistant to drought and cold weather (Bajguz and Tretyn 2003). However, whether it could be used to prevent or treat human diseases remains unknown. The present study was carried out to evaluate the effect of brassinolide on androgen-independent prostate cancer PC-3 cell viability, and explore the underlying mechanism.



2. Investigations and results

2.1. Assessment of cell viability

PC-3 cells were treated with various concentrations of brassinolide (0, 10, 20 and 40 μM) for 12, 24 and 36 h. Results showed that brassinolide induced a time and concentration-dependent cytotoxicity in PC-3 cells.

2.2. Flow cytometric analysis

Quantitative detection of apoptotic cells and analysis of cell cycle kinetics were performed by flow cytometry. The sub-G_1 peak formed with reduced DNA content represented the presence of apoptotic cells. The two major peaks represented G_0/G_1 and G_2/M phase of cell cycle.

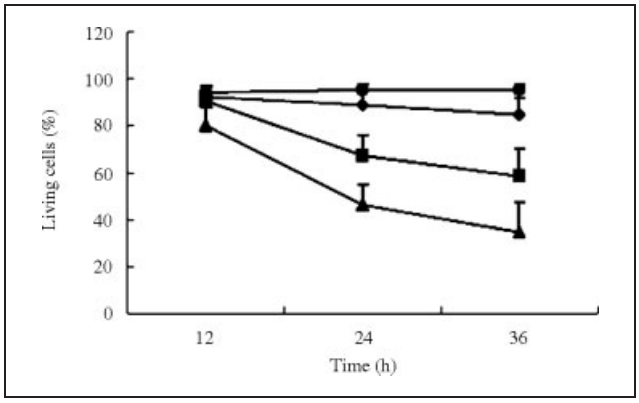


Fig. 1: Assessment of cell viability in PC-3 cells treated with brassinolide. The viable cells were counted with hemocytometer by trypan blue staining every 12 h, n = 3. Mean ± SD
—●— 0 μM; —◆— 10 μM; —■— 20 μM; —▲— 40 μM

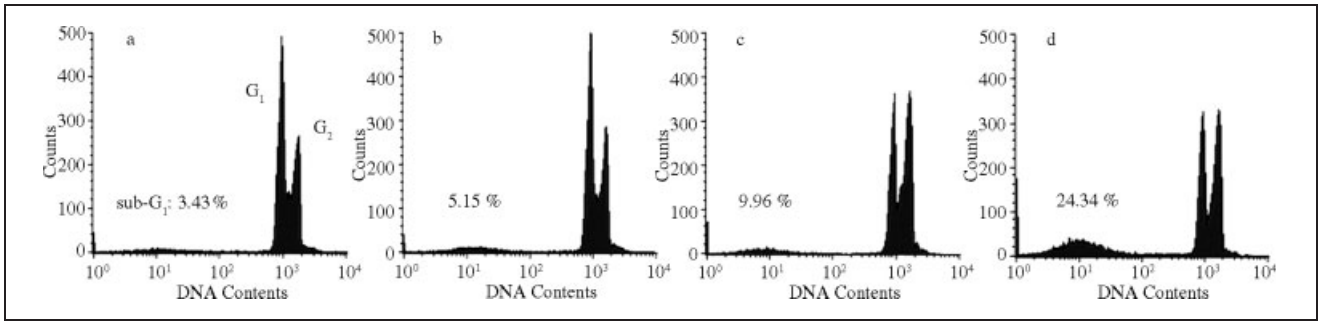


Fig. 2: Flow cytometry analyzed the DNA contents of PC-3 treated with brassinolide. a, b, c and d: the cells treated with brassinolide (0, 10, 20 and 40 μM) for 12 h respectively. The sub-G1 peak formed by cells with reduced DNA content represented the presence of apoptotic cells. The two major peaks represented G0/G1 and G2/M phase of cell cycle. n = 3. Figure data from a representative experiment

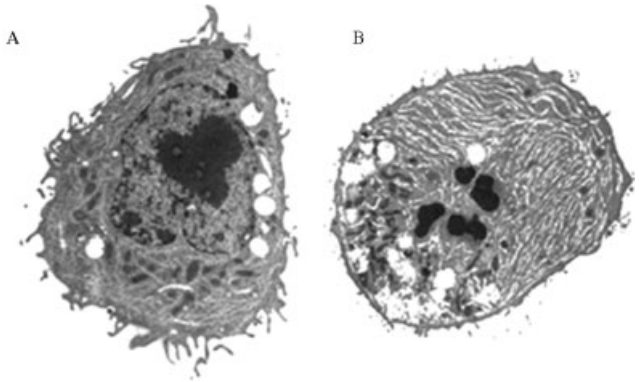


Fig. 3: Morphological changes of PC-3 cells induced by brassinolide under transmission electron microscope. A: untreated cells; cells had intact cytoplasm membranes, clear cytoplasm, intact subcellular organelles, intact nuclear membranes and normal nucleoli. B: cells treated with brassinolide 20 μM 24 h, cells showed the characteristic apoptotic alterations, shrinking cellular morphology, cell surface microvilli decrease, cytoplasmic vacuoles, chromatin condensation. (×4000)

Results showed that PC-3 cells treated with brassinolide (0, 10, 20 and 40 μM) for 12 h induced a concentration-dependent increase in the apoptotic rate and marked accumulation in G_2/M phase of cell cycle (Fig. 2).

2.3. Electronic micrography of PC-3 cells

Electronic micrographs revealed that PC-3 control cells had intact cytoplasm membranes, clear cytoplasm, intact subcellular organelles, intact nuclear membrane and normal nucleoli. In contrast, after 24 h exposure to 20 μM brassinolide, PC-3 cells showed characteristic apoptotic alterations: shrinking cellular figure, decreasing cell surface microvilli, cytoplasmic vacuoles, chromatin condensation (Fig. 3).

2.4. Fluorescent analysis

After stained with AO/EB, Fluorescent assay revealed that PC-3 untreated cells showed uniform chromatin stained with AO. In contrast, after 24 h exposure to 20 μM brassinolide, 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 EB (+), with obviously dispersed nuclear chromatin (Fig. 4).

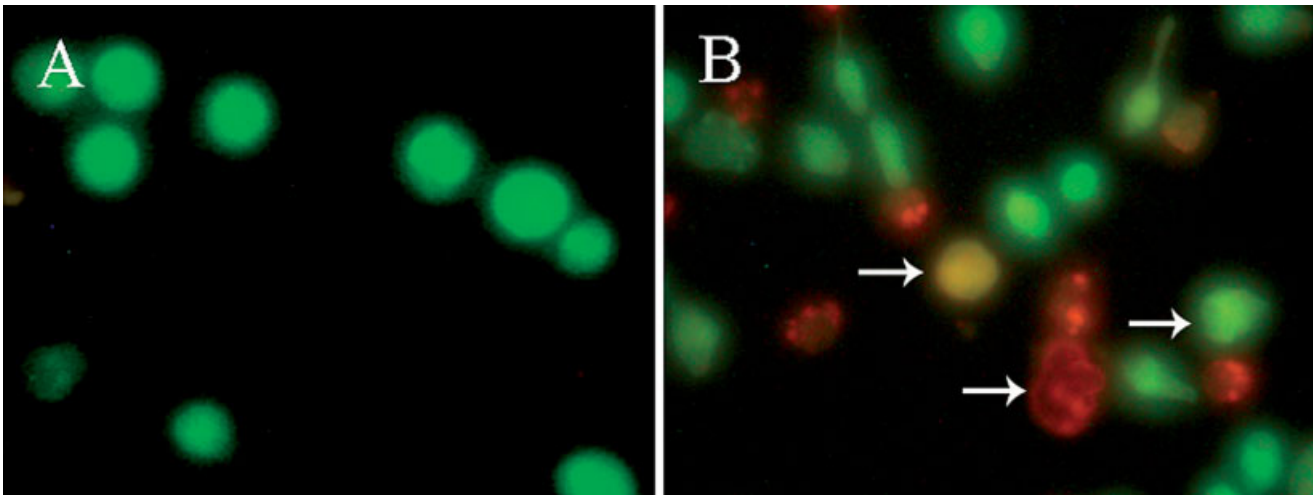


Fig. 4: Morphological changes of PC-3 induced by brassinolide under fluorescence microscope (stained by AO and EB). A: untreated normal cells showed uniform chromatin stained with AO, B: cells treated with brassinolide 20 μM 24 h. apoptotic cells displayed well-defined condensed chromatin clearly stained 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)

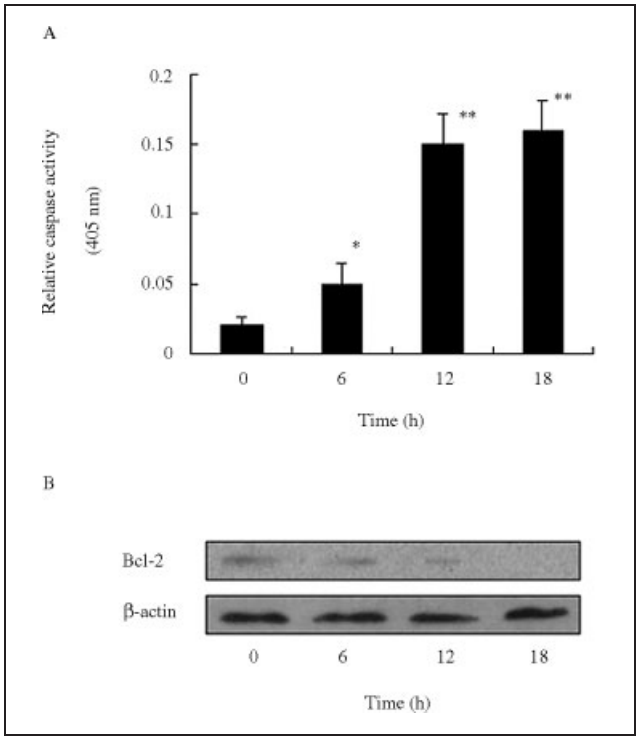


Fig. 5: PC-3 cells were treated with brassinolide 20 μM for 0, 6, 12, and 18 h. A: The activity of caspase-3 in brassinolide-treated PC-3 cells. n = 3. Mean ± SD. B: Western blot analysis of Bcl-2 expression in brassinolide-treated PC-3 cells. The expression of β-actin was loading control

2.5. Assay of caspase-3 activities

To determine whether induction of apoptotic effect of brassinolide was associated with caspases-3 activation, we measured the activity of caspase-3 in PC-3 cells after treatment with 20 μM brassinolide. Results indicated that PC-3 cells treated with brassinolide 20 μM for 0, 6, 12 and 18 h showed a time-dependent increase in the activity of caspases-3 (Fig. 5A).

2.6. Western blot analysis of Bcl-2 expression

PC-3 cells were treated with 20 μM brassinolide for 0, 6, 12, and 18 h. After 6 h, Bcl-2 expression began to decrease and was almost undetectable after 18 h (Fig. 5B).

3. Discussion

The PC-3 cell line is a prototype of cells from a highly malignant human prostate cancer that is hormone-refractory and resistant to further 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). In the present study, we investigated the effect of brassinolide on the cell viability of this cell line. Our studies show that brassinolide could induce a time and concentration-dependent cytotoxicity in PC-3 cells. The mode of cell death induced by brassinolide appeared to be apoptosis in PC-3 cells, as shown by fluorescence and transmission electron microscopy. Further studies using flow-cytometric analysis confirmed a concentration-dependent increase in apoptotic cells, together with the accumulation of G_2/M phase cells. Caspases are believed to serve as the central executioners of the apoptotic pathway. Morphologic 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). Our results show that cells treated with brassinolide resulted in a time-dependent increase in caspase-3 activity. We suggest that brassinolide is 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 (Huang et al. 1998; Petrylak 2005; Raffo et al. 1995). These findings prompted us to test the effect of brassinolide on the expression of Bcl-2 in PC-3 cells. Our results show that brassinolide could decrease the expression of Bcl-2 in a time-dependent manner. This suggests that brassinolide-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 (Kerr et al. 1994), we believe that brassinolide 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 (Kyprianou et al. 1990). However, current therapy for prostate cancer is hindered because of the disease progression from androgen-dependence to androgen- independence state (Rocchi et al. 2005). In the latter case, hormonal ablation is always ineffective, thus promoting apoptosis of androgen-independent prostate cancer cells becomes a formidable challenge. Our results show that brassinolide could induce apoptosis in the androgen-independent prostate cancer cell line PC-3, and we therefore suggest that brassinolide may be used to prevent or treat advanced prostate cancer.

Brassinolide is found in very low abundance in a variety of plants, such as *Brassica napus* L., *Arabidopsis thaliana* L. and *Dolichos lablab* L. Pollen of *Brassica napus* L. is believed to contain a relatively high level of brassinolide (100 μg/kg) and serves as one of the major natural sources of this compound (Bajguz and Tretyn 2003). However, methods for chemical synthesis of brassinolide are now readily available and a reasonable amount of this compound can be easily obtained for pharmaceutical use (Thomas et al. 1997).

4. Experimental

4.1. Materials

Trypan blue, RNase and propidium iodode were purchased from Sigma Chemical CO. (USA); acridine orange (AO) and ethidium bromide (EB) were from Fluka (USA). Brassinolide was purchased from Unida (China). F-12 (Ham), DMEM (Dulbecco's modified Eagle's medium) and RPMI-1640 from Life Technologies (USA).

Brassinolide solution (100 μM) was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Different concentrations used for different experiments were prepared by diluting the stock with culture medium, and control cultures received the carrier solvent (0.2% DMSO). In control experiments, exposure to 0.2% DMSO in the culture medium showed to have no effect on cell viability (data not shown).

4.2. Cell culture

Androgen-independent prostate cancer PC-3 cells were obtained from Institute of Biochemistry and cell Biology, CAS (Shanghai, CHINA). Cells 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. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

4.3. Assessment of cell viability

The logarithmically growing PC-3 cells were planted to 24/96-well culture plates and cultured overnight. Brassinolide was added to the wells with final concentration at 0, 10, 20 and 40 μM. After 12, 24 and 36 h treatment with brassinolide, the cells were counted with hemocytometer by exposed to 0.4% (w/v) trypan blue at 37 °C for 1 min.

4.4. Flow cytometry analysis

PC-3 cells were seeded into 50 ml culture flask and after being treated with 0, 10, 20 and 40 μM brassinolide for 12 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.

4.5. Preparation of samples for transmission electron microscopy

PC-3 cells previously treated with 20 μM brassinolide for 24 h, or untreated PC-3 cells were washed with PBS (pH 7.2) and fixed in 2.5% glutaraldehyde for 2 h. After washed with PBS, the cells were fixed in 1.5% osmium tetroxide, dehydrated through a graded alcohol, embedded in Epon 812, thin-sectioned, stained with uranyl acetate and lead citrate, examined under a JEM-1200EX transmission electron microscope (JEM, Japan), and the results were sent to the pathology and medical jurisprudence laboratory (Zhejiang University, China) for double-blind evaluation.

4.6. Fluorescent analysis

PC-3 cells were seeded into 50 ml culture flask and treated with 20 μM brassinolide 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).

4.7. Assay of caspase-3 activities

PC-3 cells were seeded into 50 ml culture flask and after being treated with 20 μM brassinolide for various times (0, 6, 12 and 18 h); the activity of caspase-3 was assayed using a caspase-3/CPP32 Colorimetric Assay kit (BioVision Research Products) according to the manufacturer's protocol.

4.8. Western blot analysis of Bcl-2 expression

PC-3 cells were treated with 20 μM brassinolide for 0, 6, 12 and 18 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 (Sigma) 0.1 g/L, leupeptin (Sigma) 0.01 g/L, then lysed in 4 °C for 1 h. After 13000 × 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 primary polyclonal anti Bcl-2 antibody (Santa Cruz Biotechnology, USA) and secondary polyclonal antibody conjugated with peroxidase (goat anti-rabbit IgG) (Santa Cruz Biotechnology, USA).

4.9. Statistical analysis

Results are reported as means ± standard deviation. All experiments were done at least three times independently. Statistical analysis was performed using the Student's t-test and one-way ANOVA.

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