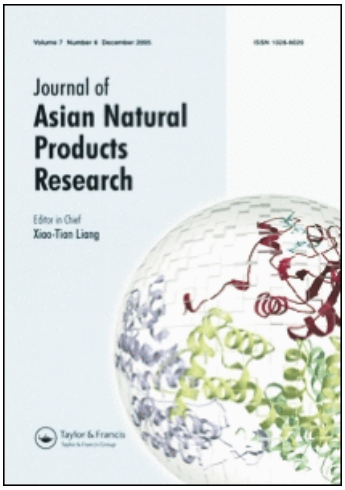

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Anti-tumor activities of andrographolide, a diterpene from *Andrographis paniculata*, by inducing apoptosis and inhibiting VEGF level

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Andrographolide (**1**), a diterpenoid lactone isolated from a traditional herb (*Andrographis paniculata*), is known to possess potent anti-inflammatory activity. In this study, we investigated the anti-tumor effect of **1** on various cancer cell lines *in vitro*. It induced apoptosis of prostate cancer (PC-3) cells (the most sensitive cell line among the cell lines screened) via the activation of caspase 3, up-regulation of bax, and down-regulation of bcl-2. Furthermore, its inhibitory activity on the level of vascular endothelial growth factor was also verified by ELISA.

Keywords: andrographolide; anti-tumor activity; apoptosis; caspase 3; bax; bcl-2

1. Introduction

Andrographis paniculata is a kind of oriental, traditional medicinal herb used in curing inflammation; the extracts from it show a wide spectrum of biological activities, and andrographolide (**1**) is the major active component of this plant. As shown in Figure 1, andrographolide has been reported to possess various bioactivities, and its anti-tumor activity has been explored in the recent years. It has been reported that "Lianbizhi" injection, which is made of andrographolide derivatives, has a strong inhibitory effect on gastric, liver, and lung cancer *in vivo*.[1] Jada found that **1** and its analogues possessed a strong inhibitory activity against several cancer cell lines *in vitro*.[2] Furthermore, **1** has been demonstrated to induce cell cycle arrest at G2/M phase and cell death in HepG2 cells.[3] Based on the previous reports, we attempted to investigate the mechanism of its anti-tumor effect.

As shown in the results of methyl thiazolyl tetrazolium (MTT) assay, **1** exhibited significant inhibition on the proliferation of human prostate cancer (PC-3) cell line, compared with human breast (MDA-MB-435s, MDA-MB-231), hepatoma (SMMC 7721), leukemia (K562), lung (A549), and gastrointestinal carcinoma (SGC 7901) cell lines. In the present study, PC-3 cell line was used to investigate the mechanism of its anti-tumor effect. The induction of apoptosis in PC-3 cells was detected by morphological analysis and DNA agarose electrophoresis. To further elucidate the mechanism involved in andrographolide-induced apoptosis in PC-3 cells, expression of proteins (caspase 3, bax, and bcl-2) that play crucial roles in apoptosis procedure was investigated by western blotting.

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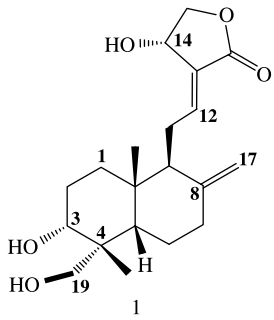

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Figure 1. Chemical structure of andrographolide (**1**).

2. Results and discussion

2.1 Inhibitory effect of andrographolide on the proliferation of PC-3 cells

The *in vitro* cytotoxic activity on various human cancer cell lines of **1** was evaluated by MTT assay. The concentrations that caused 50% inhibition on cancer cell growth are expressed as IC_{50} values that are reported in Table 1. The different cancer cell lines showed distinctive sensitivity toward **1**. The respective treatment of **1** did not significantly inhibit the proliferation of human lung (A549) and hepatoma (SMMC-7721) cancer cells. The proliferation of prostate cancer cells (PC-3) was significantly inhibited by **1** in a dose-dependent manner (IC_{50} value: 23.3 μmol/l). Compared with cisplatin (CDDP), which is a widely used anti-tumor chemotherapy in clinic, **1** exhibited an equivalent potency in the inhibitory effect on the proliferation of PC-3 cells as a natural compound.

2.2 Effect of andrographolide on morphological changes of PC-3 cells

Aiming at whether the loss of cell viability was induced by apoptosis or not, we monitored the nuclear morphology of PC-3 cells that were treated with four concentrations of **1** (0, 25, 50, 100 μmol/l, respectively). As illustrated in Figure 2, the cells treated with **1** obviously appeared apoptosis but no apoptotic nuclei were observed in the control group. Additionally, a definite sign of apoptosis is DNA fragmentation by endonuclease. The DNA ladder in Figure 3 revealed that the DNA of PC-3 cells that were treated with **1** at two concentrations (25, 50 μmol/l, respectively) was cleaved into distinctive sequential fragments. These results indicated that **1** can induce the apoptosis of PC-3 cells.

2.3 The level of caspase 3 in PC-3 cells

There are multiple ways for inducing apoptosis in cancer cells. However, caspase plays a principal role in the execution of apoptosis by a variety of stimulus. There are two well-established pathways of caspase activation for propagating death signals.[4,5] Nevertheless, both pathways result in the activation of the major downstream effector caspase 3 that cleaves various cellular targets and leads to cell death.[6] Based on these factors, we want to know whether the apoptotic pathway was induced by caspase 3. The proteolytic activity of caspase 3 was measured in terms of its ability to cleave Ac-DEVD-*p*NA, which is a specific substrate for caspase 3 and can be

Table 1. Cytotoxic activities of **1** and CDDP *in vitro*.

	Cytotoxicity (IC_{50}/μmol/l)[a]						
Compound	MDA-MB-435	MDA-MB-231	SGC7901	SMMC-7721	PC-3	K562	A549
Andrographolide	51.3	58.1	42.7	78.7	23.3	30.2	107.9
CDDP	12.8	8.16	6.97	6.25	12.6	1.51	18.7

[a] Cytotoxicity IC_{50} values are the concentrations corresponding to 50% growth inhibition.

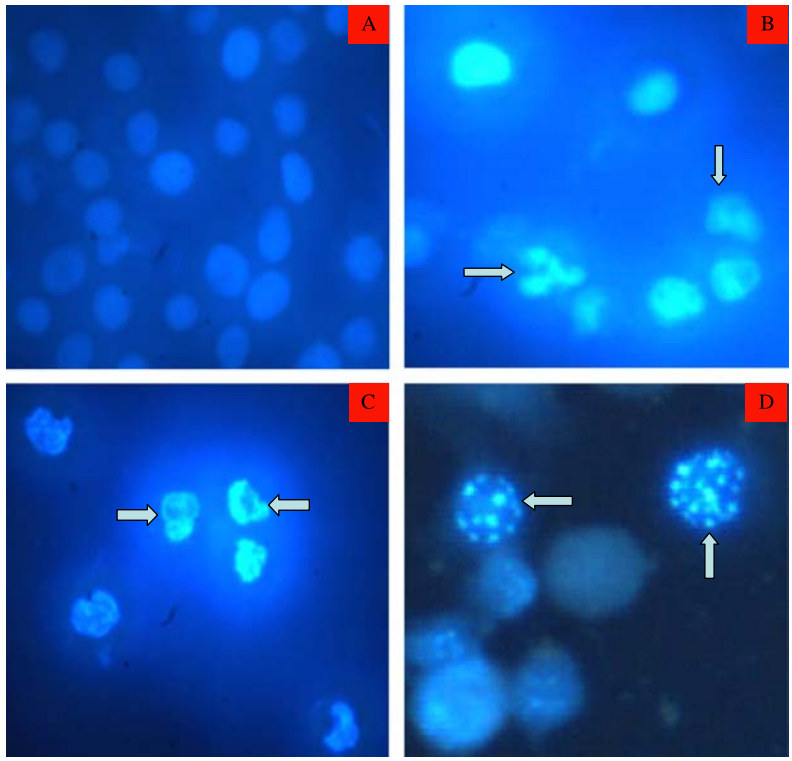

Figure 2. Effect of **1** on morphological changes of PC-3 cells. A: Control; B: **1** (100 μmol/l); C: **1** (50 μmol/l); and D: **1** (25 μmol/l). Arrow: the apoptotic bodies of PC-3 cells and nuclear cleavage (B–D).

measured at 405 nm. As shown in Figure 4, the levels of caspase 3 after being treated with different concentrations of **1** (0, 1.25, 2.5, 5, 10 μmol/l) were 9850, 50,230, 80,370, 90,630, 105,080 units, respectively (one unit is the amount of enzyme that will cleave 1.0 mmol/l of the colorimetric substrate Ac-DEVD-*p*NA per hour at 37°C under saturated substrate concentration). Compared with control group, the activation of caspase 3 in cells treated with **1** was significantly detected. Consistent with this observation, as shown in Figure 5, the level of pro-caspase 3 in cells treated with **1** decreased conspicuously compared with control group. Based on these data, it can be concluded that **1** promotes the activation of caspase 3 by motivating the cleavage of caspase 3 from precursor to its active form, which induced apoptosis subsequently.

2.4 The expression of bax and bcl-2 in PC-3 cells

The most important among apoptotic regulatory proteins is bcl-2 family that includes anti-apoptotic member bcl-2 and pro-apoptotic member bax.[7] One important underlying mechanism of the pro-apoptotic function of bax is to promote the release of pro-apoptotic factors such as cytochrome *C* from mitochondria.[8,9] It has been proposed that bax may stimulate the opening of the permeability transition pore (PTP) through interaction with the adenine nucleotide translocation. As a result of PTP opening, mitochondria would swell, leading to the rupture of the outer mitochondrial membrane and release of cytochrome *C*.[8] Contradictorily, bcl-2 is a mitochondrial protein family, which inhibits apoptotic process and promotes cell survival by heterodimerization

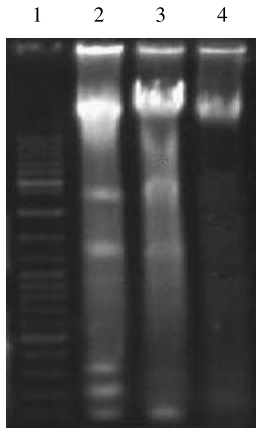

Figure 3. Analysis of internucleosomal DNA cleavage on agarose gel electrophoresis in PC-3 cells treated with **1** for 48 h. Lanes 1–4 correspond to marker, 50, 25, and 0 μmol/l of **1**, respectively.

with pro-apoptotic bcl-2 family member such as bax.[10,11] In order to elucidate the mechanism of **1** inducing apoptosis, the level of both bax and bcl-2 was investigated. As shown in Figure 5, the up-regulation of bax and the down-regulation of bcl-2 were notably observed when treated with **1**. These results suggested that **1** inhibited the proliferation of PC-3 cells by inducing apoptosis via the activation of caspase 3, up-regulation of bax, and down-regulation of bcl-2.

2.5 Effect of andrographolide on the level of VEGF in PC-3 cells

The vascular endothelial growth factor (VEGF) is a strong mitogen with special effect on vascular endothelial cells, and promotes the formation of new blood vessels. Many results from clinical research suggest that VEGF protein release is always an independent prognosis factor in cancer disease.[12] In the present study, the levels of VEGF were measured after treatment with **1** at different concentrations (0, 1.25, 2.5, 5, 10, 20 μmol/l, respectively), at which concentrations it was certified by MTT assay that PC-3 cells viability was not decreased. As shown in Table 2, a considerable reduction in VEGF level suggested the inhibitory effect of **1** on the excretion of VEGF in PC-3 cells.

In conclusion, **1** exhibited a powerful inhibition on PC-3 cells and exerted a dose-dependent inhibitory effect. The mechanism of this intensive inhibition could be ascribed to motivate the activation of caspase 3, up-regulation of bax, and simultaneous down-regulation of bcl-2, finally leading to apoptosis. Meaningfully, **1** showed a remarkable inhibitory effect on the release of VEGF in PC-3 cells that exhibited a new insight for further investigation. These findings suggested the potency of **1** as an anti-tumor agent.

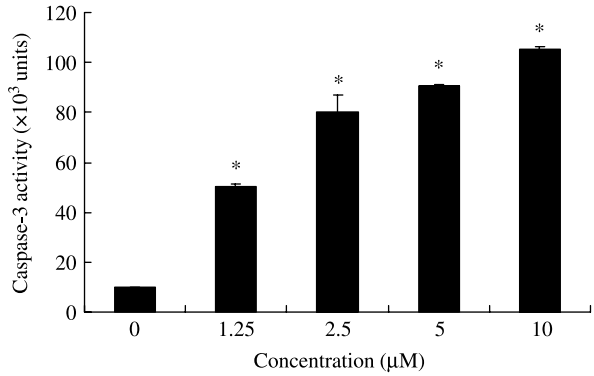

Figure 4. The activity of caspase 3 enzyme in PC-3 cells is evaluated by enzyme activity units. Values are mean ± SE for two independent experiments, in which each measurement was made in triplicate. $*p < 0.001$ *vs.* control group.

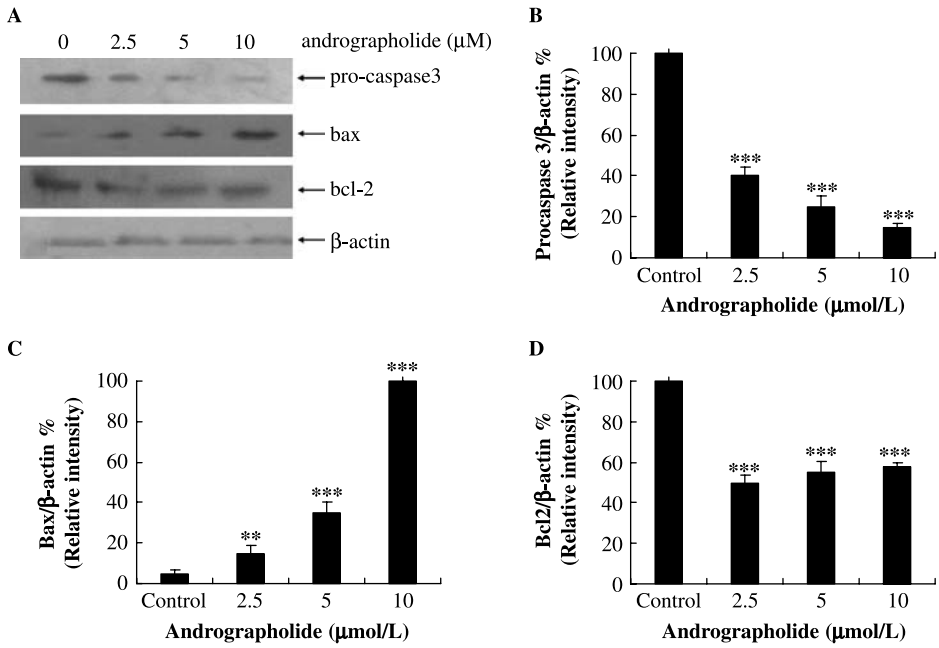

Figure 5. The levels of caspase 3, bax, and bcl-2 in PC-3 cells were detected by western blotting. Cells were treated with **1** at four concentrations (0, 2.5, 5, and 10 μmol/l) and incubated for 48 h. Detection of β-actin was carried out to confirm the equal loading of proteins. Densitometric analysis of caspase 3, bax, and bcl-2 expression represents the mean from three separate experiments. Data were normalized on the basis of β-actin levels. $*p < 0.05$ vs. control group; $**p < 0.01$ *vs.* control group; $***p < 0.001$ *vs.* control group.

3. Experimental

3.1 Plant material, cell lines, chemicals, and biochemicals

The plant of *A. paniculata* was purchased from Yantai Chinese Traditional Pharmacy (Yantai, China). Human breast (MDA-MB-435s, MDA-MB-231), hepatoma (SMMC 7721), leukemia (K562), lung (A549), gastrointestinal carcinoma (SGC 7901), and prostate (PC-3) cancer cell lines were obtained from the Chinese Academy of Science. The fetal bovine serum, RPMI 1640 medium, DMEM medium, and antibiotics were purchased from Invitrogen Co. (New York, USA). Hoechst 33258 and MTT were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Human VEGF ELISA kit was from R&D Chemical Co. (Minneapolis, USA). The caspase 3 enzyme activity detection kit and all specific monoclonal antibodies (caspase 3, bax, bcl-2) were purchased from the Beyotime Institute of Biotechnology (Haimen City, China).

Table 2. Effect of **1** on the level of VEGF in PC-3 cells.

Concentration (μmol/l)	VEGF level (pg/ml)
0	1323.15 ± 9.09
1.25	1006.36 ± 18.81*
2.5	963.50 ± 15.42*
5	945.99 ± 18.80*
10	859.45 ± 36.86*
20	689.11 ± 1.99**

The data are expressed as means ± SD of three separate experiments. $*p < 0.01$ *vs.* control group, $**p < 0.001$ *vs.* control group.

3.2 Extraction and isolation

The whole plant material was dried, powdered, and extracted according to the method of Handa and Sharma.[13] Crystals of **1** were collected by filtration and purified by recrystallization. The chemical structure was

identified by a combination of physicochemical and spectral methods (IR, UV, ^{1}H NMR, ^{13}C NMR, mass spectrum). The purity of **1** is higher than 99% (by HPLC).

3.3 Cell culture

Human breast (MDA-MB-435, MDA-MB-231) and prostate (PC-3) cancer cells were cultured in DMEM medium, while hepatoma (SMMC 7721), leukemia (K562), lung (A549), and gastrointestinal carcinoma (SGC 7901) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

3.4 Cell growth assay

Each kind of cell line was seeded onto 96-well plates at a density of 2×10^4 cells per well and incubated for 24 h. Compound **1** was added at different concentrations of 6.25, 12.5, 25, 50, 100, 150, and 200 μmol/l, respectively. Cisplatin (CDDP), as a positive control, was added at different concentrations, respectively (0.1, 0.5, 1, 2, 4, 8 μg/ml). After incubation for 48 h, 8 μl of MTT solution (5 mg/ml, the final concentration is 0.2 mg/ml) was added to each well, and incubated for another 4 h. The supernatant from the wells was gently removed and 100 μl of DMSO was added to each well. The absorbance at 570 nm was read on a universal microplate reader using 630 nm as a reference wave. The inhibition rate was calculated according to the following equation:

$$\text{Inhibition rate}\,(\%) = 100 \times (A_{\text{control}} - A_{\text{treated}})/A_{\text{control}}$$

where A_{treated} is the absorbance of the treated cells (mean value) and A_{control} is the absorbance of the untreated cells (mean value).

3.5 Hoechst 33258 staining and assessment of apoptotic cells

To detect apoptotic cells, the cells were stained with the DNA-binding dye Hoechst 33258. After the treatment on indicated conditions for 48 h, the cells were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 10 min at 4°C, and then washed with PBS. The cells were then incubated with 20 μg/ml of Hoechst 33258 for 20 min. After washing with PBS, the cells were observed under a fluorescence microscope. The cells exhibiting condensed chromatin and fragmented nuclei were scored as apoptotic cells.

3.6 DNA agarose electrophoresis

An apoptotic DNA ladder kit was used to determine the presence of DNA ladder in apoptosis induction. Briefly, the cell samples were collected in lysis buffer for 15 min on ice. The genomic DNA was subjected to electrophoresis on a 1% agarose gel containing ethidium bromide and visualized by UV.

3.7 Measurement of enzyme activity of caspase 3

The enzyme activity of caspase 3 was measured using a caspase enzyme activity assay kit. PC-3 cells were incubated with **1** at indicated concentrations and harvested at a density of 2×10^6 cells per plate by 0.25% trypsin after 14 h. After centrifuged at 600 g for 5 min, the supernatant was removed, the cells were washed with PBS, and then lysed in a lysis buffer for 15 min on ice. The protein concentration of the cell lysate was determined by the Bradford method. Twenty-five microliters of cell lysate were incubated with a caspase 3 colorimetric substrate (Ac-DEVD-*p*NA) for 4 h at 37°C. The absorbance was measured by a universal microplate reader (at 405 nm) subsequently, and the activity of caspase 3 was calculated under the description.

3.8 Western blot analysis

A general method of western blot was used to detect the protein expression. Briefly, collected cells were lysed immediately by sonication. Then the lysate was centrifuged at 12,000 *g* for 10 min, the supernatant was collected, and the protein content was assayed with a Bradford reagent. Equal amounts of protein (40 μg) were separated by 15% SDS–PAGE and then electrotransferred onto a PVDF membrane. The membranes were blocked with 8% skim milk solution in Tris-bufferred saline–Tween 20 (TBS–T) at room temperature for 2 h. After blocking, the blots were incubated with an appropriate dilution of specific monoclonal antibodies (caspase 3, bax, bcl-2) over night at 4°C. The blots were washed four times and then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The blots were again washed four times and then developed using an enhanced chemiluminescence assay. Images were collected and the bands corresponding to caspase 3, bax, bcl-2, and β-actin protein were quantitated by densitometric analysis using DigDoc100 program (AlphaEase FC software). Data of caspase 3, bax, and bcl-2 were normalized on the basis of β-actin levels. Densitometric analysis of caspase 3, bax, and bcl-2 expression represents the mean from three separate experiments.

3.9 Determination of VEGF level in PC-3 cells treated with andrographolide

PC-3 cells were treated with **1** at the concentrations of 0, 1.25, 2.5, 5, 10, and 20 μmol/l, respectively, for 48 h. Hundred microliters of supernatant were taken out for the determination of VEGF level by human VEGF ELISA kit. The assays were performed according to the manufacturer's instructions.

Acknowledgements

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