

RESEARCH ARTICLE

Induction of Cytotoxicity and Apoptosis in Human Gastric Cancer Cell SGC-7901 by Isovaltrate Acetoxyhydrin Isolated from *Patrinia heterophylla* Bunge Involves a Mitochondrial Pathway and G2/M Phase Cell Cycle Arrest

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Abstract

Background: Our previous study demonstrated cytotoxicity of a crude extract from *Patrinia heterophylla* Bunge (PHEB). In the present study, we aimed to investigate the effects of isovaltrate acetoxyhydrin (IA) isolated from PHEB on the gastric cancer cell SGC-7901, in order to explore a potential treatment for gastric cancer. **Methods:** MTT assays were employed to determine the effects of IA on cell vitality and proliferation, with monitoring of cell morphology changes and examination of apoptosis with Annexin V-PI staining. Flow cytometry was used to assess cell cycle progression and mitochondrial membrane potential. The activity of caspase 3, 9 was evaluated by spectrophotometry, and the protein levels of Bax, Bcl2 and Cyclin B1 were analyzed with Western blotting of total proteins extracted from cultured cells. **Results:** The results demonstrated direct toxicity of IA towards SGC-7901 cells. Evidence of apoptosis included blebbing and chromatin condensation. Annexin V-PI assays revealed early apoptosis, involving rapid depolarization of mitochondrial membranes and activity of caspase 3, 9 signaling pathways. Western blotting showed that Bcl2 and Bax proteins was down- and up-regulated, respectively, and cyclin B1 was up-regulated. Cell cycle analysis further indicated that IA could induce G2/M phase arrest in SGC-7901 cells. **Conclusions:** In conclusion, we believe that IA induces apoptosis of SGC-7901 cells, therefore providing a potential therapeutic agent for treatment of gastric cancer.

Keywords: *Patrinia heterophylla* Bunge - isovaltrate acetoxyhydrin - SGC-7901 - apoptosis - G2/M arrest

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Introduction

Mutouhui is a common Traditional Chinese Medicine (TCM), presented as roots and rhizomes of *Patrinia heterophylla* Bunge. This folk medicine is used to treat malignant tumors, with considerably significant effects. The biologically active components for the anti-cancer effects contain water soluble triterpenoid saponin (Zhang et al., 2008), as well as fat-soluble components of *Patrinia* (Chiu et al., 2006). Recent years, scholars found rare non-glycosides iridoids in *Patrinia*, which are presented as iridoid esters. These components are more existed in *Valeriana* Linn., showing sedative and hypnotic effects (Niamh et al., 2004). Valtrate, acevaltrate were important sedative-hypnotics chemical bulk drugs. Iridoid esters could also inhibit the growth and proliferation of human tumor cell lines (Lin et al., 2009; Lin et al., 2010), with the mechanism unknown. In addition, valtrate and its analogs are Rev-export inhibitors demonstrated other effects such as anti-HIV (Watanabe et al., 2011), and reversed tumor

resistance (Turner et al., 2012).

Isovaltrate acetoxyhydrin (IA) is a natural iridoid ester, which was first isolated and identified by Kouch U from *Valeriana alliariifolia* (Koch et al., 1985). Now, there is almost no other reports about this organic small molecular. Herein, we firstly reported its inhibition effect on gastric cancer cell SGC-7901, and then we studied the molecular mechanisms of its inhibition effect on SGC-7901, and found that it could induce apoptosis of SGC-7901 cells through Bcl2/Bax signaling pathways with the involvement of caspase and G2/M phase cell cycle arrest. Finally, we further studied the inhibitory activity of IA on other human cancer cell lines.

Materials and Methods

Plant and IA

Mutouhui (*Patrinia heterophylla* Bunge.) were purchased from Huadong medicine Co., Ltd, the producing area was Shanxi Province, People's Republic of China.

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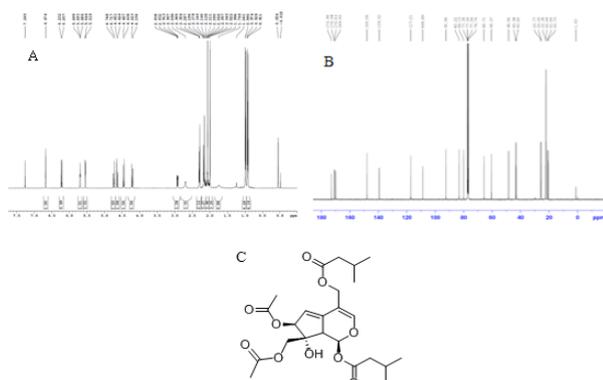


Figure 1. Isovaltrate Acetoxyhydrin (IA) and Its Nuclear Magnetic Resonance Spectra. (A) ^1H NMR of IA. (B) ^{13}C NMR of IA. ^1H NMR and ^{13}C NMR spectra were measured on Bruker DRX-400 NMR spectrometer operating at 400 MHz, and CDCl_3 was used as solvent. (C) Chemical structure of IA

IA was isolated from ethyl acetate extracts of *Patrinia heterophylla* Bunge. (PHEB), with 98% purity. The chemical structure (Figure 1C) was identified by ^1H NMR (Figure 1A) and ^{13}C NMR spectra (Figure 1B).

Biochemical reagents

DMEM, RPMI Medium 1640 (Invitrogen Life Technologies Corporation, USA), Fetal bovine serum (Hangzhou Sijiqing Biological Products Co., Ltd, China), Trypsin (Amresco, USA), MTT (Biosharp, USA), Acridine orange (AO) dye was purchased from Sigma (St. Louis, MO, USA), Primary antibodies against Cyclin B1, Bax, Bcl-2, β -actin, HRP-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), PVDF transfer membrane (PALL, USA), Casepase-3, 9 colorimetric assay kit (Nanjing KeyGen Biotech, Nanjing, China), Annexin V/PI apoptosis kit (BioVision, Mountain View, CA, USA).

Cancer cell lines and culture conditions

Human gastric cancer SGC-7901, Human acute promyelocytic leukemia NB4, Human breast cancer MCF-7, Human colon cancer COLO-205, Human oral epithelial cancer KB, Human lung cancer H1703, Human prostate cancer PC-3, and Human hepatoma HepG-2 were all obtained from Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). HepG-2 was cultured in DMEM medium and the other cells in RPMI-1640 medium, containing 10% FBS, 100 U/mL streptomycin and 100 U/mL penicillin at 37°C in humidified atmosphere with 5% CO_2 incubator.

Growth inhibition effect of IA on SGC-7901

The relations of SGC-7901 cell growth inhibition and IA concentration, exposure time was then be studied by MTT assay. Briefly, Cells were placed within 96-well culture plates (3×10^3 cells/well). After 24 h adherence, the cells were treated with 5.18, 10.4, 20.8 $\mu\text{mol/L}$ (μM) IA or 0.1% DMSO respectively for time points between 0 and 96 h. The medium was replaced at 2-day intervals. At the end of treatment, MTT (5 mg/mL) was added to each well. The cells were incubated for another 4 h, 150

μL DMSO was added to each well. Absorbance at 570 nm was measured and growth inhibition ratio was calculated. The experiment was repeated for three times.

Fluorescence microscopy examination of apoptosis

SGC-7901 cells were cultured in 6-well culture plates. Cells (5×10^5 cells/well) were incubated in RPMI-1640 medium containing 5.18, 10.4, 20.8 μM of IA for 24 h. Thereafter, the cells were stained with AO (100 $\mu\text{g/mL}$) for 15 min (Li et al., 2007), and then the nuclear morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Annexin V-PI staining for flow cytometry

The early stages of apoptosis were quantitatively analyzed with Flow cytometry (BD Biosciences, USA). Briefly, Cells (1×10^6 cells/well) were seeded in 6-well culture plates, and allowed to attach to the flask bottom over 24 h. The cells were then exposed to 2.59, 5.18, 10.4 μM IA or 0.1% DMSO for 24 h. Prior to use, 5×10^5 cells detached from each flask and washed with PBS, followed by centrifugation at 1500 rpm for 5 min. The supernatant was decanted; thereafter, the resulting pellets were resuspended in 500 μL Binding Buffer, Annexin V-FITC (5 μL) and 10 μL of propidium iodide (PI) were added to the cell suspension and this sample was well mixed followed by incubation at room temperature for 15 min in the dark. The proportion of apoptotic cells was measured by flow cytometry.

Caspase activity

To analysis caspase 3, 9 activity, the SGC-7901 cells were seeded onto 6-well cell culture plates at 3×10^6 cells/well then incubated with 10.4, 20.8 μM of IA, for 1 h prior to treatment with IA for 24 h. Cytosolic fraction proteins were prepared and determined as described previously (Reers et al., 1991; White et al., 1996). 100 μg of cytosol proteins were incubated with caspase 3, 9 specific substrates for 1 h at 37°C . The caspase activity was determined by measuring OD at 405 nm.

Mitochondrial membrane potential ($\Delta\psi_m$) measurement

The mitochondrial membrane potential changes were analyzed by flow cytometry using a mitochondriaselective dye JC-1 (Smiley et al., 1991). JC-1 is a green-fluorescent monomer at low membrane potential, with the membrane potential of energized mitochondria promoting the formation of red-fluorescent J-aggregates. The ratio of red to green fluorescence of JC-1 depends only on the membrane potential, with a decrease being indicative of membrane depolarization (Yang et al., 2004; Lee et al., 2008). For the JC-1 analysis, SGC-7901 cells (1×10^6 cells/well) were seeded in 6-well culture plates, and treated with 5.18, 10.4, 20.8 μM of IA for 24 h at 37°C , respectively. The control cells were grown in medium containing of 0.1% DMSO. Thereafter, the cells were collected by centrifugation, washed with PBS, and thereafter, cells were stained with JC-1 (2.5 $\mu\text{g/mL}$) for 15 min at 37°C , washed with PBS, and analyzed for changes in membrane potential by flow cytometer (Becton, Dickinson and Company, USA).

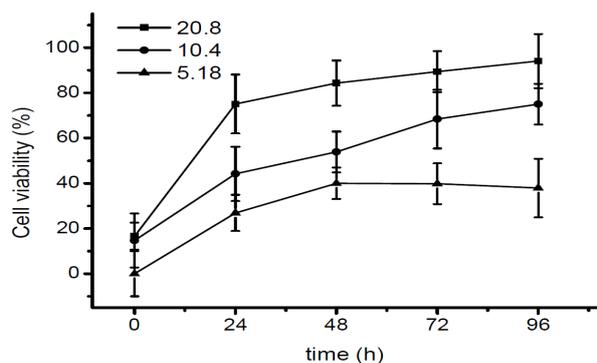


Figure 2. IA Inhibited Proliferation Against SGC-7901. SGC-7901 cells were treated with 0.1% DMSO and 5.18, 10.4, 20.8 μM IA for the indicated time periods, and the inhibitory ratio was measured. The inhibitory ratio shown are mean \pm SD (n=3 of individual experiments)

Cell cycle analysis

Cell cycle progression was monitored using flow cytometry. For cell cycle analysis, SGC-7901 cells (1×10^6 cells/well) were incubated with 5.18, 10.4, 20.8 μM IA or 0.1% DMSO in 6-well culture plates for 24 h. The cells were harvested by trypsinization, washed with phosphate buffered saline (PBS) and fixed in ice-cold 75% ethanol. The fixed cells were harvested by centrifugation and re-suspended in 500 μL of PBS containing 50 mg/mL RNase. After 30 min incubation at 37 $^{\circ}\text{C}$, the cells were stained with 50 mg/mL propidium iodide (PI) (Sigma, St. Louis, MO, USA) at 4 $^{\circ}\text{C}$ in dark for 30 min. Then the samples were analyzed by FACScan flow cytometry (Becton, Dickinson and Company, USA).

Western-blot analysis

SGC-7901 Cells were treated with 0.1% DMSO (control) and with different concentrations of IA (10.4, 20.8, 31.2 μM). After 24 h of treatment, floating cells and adherent cells were collected and washed three times with ice-cold PBS and harvested in lysis buffer. The lysate was centrifuged at 13,000 rpm for 10 min at 4 $^{\circ}\text{C}$, and the supernatant was collected. The protein concentration of lysates was determined by Bradford method (Bradford, 1976). The protein extracts (70 μg) from each sample were separated by electrophoresis on 8~12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDSPAGE) and transferred to a PVDF membrane. The membranes were incubated in 1 X PBS containing 5% non-fat dry milk for 1 h to block non-specific binding sites. The blots were incubated with 1:200 dilutions of rabbit polyclonal antibody for Bcl-2, Bax, Cyclin B1 overnight at 4 $^{\circ}\text{C}$. The blots were washed three times with high salt buffer (2.18 g NaH_2PO_4 , 7 g Na_2HPO_4 , 23.37 g NaCl and 200 μL Triton X-100 in 400 mL distilled water) followed by low salt buffer (2.18 g NaH_2PO_4 , 7.03 g Na_2HPO_4 , 1.2 g NaCl and 200 μL Triton X-100 in 400 mL distilled water). The blots were then incubated with 1:5000 dilutions of horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. After extensive washes with high and low salt buffers, the immunoreactive proteins were visualized using enhanced chemiluminescence (ECL) detection reagents (Sigma, USA), and blots were exposed to radiographic film.

Growth inhibition of IA on other cancer cell lines

MTT assay was used to measure the cell viability as previously described (Wahab et al., 2009). Briefly, NB4, MCF-7, COLO-205, KB, H1703, PC-3, and HepG-2 cells ($2 \sim 3 \times 10^3$ cells/well) were seeded in 96-well plate and cultured for 24 h, then treated with IA ranged from 1 to 40 μM for 72 h. Then, MTT was added and incubated for another 4 h. The purple formazan crystals were dissolved in 150 μL DMSO (Dimethyl sulfoxide). Finally the plates were read on a Microplate Reader (Model 680, BIO-RAD, USA) at 570 nm. The percentage of cell growth inhibition was calculated as follow: inhibition ratio % = $[\text{A570 (control)} - \text{A570 (IA)}] / \text{A570 (control)} \times 100\%$, and the half inhibition concentration (IC_{50}) of IA on the above tumor cell lines was calculated. The experiment was repeated three times, IC_{50} was expressed as mean \pm SD.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 soft-ware (Chicago, US). All results were expressed as mean \pm SD, and the Dunnett t test was used to evaluate statistical significance. In all statistical analyses, p values less than 0.05 were considered statistically significant.

Results

IA inhibited proliferation of SGC-7901 cells

With MTT assay, we examine the effects of IA on cell viability towards SGC-7901 cells. SGC-7901 cells were treated with IA at 5.18, 10.4, and 20.8 μM . The cell viability was determined by MTT method at 0, 24, 48, 72 and 96 h later. As shown in Figure 2, IA reduces SGC-7901, inhibition effect increased in a dose- and time-dependent manner.

IA induced SGC-7901 apoptosis

Apoptosis was confirmed by examining the nuclear morphology with AO staining. As shown in Figure 3A, after treatment with IA or 0.1% DMSO (control) for 24 h, the nuclei in control group were stained homogeneously with AO, whereas exposure to IA resulted in marked chromatin condensation, membrane blebbing, and nuclear fragmentation in SGC-7901 cells, the hallmarks of apoptosis. These morphological features changed in a dose-dependent manner.

The Annexin V-PI assay revealed the induction of apoptosis in SGC-7901 cells started within 24 h IA treatment. Negative control cells showed 0.5% in early apoptosis, whereas after treatment with 2.59 μM IA, cells showed 17.5% in early apoptosis. With the treatment dose increased to 5.18 and 10.4 μM , the percentage of early apoptotic cells continued to increase substantially (Figure. 3B). This result provided evidence that treatment of SGC-7901 cells with IA showed the presence of early apoptosis on SGC-7901 in a concentration-dependent manner.

IA induced apoptosis through caspase activation

To examine whether the caspase activation was involved in IA-induced apoptosis, we examined the activity of caspase 3, 9 and expression levels of Bcl-2

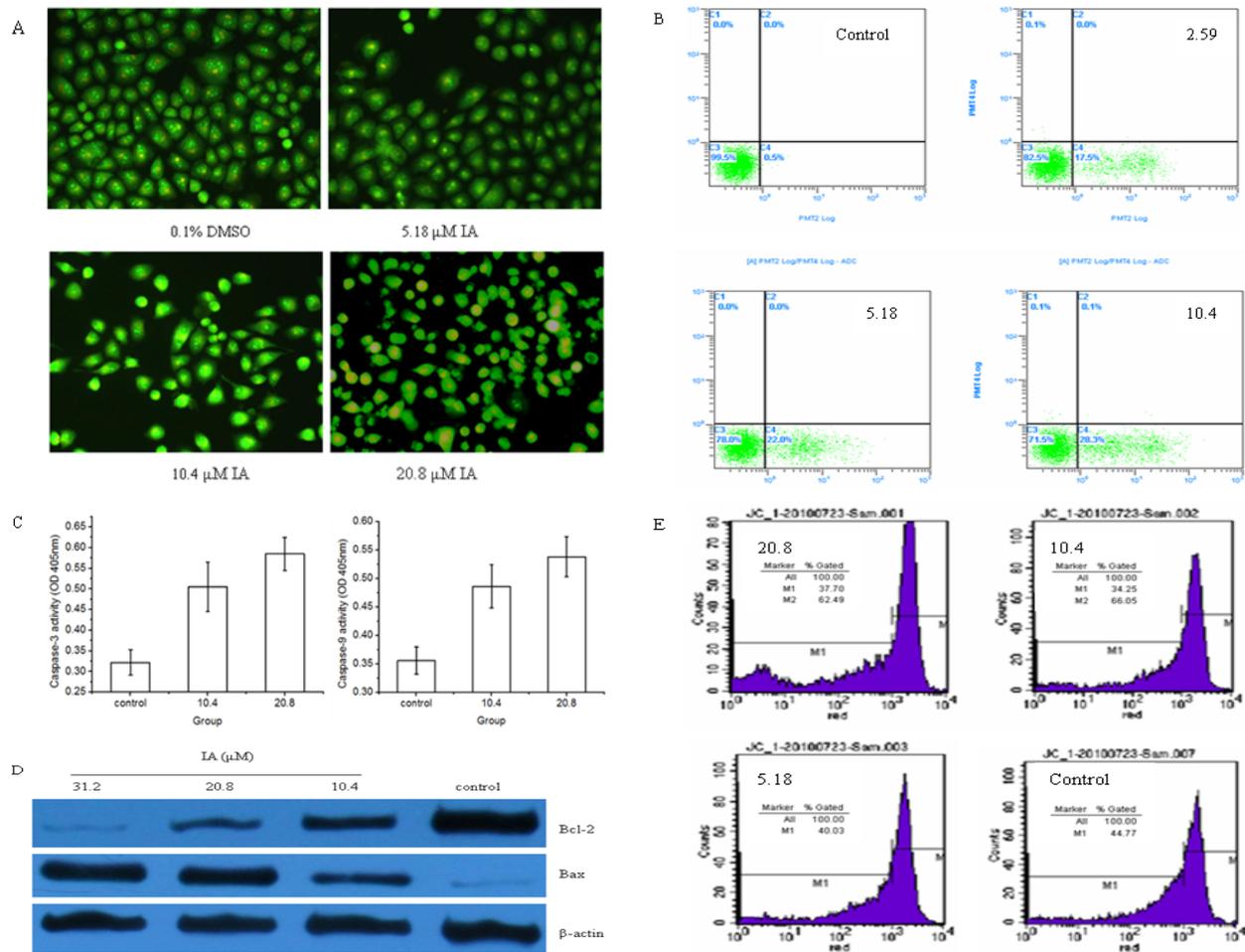


Figure 3. IA Induced Apoptosis in SGC-7901 Cells Through Activated Caspases and Increased Bax Expression, and It Disrupts Normal Mitochondrial Membrane Potential. (A) SGC-7901 cells were treated with IA (5.18, 10.4, 20.8 μ M) for 24 h then the morphological changes were examined by AO staining. (B) SGC-7901 cells were incubated with IA (2.59, 5.18, 10.4 μ M) for 24 h and apoptosis was analyzed by Annexin V-PI staining. (C) SGC-7901 cells were treated with IA (10.4, 20.8 μ M) and 0.1% DMSO (control) for 24 h, the activity of caspase 3, 9 was measured by spectrophotometry at 405 nm. (D) SGC-7901 cells were treated with IA (10.4, 20.8, 31.2 μ M) for 24 h, then collected and lysed, Bcl-2 and Bax were determined by Western blot analysis. β -Actin was used as internal control. (E) SGC-7901 cells were treated with IA (5.18, 10.4, 20.8 μ M) and 0.1% DMSO (control) for 24 h and were then stained with JC-1 to access the mitochondrial transmembrane potential by flow cytometry. Results are representative of one of three independent experiments

Table 1. Inhibition Effects of IA on Various Human Cancer Cell Lines for 72 h

Cell type	IC ₅₀ \pm SD (μ M)
PC-3	2.49 \pm 0.44
NB-4	4.79 \pm 0.21
H1703	8.93 \pm 0.30
COLO-205	11.13 \pm 0.32
HepG-2	8.85 \pm 0.12
KB	13.34 \pm 0.52
MCF-7	7.44 \pm 0.11

family proteins (Bax, Bcl-2). Firstly, we investigated the IA-treated SGC-7901 cells for the caspase 3, 9 activities by caspase activity assay. Both caspase 3 and caspase 9 activities increased at IA concentrations of 10.4 and 20.8 μ M after 24 h treatment with IA (Figure 3C), denoting caspases involvement in IA-triggered apoptosis. As shown in Figure 3D, Bcl-2 was down-regulated and Bax was up-regulated after 24 h treatment with IA on SGC-7901 cells at a concentration of 10.4, 20.8, 31.2 μ M.

IA induced disruption of mitochondria

Mitochondrial changes, including variations in mitochondrial membrane potential ($\Delta\psi$ m), are the key events during drug-mediated apoptosis (Zamzami et al., 1995). To delineate this mechanism, the JC-1 was chosen to determine whether IA induces alterations in the $\Delta\psi$ m. As compared to untreated cells, 5.18 μ M IA-treated cells exhibited a decrease in the population of cells with J-aggregates (40.03%) and subsequent increase in the monomeric form (60.31%), indicating rapid depolarization of the mitochondrial membranes. The alterations in the $\Delta\psi$ m were evident in a concentration-dependent manner, as shown in Figure 3E.

IA induced G2/M phase arrest and change of related protein expression

Cell cycle distribution of IA-treated cells was analyzed by PI staining and flow cytometry. SGC-7901 cells were exposed to IA at a series of concentrations for 24 h, Figure. 4A, B demonstrated that IA resulted in concentration-dependent accumulation of SGC-7901 cells in the G2/M

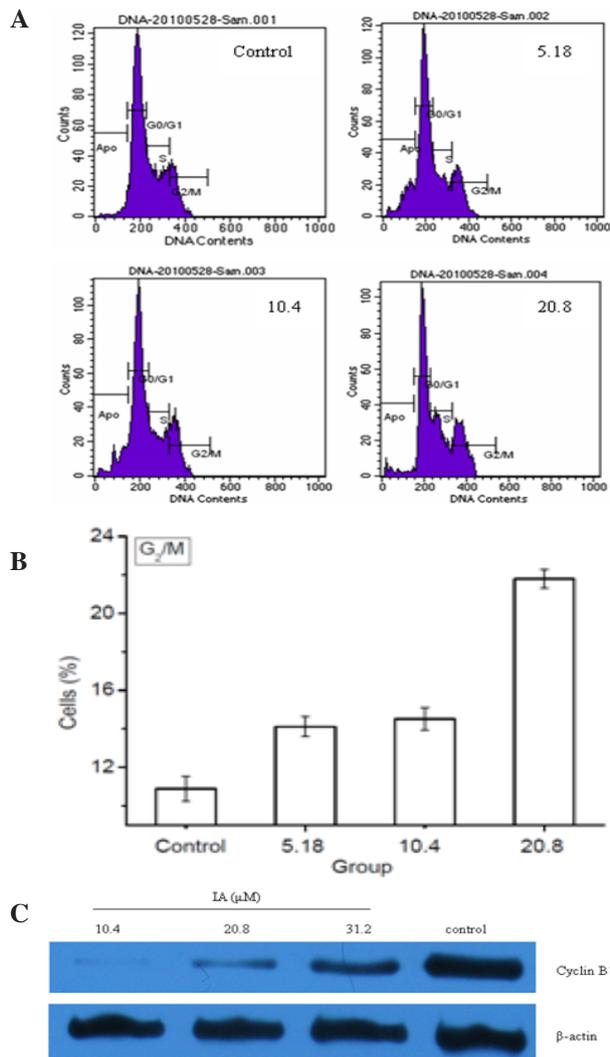


Figure 4. IA Induced G2/M Phase Arrest in SGC-7901 Cells. (A, B) IA caused G2/M phase arrest. Cells were treated with 5.18, 10.4, 20.8 μM IA and 0.1% DMSO for 24 h, then stained with PI and subjected to flow cytometric analysis. The values shown are mean \pm SD (n=3 of individual experiments). (C) Changes of the Expression of Cyclin B1 in SGC-7901 Cells with IA Treatment. SGC-7901 cells were treated with 10.4, 20.8, 31.2 μM IA and 0.1% DMSO for 24 h, then were harvested and lysed for detection of Cyclin B1 and the internal control β -actin

phase. The percentages of G2/M population increased from 13.92% to 22.83% in a concentration from 5.18 to 20.8 μM IA -exposed cells, while only about 11.69% cells were in G2/M phase in non-treated group (Figure 4A). To expose the mechanisms of IA induced G2/M arrest, we investigated the protein expressions of Cyclin B1 by Western blotting, as shown in Figure 4C, IA causes a dose-dependent increase in cyclin B1. Thus, these results indicate that IA can induce cells arrest in G2/M phase through affecting the G2/M related regulatory protein expression.

IA inhibits the proliferation of other cancer cell lines

We further found that IA inhibit the proliferation on different other cancer cell lines with MTT assay. IA showed inhibition effects towards NB4, MCF-7, COLO-205, KB, H1703, PC-3, and HepG-2. IC_{50} of IA on seven tested human cancer cell lines was shown in Table 1.

Discussion

Mutouhui is commonly used in TCM to clear heat, remove dampness, stop bleeding, arrest leucorrhea, and prevent malaria treat. Our previous studies have shown that Mutouhui fat-soluble effective part has significant cytotoxicity against various cancer cell lines (Yang et al., 2011). However, few studies have been carried out on the pure liposoluble active compounds derived from this Chinese herb. In this study, we investigated the anti-cancer activity of Isovaltrate acetoxyhydrin (IA), which was isolated from *Patrinia heterophylla* Bunge., and we also studied its underlying mechanism against SGC-7901.

MTT assay revealed that IA induced a significantly inhibit cell proliferation against SGC-7901 cells in a dose- and time-dependent manner. Thus, in the present study, we focused on investigating the molecular mechanism of IA against SGC-7901 cells.

We firstly confirmed the involvement of apoptosis in SGC-7901 cells upon treatment with IA in a dose-dependent pattern. Examining the nuclear morphology with AO staining and results from the Annexin V-PI assay all confirmed this.

The mitochondria-mediated signaling pathway plays a pivotal role in apoptosis, the event of the change in membrane permeability and subsequent loss of membrane potential is involved in it (Kim et al., 2005). Here, the changes in mitochondrial membrane potential were analyzed by the JC-1 dye, IA-induced apoptosis is mediated by a rapid dissipation of $\Delta\psi\text{m}$ within 24 h (Figure 3E), which changes mitochondrial permeability that triggers activation of the caspase, in this study, we observed the induction of caspase 3, 9 specific activities by IA. We suggested that IA-induced apoptosis in SGC-7901 cells is mediated through the activation of caspase cascades, and mitochondria-dependent pathway.

Bcl-2 family members have been reported in the regulation of mitochondria-mediated apoptotic pathways (Park et al., 2008). Among these, Bcl-2 can stabilize the mitochondria permeability; in contrast, Bax increases the membrane permeability through interactions with pore proteins on the mitochondrial membrane (Evans et al., 1994). It is also reported that an increased ratio of Bax/Bcl-2 triggered the mitochondria-mediated apoptosis (Zamzami et al., 1998). Here, we observed that IA induced down-regulation of Bcl-2 and up-regulation of Bax (Figure 3D). It suggested that IA induced apoptosis through mitochondria pathway was due to, at least in part, the disruption of a balance between Bax and Bcl-2.

Following apoptosis analysis, we further investigated the influence of IA on cell cycle arrest. Cell cycle arrest is one of the typical responses exhibited by proliferating eukaryotic cells when exposed to a variety of DNA damaging agents (Szegezdi et al., 2009). Cell cycle arrest gives an opportunity for DNA damaged cells to either undergo DNA repair mechanisms or follow the apoptotic pathway (Zou et al., 1999). Targeting cell cycle and apoptotic pathways has emerged as an attractive approach for cancer treatment. To determine if the inhibition of IA on SGC-7901 cells involved the cell cycle arrest, we examined cell cycle phase distribution of the treated

cells by flow cytometry. We observed that IA induced a G2/M cell cycle arrest at 24 h of treatment. Cell cycle arrest at G2/M phase has been suggested to be involved in apoptosis induction in a variety of proliferating cells (Kelly et al., 2011). To better understand the mechanisms of IA-induced G2/M arrest, we examined G2/M phase related regulatory proteins. As shown in Figure 4C, IA up-regulated the expression of Cyclin B1.

In this current study, the results that we gather demonstrated that IA induced apoptosis of SGC-7901 cells through Bcl2/Bax signaling pathways with the involvement of caspases and G2/M phase cell cycle arrest.

To prove whether there is a broad spectrum of human cancer cell lines, MTT assay was employed, our results also revealed that IA possesses different degrees of inhibition effects against various human cancer cell lines including NB4, MCF-7, COLO-205, KB, H1703, PC-3, and HepG-2 (Table 1).

The current findings also warrant further research on IA as a novel chemotherapeutic agent for gastric cancer intervention including studies in nude mice Xenografts models.

Acknowledgements

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