Full-length article

Oridonin induces apoptosis via PI3K/Akt pathway in cervical carcinoma HeLa cell line[1]

Hong-zhen HU[2], Yue-bo YANG[3], Xiang-dong XU[4], Hong-wei SHEN[5], Yi-min SHU[6], Zi REN[7], Xiao-mao LI[3], Hui-ming SHEN[3], Hai-tao ZENG[3,8]

[2]*Department of Gynecology and Obstetrics, Yijishan Hospital,Wannan Medical College, Wuhu 241001, China;* [3]*Department of Gynecology and Obstetrics, the Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China;* [4]*Department of Surgery, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China;* [5]*Department of Gynecology and Obstetrics, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China;* [6]*Department of Obstetrics and Gynecology, IVF Program, Stanford University Medical Center, Palo Alto, CA 94304, USA;* [7]*Department of Gynecology and Obstetrics, Guangdong Provincial People's Hospital, Guangzhou 510080, China*

Key words

Abstract

Aim: To investigate the apoptosis-inducing effect of oridonin, a diterpenoid isolated from *Rabdosia rubescens*, in the human cervical carcinoma HeLa cell line. **Methods:** A morphological analysis, nuclear condensation, and fragmentation of chromatin were monitored using Hoechst 33342 staining. Cell viability was assessed using the 3-(4, 5-dimethylthiazol-(2)-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Cell apoptosis and the apoptosis-related activation in the HeLa cell line were evaluated by flow cytometry and Western blotting. **Results:** Oridonin suppressed the proliferation of the HeLa cell line in a dose- and time-dependent fashion. Oridonin treatment downregulated the activation of protein kinase B (Akt), the expression of forkhead box class O (FOXO) transcription factor, and glycogen synthase kinase 3 (GSK3). Oridonin also induced the release of cytochrome *c* accompanied by the activation of caspase-3 and poly-adenosine diphosphate–ribose polymerase cleavage. In addition, Z-D(OMe)-E(OMe)-V-D(OMe)-FMK (z-DEVD-fmk), an inhibitor of caspases, prevented caspase-3 activation and abrogated oridonin-induced cell death. Finally, oridonin treatment of the HeLa cell line downregulated the expression of the inhibitor of the apoptosis protein. **Conclusion:** Our results showed that oridonin-induced apoptosis involved several molecular pathways. Oridonin may suppress constitutively activated targets of phosphatidylinositol 3-kinase (Akt, FOXO, and GSK3) in the HeLa cell line, inhibiting the proliferation and induction of caspase-dependent apoptosis.

Introduction

Cervical cancer is the second most common cancer in women worldwide, but it is the leading malignancy among women in many developing countries[1]. Traditional herb formulations have been re-evaluated by clinicians because these medicines have fewer side-effects and are more suitable for long-term use compared with chemically synthesized medicines. New therapeutic strategies must be evaluated to improve survival. Clinical responses can be improved by identifying therapies that are particularly effective in activating apoptosis *in vivo* among cervical carcinoma cells.

Oridonin, a traditional herb formulation, has been successfully used for women's diseases. Oridonin is a natural, biologically active substance isolated from *Rabdosia rubescens*[2], one of several Chinese herbs in the once popular dietary supplement called PC–SPES. PC–SPES, which contains 7 Chinese and 1 North American herbal supplements, has potent inhibitory activities against a variety of cancer cells[3]. The use of the entire plant of this Chinese herb

purportedly offers digestive benefits, and anecdotal evidence suggests its efficacy in the treatment of esophageal carcinoma with little toxicity[4]. Oridonin, the main active component of *Rabdosia rubescens*, is a tetracyclic diterpenoid compound[5]. Tetracyclic diterpenoids are natural, biologically-active substances with various physiological and pharmacological properties, including antitumor activities[6].

In recent years, apoptosis has emerged as the major mechanism by which anticancer agents eliminate preneoplastic or neoplastic cells. Oridonin induces apoptosis in neoplastic cells from the breast[7], liver[8], lung[9] and osteosarcoma[10]. Oridonin causes apoptosis of cancer cells by inhibiting the activation of NF-κβ, signal transducer and activator of transcription 3 and protein kinase B (Akt)[11]. Oridonin also downregulates the expression of various genes that NF-κβ regulates, including Bcl-2, cyclooxygenase-2 (COX-2), cyclinD1, and adhesion molecules[12]. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays an important role in various cellular processes including cell growth, survival, and motility[13]. In addition, the abnormal expression of the PI3K/Akt pathway has been reported for many human tumors[14]. Phosphatase and tensin homolog deleted on chromosome 10 and Src homology 2-containing 5' phosphoinositol phosphatases, which serve as negative regulators of the PI3K/Akt pathway, are frequently mutated in a number of human cancers[15,16]. The principal target of the PI3K pathway is the activation of Akt (protein kinase B), which directly effects apoptosis by targeting the pro-apoptotic Bcl-2-related protein Bad. In addition, PI3K plays a role in regulating the proliferation of many cell types[17]. Akt prevents apoptosis by generating anti-apoptotic signals by phosphorylating Bad, glycogen synthase kinase 3 (GSK3), and caspase-9, and by activating transcriptional factors, such as forkhead box class O (FOXO)-1 and NF-κβ [18–22]. Therefore, the deregulated signaling functions in the PI3K/Akt pathway make it a potential target for chemotherapeutic agents.

Cervical carcinoma accounts for about 10% of all newly diagnosed cancers in women worldwide[1]. Cervical carcinoma in patients with a poor prognosis is characterized by rapid cellular proliferation and strong expression of anti-apoptotic genes. These features may be due to incomplete cell cycle arrest and apoptosis resistance to conventional therapies. The purpose of this study was to explore the mechanism of the chemopreventive effects of oridonin in the human cervical carcinoma HeLa cell line. We examined the involvement of Akt and its substrates FOXO and GSK3 in HeLa cells, the role of members of the Bcl2 and inhibitor of apoptosis protein (IAP) families, the effect of oridonin on

mitochondrial-mediated apoptosis involving cytochrome *c* release, and the activity of caspases-3 and -9 in propagating death signals.

Materials and methods

Reagents The Kunming Institute of Botany, the Chinese Academy of Sciences (Kunming, China) provided oridonin (purity 99.7%) isolated from the aerial parts of *Rabdosia rubescens*. The method for isolation of oridonin was the same as that described previously[23]. Stock solution for oridonin (10 mmol/L) was prepared in DMSO and protected from light at 4 °C, then diluted to a appropriate concentration with medium immediately before use. Bisbenzimide (Hoechst H33342), ethidium bromide, rhodamine, monoclonal mouse anti-β-actin antibody, and heat-inactivated fetal bovine serum were purchased from Sigma-Aldrich (St Louis, MO, USA). Z-D(OMe)-E(OMe)-V-D(OMe)-FMK (z-DEVD-fmk), (EMD Chemicals, Inc. San Diego, CA, USA) were prepared in 100 mmol/L in DMSO stock solution. Propidium iodide was purchased from Bio Basic (Toronto, Canada). *L*-Glutamine (200 mmol/L) and a mix of penicillin-streptomycin (10000 IU/mL and 10000 mg/mL, respectively) were purchased from Mediatech (Herdon, VA, USA). Antiphospho-Akt antibodies (Ser$_{473}$, Thr$_{308}$), anti-poly-adenosine diphosphate–ribose polymerase (PARP) and anti-caspase-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-GSK3, anti-phospho-Forkhead Drosophila homolog rhabdomyosarcoma like 1 (FKHRL1), anti-cleaved caspase-3, anti-cIAP1, anti-X-linked inhibitor of apoptosis protein (XIAP), and antisurvivin antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). The anticaspase-9 antibody was obtained from R&D Systems (Minneapolis, MN, USA). Secondary horseradish peroxidase-labeled goat antimouse and antirabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Polyvinylidene fluoride membrane was purchased from Millipore (Billerica, MA, USA). Plastic dishes were obtained from Corning-Costar (Cambridge, MA, USA), and all other tissue culture plastics were from Falcon Plastics (Los Angeles, CA, USA). The Bio-Rad protein assay based on the Bradford dye-binding procedure was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The enhanced chemiluminescence detection system was purchased from Amersham (Piscataway, NJ, USA). All other reagents were of analytical reagent quality.

Cell culture and treatment The clonal human cervical carcinoma HeLa cells, obtained from American Type Culture

Collection (ATCC, Manassas, VA, USA), were grown in Dulbecco's modified Eagle's medium supplemented with 10% (*v/v*) fetal calf serum, 100 µg/mL streptomycin, and 100 U/mL penicillin. Cultures were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO_2. The cells were exposed to oridonin at different concentrations and for different times. The cells grown in medium containing equivalent amounts of DMSO without oridonin served as the controls.

Cell viability assay The viability of the HeLa cells was assessed by using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which was based on the reduction of MTT by the mitochondrial dehydrogenase of intact HeLa cells to a purple formazan product. A total of 2×10^4 HeLa cells were plated on a 96-well plate. After incubation for 24 h, they were treated with oridonin at different concentrations for different intervals. After treatment, the medium containing oridonin were carefully aspirated. 100 µL of 0.5 mg/mL MTT was added to the cell culture medium in the wells and incubated for 4 h, as described previously[24]. We added 100 µL of 1% SDS to each well after 4 h incubation. The plates were covered with aluminum foil and kept in an incubator for 12 h to allow the formed formazan crystals to dissolve. An ELISA reader was used to measure the absorbance at 570 nm, and the half maximal inhibitory concentration (the concentration that caused 50% inhibition of cell proliferation) was assessed by using the Bliss method[9].

Hoechst 33342 staining Apoptotic morphology was studied by staining the cells with Hoechst 33342. The HeLa cells were seeded on coverslips on a 6-well plate and treated with 25 µmol/L oridonin. After 24 h, the cover glasses were carefully washed with phosphate-buffered saline (PBS) and stained with 20 µg/mL Hoechst 33342 for 10 min. Thereafter, the HeLa cells were washed in PBS and observed under a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany).

Flow cytometry analysis Flow cytometry (FCM) was used to quantitatively assess the rates of apoptosis. A total of 1×10^6 HeLa cells were plated onto 10 cm tissue culture dishes 1 d before treatment. They were then treated with 25 µmol/L oridonin for 6, 12, and 24 h. At the indicated time, floating and attached HeLa cells were harvested, washed with PBS, fixed in 70% ethanol overnight at 4 °C, and stained with 50 mg/mL propidium iodide. The sub-G_1 peak (DNA content <2 N) was measured by means of FCM (FACScan; Becton Dickinson and Company, Franklin Lakes, NJ USA) and analyzed by using CellQuest software (BD, Franklin Lakes, NJ USA, USA).

Assay for cytochrome *c* release The release of cytochrome *c* from mitochondria was assayed as described elsewhere[4]. In brief, the HeLa cells were treated with and without oridonin and centrifuged at $1000 \times g$. The cell pellets were resuspended in 5 volumes of a hypotonic buffer containing 20 mmol/L HEPES-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L DL-Dithiothreitol (DTT), 20 µg/mL leupeptin, 10 µg/mL aprotinin, and 250 mmol/L sucrose, and incubated for 15 min on ice. The HeLa cells were homogenized by passing them 15–20 times through a 22 gauge, 1.5 inch-long needle. The lysates were centrifuged at $1000 \times g$ for 5 min at 4 °C to create a pellet of nuclei and unbroken cells. The supernatants were collected and centrifuged at $12\,000 \times g$ for 15 min. The resulting mitochondrial pellets were resuspended in lysis buffer. The supernatants were transferred to new tubes and centrifuged again at $12\,000 \times g$ for 15 min, and the resulting supernatants, which represented cytosolic fractions, were separated. We analyzed 20–25 µg of protein from the cytosolic fraction of each sample by performing immunoblotting using an antibody against cytochrome *c*.

Measurement of mitochondrial membrane potential After a fluorescent probe was incorporated, the HeLa cells were incubated for up to 4 h with or without 25 µmol/L oridonin. At first, 1×10^6 HeLa cells/mL was incubated with 10 µmol/L rhodamine 123 for 10 min at 37 °C. At the end of the incubation, the cells were washed twice with PBS, harvested by means of centrifugation, and resuspended in 1.5 mL PBS. The fluorescent intensity of each cell suspension was measured at an excitation wavelength of 480 nm and an emission wavelength of 530 nm in a L15B fluorescence spectrophotometer (Perkin-Elmer, Waltham, MA, USA). The fluorescence intensity was recorded in arbitrary units representing the mitochondrial transmembrane potential.

Western blot analysis The HeLa cells were treated with oridonin and lysed, as previously described[25]. In brief, the cell pellets were resuspended in phosphorylation lysis buffer (0.5%–1.0% TritonX-100, 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 200 µmol/L sodium orthovanadate, 10 µmol/L sodium pyrophosphate, 100 µmol/L sodium fluoride, 1.5 mmol/L magnesium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin). Protein concentrations were assessed using the Bradford assay before the samples were loaded. Equal amounts of proteins were separated by means of SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed with different antibodies and visualized using an enhanced chemiluminescence method.

Statistical analysis Data were expressed as mean±SD. Statistical analysis was performed by applying a one-way

ANOVA followed by the Student's *t*-test. *P*-values of less than 0.05 indicated statistical significance.

Results

Cytotoxicity of oridonin in HeLa cells and apoptosis of HeLa cells When we investigated the effect of oridonin on HeLa cell survival, the cells were treated for 24 h in medium containing various oridonin concentrations (5–50 μmol/L). The cells were counted by performing MTT studies. Oridonin had a potent cytotoxic effect in HeLa cells in a dose- and time-dependent manner and was expressed as a percentage of cell viability (Figure 1A). The survival rate of the HeLa cells treated with 25 μmol/L oridonin started to decrease when treated for 6 h and sharply dropped after 12 h incubation (Figure 1B). When we examined several hallmarks of apoptosis (nuclear chromatin condensation and fragmentation of DNA by Hoechst 33342), oridonin induced apoptosis in human HeLa cells. In contrast to the control cells, the cells exposed to 25 μmol/L oridonin provoked nuclear chromatin condensation and fragmentation (Figure 2). On morphological examination, the oridonin-treated cells had
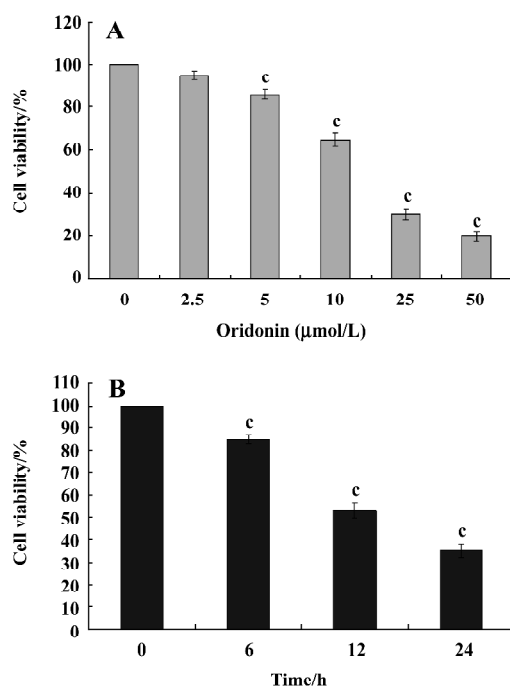

Figure 1. Cytotoxicity of oridonin on human HeLa cells. HeLa cells were incubated with oridonin at concentrations of 0, 2.5, 5, 10, 25, and 50 μmol/L, respectively, for 24 h (A). Cells were incubated with 25 μmol/L oridonin for various periods (B). At the end of incubation, the cell survival rate was determined by MTT assay. Cytotoxicity is expressed as the percentage of cell survival compared with the control. Data are expressed as mean±SD. *n*=3–5.
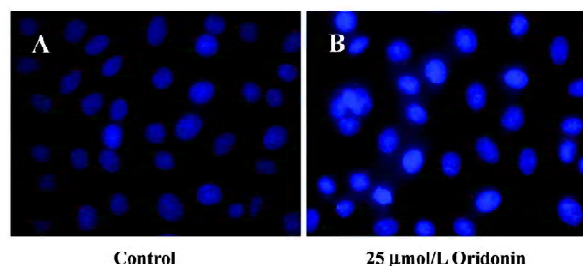

Figure 2. Hoechst 33342 fluorescence staining to detect apoptosis in cervical carcinoma cells treated with 25 μmol/L oridonin for 24 h (B) compared with untreated cells (A).

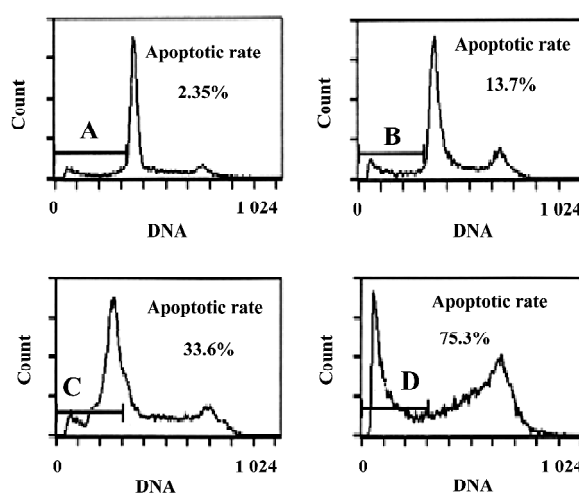**Figure 3.** After being cultured in medium alone (A) or treated with 25 μmol/L oridonin for 6 h (B), 12 h (C), and 24 h (D), HeLa cells were harvested and washed with PBS, fixed with ice-cold 70% ethanol, stained with propidium iodide, and treated with RNase A. DNA content was measured during FCM. Apoptotic population was measured as the percentage of total cell populations with sub-G_1 DNA content. Results shown represent 5 independent experiments.

changes typical of apoptosis, such as cell shrinkage, nuclear fragmentation, and formation of apoptotic bodies. When we quantified the apoptotic cells after oridonin treatment with FCM and propidium iodide staining, we identified apoptotic nuclei in the subdiploid region (sub-G_1 peak) of the histograms. Figure 3 shows the rates of apoptosis in the control group compared with the cells treated with 25 μmol/L oridonin for 6, 12, and 24 h. These results suggested that oridonin treatment may suppress the growth of cervical carcinoma cells by inducing apoptosis.

Induced dephosphorylation of Akt and its substrates in HeLa cells Several lines of evidence appear to suggest that Akt may be a critical target for the discovery of novel anticancer drugs[26]. Oridonin inhibits the activation of Akt in

other cell systems[27]. When we determined the expression and phosphorylation level of Akt in cervical carcinoma cells after treatment with various concentrations of oridonin, Akt was constitutively activated in HeLa cells, as determined by the expression of phosphorylated Akt at Ser_{473} and Thr_{308} (Figure 4A). Oridonin treatment dephosphorylated Akt at both sites. These findings suggested that Akt plays an important role in the survival of cervical carcinoma HeLa cells and their inactivation lead to their growth inhibition and apoptosis.

The forkhead family of transcription factors is reported as a downstream target of Akt-mediated apoptosis in other systems. Active transcription factors in the FKHR family promote the transcription of genes in cell cycle arrest and apoptosis[28]. One mechanism by which Akt promotes cell survival is by phosphorylating the transcription factors of the FKHR family, inactivating them, and preventing apoptosis. Because forkhead proteins are implicated as both Akt targets and a potential regulator of Fas ligand[29], we studied the status of forkhead phosphorylation in oridonin-treated and non-treated cervical carcinoma cell lines by performing Western blotting. The constitutive phosphorylation of FKHR was seen in the HeLa cell line (Figure 4B). Treatment with oridonin inhibited this phosphorylation in a dose-dependent manner.

We further investigated the effect of oridonin on the activation of GSK3 in the HeLa cell line. GSK3 is reported as a target of Akt and is involved in the promotion of cell survival [30]. The cervical carcinoma HeLa cell line treated with oridonin showed dephosphorylation of GSK3 (Figure 4C). These results suggested that HeLa cells expressed a high level of constitutive Akt and its downstream targets FOXO transcription factors and GSK3. The treatment of oridonin inactivated Akt and its downstream targets and thereby inhibited

HeLa cell growth.

Changes of mitochondrial membrane potential and release of cytochrome *c* from mitochondria Previous studies have demonstrated that Akt activity inhibits the release of cytochrome *c* from mitochondria after UV irradiation[25]. Therefore, we sought to determine whether oridonin treatment causes the release of cytochrome *c* from mitochondria to the cytosol. This release was examined with Western blot analysis in HeLa cells after treatment with 25 μmol/L oridonin for 24 h. Cytosolic-specific, mitochondria-free lysates were prepared. Equal amounts of protein from the control and oridonin-treated samples were separated on SDS-PAGE and immunoblotted with an antibody against cytochrome *c*.

Oridonin induced the translocation of cytochrome *c* from the mitochondria to the cytosol in the HeLa cells (Figure 5A). When we further tested the effect of oridonin on the mitochondrial membrane potential in the HeLa cells, oridonin induced mitochondrial transmembrane depolarization, which was represented as a decrease in the potential. Oridonin-induced cytochrome *c* release was also observed in the HeLa cells; this was represented as a significant increase in the cytosolic cytochrome *c* concentration (Figure 5B). Cytochrome *c* release and mitochondrial membrane potential was analyzed spectrophotometrically. The data suggested that a loss of mitochondrial membrane potential may be required for oridonin-induced cytochrome *c* release into the cytosol, which later triggered the cleavage and activation of mitochondrial downstream caspases and the onset of apoptosis.

Activation of caspases-9 and -3 and PARP cleavage in HeLa cells We investigated whether the oridonin treatment of HeLa cells activates caspases-9 and -3 and promotes cleavage of PARP further downstream in the apoptotic pathway. Oridonin treatment activated caspases-9 and -3 in HeLa cells (Figure 5). On caspase-3 activity assay after treatment for 24
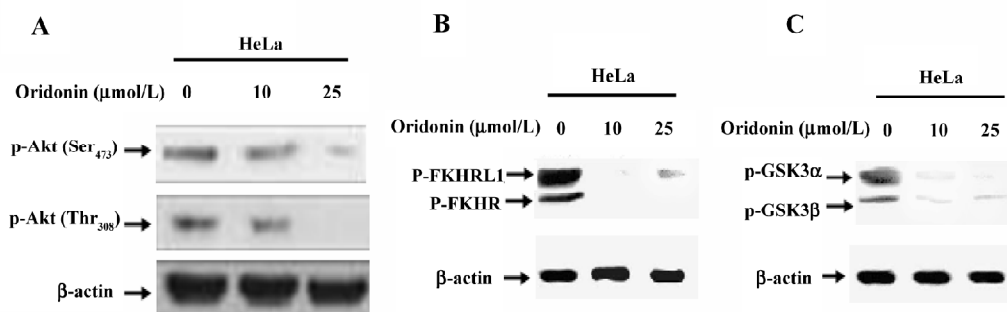

Figure 4. Oridonin inhibited constitutively-active proteins of the PI3K/Akt and mitochondrial apoptotic pathways in HeLa cells. HeLa cells were treated with various doses of oridonin as indicated, and cytoplasmic extracts were prepared. Equal amounts of protein from each sample were separated on SDS–PAGE and immunoblotted with phospho-Akt (Ser_{473}) and phospho-Akt (Thr_{308}) (A), phospho FKHR (B), or phospho-GSK3-α/β (C).

A
HeLa

Oridonin   —   +
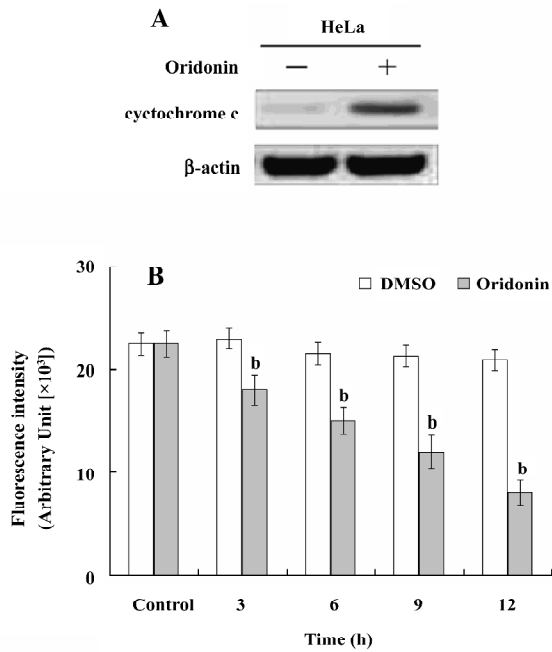
cytochrome c

β-actin

Figure 5. Measurement of mitochondrial membrane potential and cytoplasmic cytochrome *c* release in HeLa cells after oridonin treatment. Oridonin induced the release of cytochrome *c* (A). Mitochondrial membrane potential was measured (B). Data are expressed as mean±SD. *n*=5. [b]*P*<0.01 *vs* the group treated with DMSO.

h, 25 μmol/L oridonin activated caspase-3 in HeLa cells. These results were consistent with the data on cytochrome *c* release and indicated that the activation of effector caspases participate in oridonin-induced apoptosis in HeLa cells. When we used the cell permeable caspase-3 inhibitor (z-DEVD-fmk), oridonin activated caspase-3, and cleaved PARP. However z-DEVD-fmk pretreatment abolished this induced activation and cleavage (Figure 6).
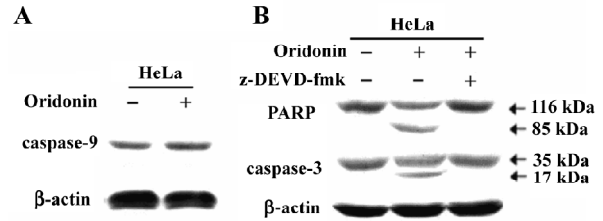
A
HeLa

Oridonin   —   +

caspase-9

β-actin

B
HeLa

Oridonin   —   +   +
z-DEVD-fmk —   —   +

PARP   ← 116 kDa
                        ← 85 kDa

caspase-3   ← 35 kDa
                        ← 17 kDa

β-actin

Figure 6. (A) oridonin-induced activation of caspases-9 and -3, and cleavage of PARP in HeLa cells. (B) effect of z-DEVD-fmk on the oridonin-induced activation of caspase-3. HeLa cells were pre-treated with 25 μmol/L z-DEVD-fmk for 2 h and subsequently treated with 25 μmol/L oridonin for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and immunoblotted with antibodies against pro-caspase-3 and PARP.

Modulation of the IAP protein family in oridonin-induced apoptosis in the HeLa cell line IAP are physiological substrates of Akt that are stabilized to inhibit programmed cell death. IAP have direct effects on caspases-9 and -3[31]. Therefore, we also determined whether oridonin induced cell death by modulating the expression of IAP family members that ultimately determine the cellular response to apoptotic stimuli. The HeLa cells lines were treated with 25 μmol/L oridonin for 24 h, and the expression of cIAP1, XIAP, and survivin were determined by Western blotting. Oridonin treatment caused the downregulation of cIAP1, XIAP, and survivin (Figure 7). These results suggested that Akt may modulate these survival proteins for the survival of HeLa cells.

Discussion

Apoptosis plays an important role in the maintenance of tissue homeostasis by selectively eliminating excessive cells.
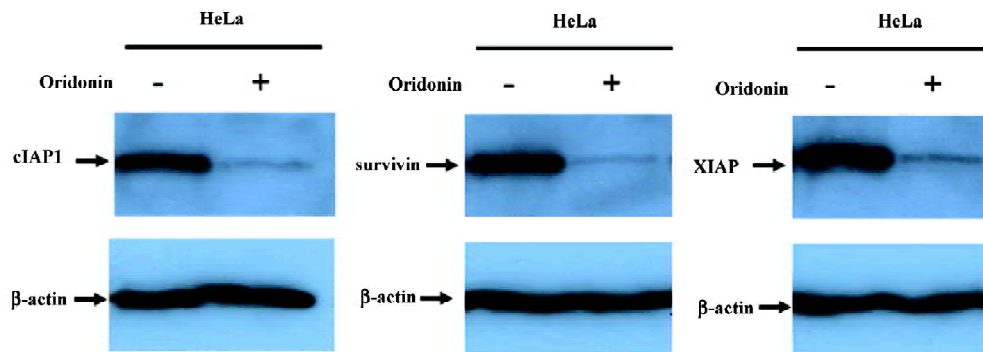

Figure 7. Oridonin-induced downregulation of cIAP1, XIAP, and survivin expression. HeLa cells were treated with or without 25 μmol/L oridonin for 24 h. Cells were lysed and equal amount of proteins were separated with SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and immunoblotted with antibodies against cIAP1, XIAP, survivin, and actin.

At present, many researchers focus on the ability of natural and dietary agents to induce cancer cell apoptosis to seek new anticancer drugs. In the present study, we demonstrated that oridonin significantly inhibited HeLa cell growth and induced apoptosis in a time- and dose-dependent manner. HeLa cells treated with oridonin exhibited characteristic morphological features of apoptosis, such as membrane shrinkage, chromosomal condensation, and increased caspase-3 activity. Moreover, the pre-incubation of cells with z-DEVD-fmk, a specific caspase-3 inhibitor, effectively inhibited caspase-3 activity and prevented oridonin-induced cell death. Oridonin induced apoptosis in HeLa cells in a caspase-dependent apoptotic pathway.

Signal transduction pathways involved in growth and apoptosis are potential targets for chemopreventive agents. Oridonin, which is potently antitumorigenic, has well-documented pro-apoptotic properties in a variety of cell types[6–9]. The oridonin inhibition of NF-$\kappa\beta$ activity is accompanied by apoptosis in various types of cancer cells[13]. Apoptosis is a multistep process, and an increasing number of genes involved in the control or execution of apoptosis has been identified. Our data showed that oridonin-induced HeLa cell growth inhibition was accompanied by the inhibition of Akt activity. Oridonin inhibited the Akt phosphorylation at Ser_{473} and Thr_{308}. For the full activation of Akt, cytokines and growth factors must phosphorylate Akt at these sites[32]. This observation further supports our hypothesis that the inhibition of Akt activity by oridonin plays a critical role in inducing growth inhibition by means of apoptosis. Studies have also demonstrated that Akt activity inhibits the release of cytochrome *c* from mitochondria after UV irradiation[25]. In addition, constitutively activated Akt significantly protected distal hereditary motor neuronopathy (HMN1) cells from apoptosis induced by C2-ceramide[26]. Our results are in agreement with the finding that oridonin inhibited the constitutive activity of Akt in HeLa cells accompanied with the release of cytochrome *c* from mitochondria into the cytoplasm. This result suggested a link between mitochondria and oridonin-induced cell growth inhibition of cervical carcinoma HeLa cells.

On exposure to oridonin, we also observed a progressive decrease in the mitochondrial membrane potential and release of cytochrome *c* into the cytosol in HeLa cells. In many *in vitro* systems, apoptosis is associated with a loss of mitochondrial membrane potential, which may correspond to the opening of an outer membrane pore. Therefore, some have suggested that this event is responsible for the cytosolic release of cytochrome *c* from mitochondria[33]. In this study, the accumulation of cytosolic cytochrome *c* in oridonin-induced human HeLa cells was probably the consequence of the loss of mitochondrial membrane potential, which finally led to cell death. Members of the Bcl-2 homology family of proteins facilitate mitochondrial-mediated apoptosis[34]. Reduced mitochondrial membrane potential leads to the release of intermembrane proteins, such as cytochrome *c* and apoptosis-inducing factors into the cytosol and induces apoptosis[35].

In the cytosol, cytochrome *c* binds to Apaf-1 and triggers Apaf-mediated caspase-9 activation, which activates the death signal by activating caspase-3 and other caspases[36]. Our data demonstrated that oridonin-mediated cytochrome *c* release activated caspases-9 and -3 and cleaved PARP. The expression of IAP is correlated with resistance to apoptosis in transformed cell types and in a variety of human tumors cells[37,38]. Akt regulates the expression of many survival genes, including those encoding IAP[39]. Our results showed that cervical carcinoma cells expressed IAP, including cIAP1, XIAP, and survivin, and that oridonin treatment decreased the expression level of these molecules. This finding suggested that the downregulation of IAP may be involved in the activation of caspases-9 and -3 in oridonin-induced apoptosis.

In conclusion, oridonin induced apoptosis in cervical carcinoma HeLa cells. The treatment of HeLa cells with oridonin activated a cell-death pathway that regulated mitochondrial membrane permeability by downregulating Akt kinase signaling, triggering the release of cytochrome *c* from the mitochondria into the cytosol. Loss of mitochondrial membrane potential may be required for oridonin to induce the release of cytochrome *c*, which led to the activation of the downstream caspases-9 and -3 and the downregulation of the survival proteins (cIAP1, XIAP, and survivin), resulting in the death of cervical carcinoma cells. Taken together, our data suggests that oridonin may be a promising candidate as an antitumor agent against human cervical carcinoma.

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