Oridonin induces apoptosis of HeLa cells via altering expression of Bcl-2/Bax and activating caspase-3/ICAD pathway

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ABSTRACT

AIM: To study the mechanisms by which oridonin inhibited HeLa cell growth in vitro. METHODS: Viability of oridonin-induced HeLa cells was measured by MTT assay. Apoptotic cells with condensed nuclei were visualized by phase contrast microscopy. Nucleosomal DNA fragmentation was assayed by agarose gel electrophoresis. Caspase activity was assayed using fluorometric protease assay. ICAD, Bcl-2, and Bax proteins expression were detected by Western blot analysis. RESULTS: Oridonin induced oligonucleosomal fragmentation of DNA and increased caspase-3 activity, on the other hand, reduced the expression of inhibitor of caspase-3-activated DNase (ICAD), a caspase-3 substrate, at 12 h in HeLa cells. Oridonin-induced DNA fragmentation, caspase-3 activation and down-regulation of ICAD expression were effectively inhibited by a caspase-3 inhibitor, z-DEVD-fmk (z-Asp-Glu-Val-Asp-fmk). However, pretreatment with an inhibitor of poly (ADP-ribose) polymerase (PARP), 3, 4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ), did not suppress oridonin-induced HeLa cell death. In addition, oridonin-induced apoptosis was associated with an increase in the expression of the apoptosis inducer Bax, and a significant reduction in expression of the apoptosis suppressor Bcl-2 in mitochondria. CONCLUSION: Oridonin induces HeLa cells apoptosis by altering balance of Bcl-2 and Bax protein expression and activation of caspase-3/ICAD pathway.

INTRODUCTION

Herbal medicine, Donglingcao (rabdosia rubescens), has been traditionally used in China for the treatment of various diseases such as leukemia. Oridonin (Fig 1) is a diterpenoid compound isolated from Rabdosia rubescens (hemsl). It has various pharmacological and physiological effects such as anti-inflammatory, anti-bacteria, anti-tumor[1-3] and has been used for the treatment of human cancers, especially...
esophageal carcinoma\cite{4}. This compound has been observed to block DNA synthesis in L1210 tumor and bone marrow cells\cite{5}, and induce HeLa, K562 and HL-60 cell death\cite{6,8}. However, the mechanisms of oridonin-induced HeLa cell apoptosis are still unclear.

Apoptosis is an essential and highly conserved mode of cell death that is important for normal development, host defense and suppression of oncogenesis. Faulty regulation of apoptosis has been implicated in degenerative conditions, vascular diseases and cancer\cite{9,10}. Some anticancer drugs and a variety of cell differentiation inducers have been shown to induce apoptosis in susceptible cancer cells\cite{11,12}. Among the numerous proteins and genes involved, members of the caspase family and the Bcl-2 family play important roles in inhibiting or promoting apoptosis\cite{13-15}. It is well known that in caspase family, caspase-3 plays the central role. Once activated, caspase-3 performs a number of executioner functions, including the activation of a latent cytosolic endonuclease, poly (ADP-ribose) polymerase (PARP) and inhibitor of the caspase-activated DNase (ICAD). The ICAD cleavage is consistent with DNA degradation, which is a marker for apoptotic cell death\cite{16}.

The Bcl-2 family proteins constitute an important control mechanism in the regulation of apoptosis. Some of them suppress apoptosis, including Bcl-2 and Bcl-X\textsubscript{L}, and others promote apoptosis, such as Bax and Bid, and the balance between these two groups determines the fate of cells in many apoptotic systems\cite{17}. Bcl-2 is the prototypic family member and is homologous to the nematode Caenorhabditis elegans cer-9 product. Bcl-2 binds to the adapter CED-4 to prevent it from activating caspase-3\cite{18}, it is widely believed that the Bcl-2 protein regulates mitochondrial membrane pore size that release cytochrome c and other apoptogenic factors\cite{19,20}. The Bax gene, a family member that promotes apoptosis to limit cell numbers in tissues, may be negatively selected during progression of some human colon cancers, implying that it may function as a tumor suppressor, dependent of p53\cite{21}.

Previous papers have reported that HeLa cell growth was inhibited by some cytokines, including TNF-related apoptosis-inducing Ligand (TRAIL) and FasL, but not TNF\textalpha\cite{22}. Moreover, experiments using TRAIL suggested the activation of caspase-8 and caspase-3. In view of the roles of caspase and Bcl-2 families in the apoptotic pathway, we tried to identify the key caspase(s) and to examine the expression of Bcl-2 proteins in oridonin-induced apoptosis.

**MATERIALS AND METHODS**

**Chemicals** Oridonin was obtained from Prof Han-dong SUN (Kunming Institute of Botany, The Chinese Academy of Sciences). The structure of oridonin was assigned as following: comparing the chemical and spectral data (\textit{1H-NMR, 13C-NMR}) with those reported in the literature\cite{23,24}. The purity of the oridonin was determined by HPLC and determined to be 99 % and oridonin was dissolved in dimethyl sulfoxide (Me\textsubscript{2}SO) to make a stock solution. Me\textsubscript{2}SO concentrations were kept to below 0.05 % in all the cell culture and did not exert any detectable change in the cell growth or apoptosis. Pan-caspase inhibitor, z-Val-Ala-Asp (OMe)-FMK (z-VADfmk), and caspase-8 inhibitor, z-Ile-Glu (OMe)-ThrAsp (OMe)-FMK (z-DEVD-fmk), were from Enzyme Systems (CA, USA). Caspase-3 inhibitor, Z-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk), was from Calbiochem (CA, USA). Caspase-1 inhibitor, Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk), was obtained from Bachem (Bubendorf, Switzerland).

**Cell culture** The HeLa cell line was purchased from the American Type Culture Collection (ATCC, #CRL 1872, Rockville, MD). The cells were cultured in RPMI-1640 medium (GIBCO, NY) supplemented with 10 % fetal bovine serum (FBS) and 0.03 % L-glutamine (GIBCO, NY) and maintained at 37 ºC with 5 % CO\textsubscript{2} in a humidified atmosphere.

**Cell growth assay** The cytotoxic effect of oridonin on HeLa cells was measured by MTT assay as described\cite{25}. The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10\textsuperscript{4} cells per well. After 12 h incubation, they were treated with various concentrations of oridonin and caspase inhibitors, followed by 12 h cell culture. Four hours before the end of incubation, 20 µL MTT solution (5.0×10\textsuperscript{3} mg/L) was added to each well. Resulting crystals were dissolved in Me\textsubscript{2}SO. Absorbance was measured with an ELISA reader (TECAN SPECTRA, Wetzlar, Germany). The cytotoxic effect was expressed as a relative percentage of inhibition calculated as follows:

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\text{Relative inhibition}=\frac{(A_{\text{control}}-A_{\text{exp}})}{(A_{\text{control}}-A_{\text{blank}})}\times100\%
\]

**Observation of morphological changes** HeLa cells in RPMI-1640 containing 10 % FBS were seeded into 6-well culture plates and cultured for 8 h. Oridonin (68.7 µmol/L) was added to the cell culture and the
cellular morphology was observed using phase contrast microscopy at 12 h (Leica, Wetzlar, Germany).

**DNA extraction and detection of DNA fragments**

HeLa cells (1×10⁶ cells) were collected by centrifugation at 150×g for 5 min and washed once with Ca²⁺-and Mg²⁺-free phosphate buffered-saline (PBS). The cell pellet was suspended in 100 µL cell lysis buffer pH 8.4 (Tris-HCl 10 mmol/L pH 7.4, edetic acid 10 mmol/L pH 8.0, Triton X-100 0.5 %) and kept at 50 °C for 2 h. The lysate was centrifuged at 7200×g for 20 min. The supernatant was incubated with 2 µL RNase A (2.0×10⁴ mg/L) at 37 °C for 60 min, then incubated with 2 µL proteinase K (2.0×10⁴ mg/L) at 37 °C for 60 min. The supernatant was again mixed with NaCl 0.5 mol/L and 50 % isopropyl alcohol overnight at -20 °C, followed by centrifugation at 7200×g for 15 min. After drying, DNA was dissolved in TE buffer pH 7.8 (Tris-HCl 10 mmol/L pH 7.4 and edetic acid 1 mmol/L pH 8.0) and separated by 2 % agarose gel electrophoresis at 100 V for 40 min and stained with 0.1 mg/L ethidium bromide.[26, 27]

**Fluorometric assay for caspase-3 activity**

The activity of caspase-3 was measured by Apoptosis Detection Kit (Santa Cruz Biotec, CA, USA). In brief, the caspase-3 fluorometric protease assay utilizes a synthetic substrate, DEVD-AFC, to determine the activity of caspase-3. HeLa cells (2×10⁶ cells) was collected at 250×g for 5 min, then the cell pellet was washed with PBS, and added by cell lysis buffer at 1 mL per 2×10⁶ cells for 10 min on ice. For each reaction, 100 µL of cell lysate was aliquoted. The reaction buffer (provided) was diluted to 1-fold and added with DTT to a final concentration of 10 mmol/L, prior to use. Reaction mixtures were prepared by adding 1 mL this buffer and 10 µL of DEVD-AFC substrate to each aliquot of cell lysate. Then, the reaction mixture was incubated for 1 h at 37 °C. The level of free AFC (7-amino-4-trifluoromethyl coumarin) was measured by a spectrofluorometer with an excitation wavelength at 400 nm and an emission wavelength at 505 nm. Caspase-3 activation was determined by the levels of free AFC.

**Western blot analysis**

HeLa cells were treated with oridonin 68.7 µmol/L for 0, 6, 12, and 24 h. Both adherent and floating cells were collected. Then Western blot analysis was performed as previously described [28] with some modification. 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, PMSF 1 mmol/L, aprotinin (Sigma, MO, USA) 10 mg/L, leupeptin (Sigma, MO, USA) 10 mg/L, and lysed at 4 °C for 60 min. After 13 000×g centrifugation for 15 min, the protein content of supernatant were determined by 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. Proteins were detected using monoclonal antibody Bcl-2 (rabbit IgG, Oncogene), Bax (rabbit IgG, Oncogene) and ICAD (rabbit IgG, Onco-gene), and was visualized by using anti-rabbit IgG conjugated with peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

**Statistical analysis of the data**

The data are expressed as means±SD. Statistical comparisons were made by t-test. P<0.05 was considered significant.

**RESULTS**

**Inhibition of cell growth by oridonin**

Oridonin induced HeLa cell death in a time- and concentration-dependent manner. Treatment of HeLa cells with oridonin 68.7 µmol/L for 12 h induced approximately 56 % of the cell death. Thus, 12 h incubation seemed to be sufficient for the half induction of death. When HeLa cells were treated with oridonin 275 µmol/L for 3 h, about 50 % cells died (Fig 2).

**Oridonin induces apoptotic cell death in HeLa cells**

When tumor cells were cultured with oridonin 68.7 µmol/L for 6 and 12 h, marked morphological changes were observed compared with the untreated controls.

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**Fig 2.** Time courses of cell death by oridonin. HeLa cells were treated with oridonin at various doses for 12, 24, 36 and 48 h. (●: 8.6, ■: 17.2, ▲: 34.3, ○: 68.7 µmol/L). n=3. Mean±SD.
control (Fig 3). Oridonin-treated HeLa cells underwent retraction of cellular processes and became round in shape at 6 h (Fig 3B). By 12 h, the majority of HeLa cells had become round with shrunken nuclei. Some of these cells showed membrane blebbing and nuclei were fragmented into apoptotic bodies (Fig 3C, 3D). Untreated cells did not show these apoptotic characteristics (Fig 3A). After treatment with oridonin 68.7 µmol/L for 12 h, HeLa cells began to generate DNA fragmentation that is a hallmark of apoptosis (Fig 4). It was reported that the ratio of LDH (lactate dehydrogenase) released from viable cells, floating dead cells and the culture medium might be used to distinguish the number of apoptotic and necrotic cells[29]. In our previous study, in the presence of oridonin (68.7 µmol/L), the numbers of apoptotic cells increased from 22.3 % to 28.7 % at 12 h, the necrotic cells were still below 19.2 %, however, oridonin 137.4 µmol/L induced 37.4 % HeLa cells necrosis and 25.6 % cell apoptosis[30]. These results demonstrated that treatment with oridonin 68.7 µmol/L induced the majority of HeLa cell apoptosis.

**Activation of caspase-3/ICAD pathway is required for oridonin-induced apoptosis** To assess whether caspase-like proteases is participated in the apoptotic pathway, four caspase inhibitors were applied. HeLa cells were pretreated with different dose of z-VAD-fmk, Ac-YVAD-cmk, z-DEVD-fmk, or z-IETD-fmk for 60 min, then cultured with oridonin 68.7 µmol/L for 12 h. This growth-inhibitory effect was also effectively suppressed by z-VAD-fmk from 75.4 %±1.7 % to 32.8 %±8.1 % (Fig 5A). When HeLa cells were pretreated with z-DEVD-fmk 20 µmol/L, the oridonin-induced loss of cell viability was markedly reduced from 79.2 %±0.9 % to 16.7 %±3.6 % (Fig 5B). In contrast, oridonin-induced HeLa cells death was only partly reduced by up-stream caspase-8 inhibitor, z-IETD-fmk and caspase-1 inhibitor, Ac-YVAD-cmk (Fig 5C, D). Moreover, z-DEVD-fmk 20 µmol/L effectively reduced oridonin-induced DNA fragmentation (Fig 6). In view of these results, we proceeded to determine the caspase-3 activity in oridonin-treated HeLa cells.

Fluorometric assay was used to measure cleavages of the caspase-3-specific fluorogenic substrate (DEVD-AFC), and the results were considered to represent caspase-3 activity. HeLa cells were cultured with oridonin 68.7 µmol/L for 0, 6, 12, and 24 h, then the
maximum linear rate of AFC release was measured. Oridonin increased caspase-3 activity at 12 h from 1.6±0.1 to 3.7±0.15, then, by 24 h, the caspase-3 activity declined to 2.7±0.1 (Fig 7). The specific caspase-3 inhibitor, z-DEVD-fmk 20 µmol/L, reduced the caspase-3 activity at 12 h to the base line level. These findings suggest that activation of caspase-3 is involved in oridonin-induced HeLa cell apoptosis. In fact, caspase-3 is responsible for ICAD and PARP cleavage, with these nucleases giving rise to the typical apoptotic nuclei[31]. In this study, inhibitor of PARP, caspase-3 substrate, DPQ, was applied to assess whether cleavage of PARP was required for oridonin-induced
apoptosis. The result showed that high dose of DPQ (40 µmol/L) did not prevent oridonin-induced cells apoptosis (Fig 8). Then, the expression of ICAD protein, another caspase-3 substrate, was further examined by Western blot. ICAD protein decreased with culturing time, and this decline was effectively blocked by z-DEVD-fmk at 12 h (Fig 9). Together, these observations indicated that oridonin promoted apoptosis in HeLa cells involving caspase-3 activation and cleavage of its substrate ICAD.

Different expression of Bcl-2 and Bax proteins in oridonin-treated HeLa cells  Bcl-2 family play an essential role in HeLa apoptosis[32]. To confirm whether such a mechanism is involved in oridonin-induced apoptosis, HeLa cells were incubated with oridonin for 0, 6, 12, and 24 h, and then Bcl-2 and Bax expression were detected. The expression of Bcl-2 protein began to decline at 12 h after treatment of oridonin, while that of Bax protein began to increase. The observation suggests that the balance between Bcl-2 and Bax expression is essential for oridonin-induced apoptosis (Fig 10).
Mitochondrial oncogene products, Bcl-2 and Bax, are known to function upstream of caspase-3 to regulate apoptosis induction by various signals\[36\]. Our results showed that at 12 h after oridonin treatment, the expression of Bcl-2 protein began to decrease, and simultaneously, expression of Bax began to increase. At the same time, caspase-3 activity was increased. It is possible that balance between Bcl-2 and Bax expression and the caspase-3 activation are closely linked. Indeed, Bcl-2 gene expression prevented caspase-3 activation during a variety of proapoptotic conditions\[37\]. It was shown that Bcl-2 suppressed caspase-3 activation by preventing cytochrome c release from mitochondria and released cytochrome c bound Apaf-1, and then this compound activated caspase-9, triggering caspase-3 activation in the presence of ATP\[37\]. Down-regulation of Bcl-2 expression by oridonin might result in an increase of cytochrom c release and an increased ratio of Bax/Bcl-2 expression, which might be tightly linked to oridonin-induced apoptosis. Taken together, oridonin promotes HeLa cells apoptosis via pathways involving the Bcl-2/Bax proteins balance and caspase-3/ICAD activation.

REFERENCES


