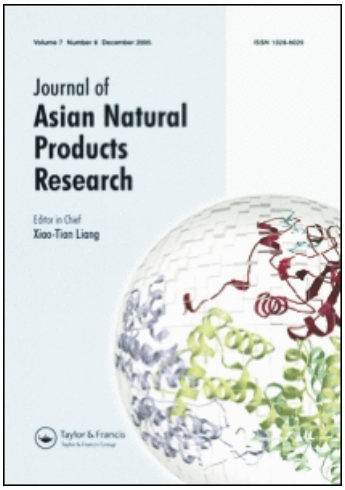

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Oridonin induced A375-S2 cell apoptosis VIA BAX-regulated caspase pathway activation, dependent on the cytochrome *C*/CASPASE-9 apoptosome

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ORIDONIN INDUCED A375-S2 CELL APOPTOSIS VIA BAX-REGULATED CASPASE PATHWAY ACTIVATION, DEPENDENT ON THE CYTOCHROME *C*/CASPASE-9 APOPTOSOME

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Two diterpenoids, oridonin (1) and ponicidin (2), were isolated from the 95% ethanol extract of *Rabdosia rubescens* and were evaluated for antiproliferative activity on cancer cells and human peripheral blood mononuclear cells (PBMC) *in vitro*. Oridonin has much more potent cytotoxic effects on four tumor cells (human melanoma A375-S2, human cervical cancer HeLa, human breast adenocarcinoma MCF-7, murine fibrosarcoma L929) than does ponicidin. The growth-inhibitory activity of oridonin for A375-S2 cells was more potent than that for the other cell lines, with an IC_{50} of $15.1 \pm 1.2\,\mu mol\,L^{-1}$. Treatment with oridonin ($34.3\,\mu mol\,L^{-1}$) for 12 h significantly inhibited A375-S2 cell growth, and showed weaker cytotoxicity against PBMC. By contrast, ponicidin markedly inhibited the growth of PBMC under the same conditions. When caspases-3 and -8 were activated at early stages after treatment of A375-S2 cells with oridonin ($34.3\,\mu mol\,L^{-1}$), apoptotic bodies were formed, nuclear damage was observed by Hoechst 33258 staining and DNA fragmentation was exhibited. In addition, oridonin increased the expression of the apoptosis inducer, Bax, promoted the release of cytochrome *c* without affecting Bcl-2 expression, and activated down-stream caspase-9 in the mitochondrial pathway. These observations indicated that an appropriate dose of oridonin gave an initial premitochondrial phase that involved the Bcl-2 family of the pro-apoptotic protein Bax that required the participation of caspase-9 and caspase-3. However, on treatment with oridonin ($137.4\,\mu mol\,L^{-1}$) for 12 h, the majority of A375-S2 cells underwent necrosis as measured by an LDH activity-based assay. Our results suggest that oridonin induces A375-S2 cell death on the balance of apoptosis and necrosis.

Keywords: Oridonin; A375-S2; Apoptosis; Necrosis; Caspase; Bax

INTRODUCTION

Dong Ling Cao, the aerial parts of *Rabdosia rubescens*, is a popular herb for the treatment of various diseases in China. Diterpenoids are the major constituent of *R. rubescens*; they have various pharmacological and physiological functions such as anti-tumor,

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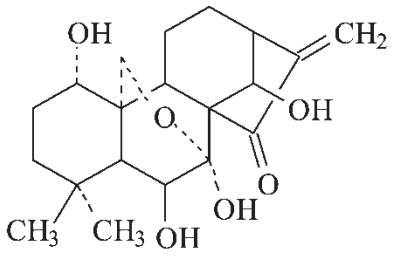

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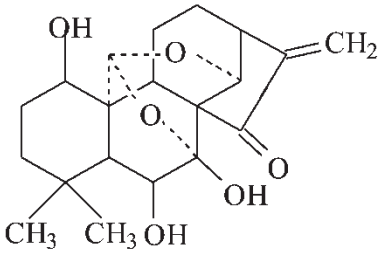

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FIGURE 1 Structures of oridonin (**1**), ponicidin (**2**).

anti-inflammation and anti-bacterial [1–4] and have been used to treat cancer, especially esophageal carcinoma [5]. However, the mechanisms of the diterpenoids-induced tumor cell death are still unclear. In this study, we have isolated the major active compounds from *R. rubescens* and investigated their mechanisms of inducing tumor cell death (Fig. 1).

Many natural products have been reported to exert their antitumor effects by induction of cancer cells apoptosis [6,7]. Apoptosis is a cell-autonomous death process that requires the active participation of endogenous cellular enzymes and oncogene expression. Morphologically, this process is characterized by a dramatic execution phase that induces loss of cell volume, plasma membrane blebbing, chromatin condensation, DNA degradation and formation of apoptotic bodies [8]. In this study, the anti-proliferative activity of oridonin and ponicidin against four cell lines and PBMC were examined. Oridonin induced A375-S2 cell death through distinct mechanisms and pathways, including apoptosis and necrosis in which caspase cascades and mitochondrial pathway are involved.

RESULTS AND DISCUSSION

Cytotoxicity of Oridonin and Ponicidin

Cytotoxic effects of oridonin and ponicidin on four tumor cell lines and PBMC were detected by MTT assays. As shown in Table I, the growth-inhibitory activity of oridonin was much more potent than that of ponicidin. The IC_{50} values of ponicidin against the four tumor cell lines range from 34.3 ± 4.1 to $150.2 \pm 3.5\,\mu mol\,L^{-1}$, approximately 3-fold greater than those of oridonin. These diterpenoids' effects on PBMC were further examined.

TABLE I IC_{50} of oridonin and ponicidin on A375-S2, HeLa, MCF-7 and L929 cells

Cell lines	*A375-S2*	*HeLa*	*MCF-7*	*L929*
Groups				
Oridonin ($\mu mol\,L^{-1}$)	15.1 ± 1.2	49.2 ± 3.1	50.1 ± 2.5	35.6 ± 4.2
Ponicidin ($\mu mol\,L^{-1}$)	34.3 ± 4.1	150.2 ± 3.5	130.2 ± 1.2	68.7 ± 2.3

$n = 3$ $\bar{x} \pm s$.

As shown in Fig. 2, although human normal mononuclear cells were more resistant to oridonin than A375-S2 cells, the growth of PBMC was inhibited as doses of oridonin increased. Oridonin (34.3 $\mu mol\,L^{-1}$) inhibited 25% PBMC growth for 24 h whereas, under the same conditions, oridonin induced approximately 85% A375-S2 cell death. But at the same concentration, ponicidin (34.3 $\mu mol\,L^{-1}$) induced 43% PBMC death. The stronger activity of oridonin than of ponicidin was suggested to be attributable to the hydrogen bonding between the C-6 hydroxyl group and the C-15 carbonyl group in the oridonin molecule [9]. The ester side chain in oridonin 14-O-acyl derivatives may play an important role in the process interaction with cells [10]. In the present study, ponicidin showed stronger cytotoxicity against PBMC than oridonin. In general, PBMC containing monocytes and neutrophils was more susceptible to some cytokines and chemokines; it was observed that neutrophils stimulated with TNFα underwent rapid apoptosis [11]. Ponicidin, but not oridonin, might promote some cytokines release or directly bind to these cytokines receptor(s), then induce PBMC apoptosis. Since A375-S2 cells were more susceptible to oridonin than other tumor cells (Table I), we further investigated the potential mechanisms by which oridonin inhibited A375-S2 cell growth *in vitro*.

Different Types of Cell Death Identified with LDH Released Assay

It was reported that the ratio of LDH released from viable cells, floating dead cells and the culture medium might be used to distinguish the number of apoptotic and necrotic cells [12]. In the presence of oridonin (137.4 $\mu mol\,L^{-1}$), the numbers of necrotic cells increased from 13.8 to 53% OVER 12 h; however, the apoptotic cells were still below 25% (Fig. 3). These observations suggested that inhibition of A375-S2 cell growth was based on the balance of apoptosis and necrosis, since treatment of A375-S2 cells with 34.3 $\mu mol\,L^{-1}$ oridonin for 12 h induced 70% cell death (Fig. 4). After treatment with oridonin (34.3 $\mu mol\,L^{-1}$) for 12 h, A375-S2 cells became round, and generated apoptotic bodies at the later stages (Fig. 5a. b). Morphological changes were further confirmed by Hoechst 33258 staining of cell nuclei (Fig. 6a, b). In a control group, nuclei of A375-S2 cells were round and stained homogeneously; however, 34.3 $\mu mol\,L^{-1}$ oridonin-treated cells showed marked blebbing of nuclei and granular apoptotic bodies. But at a high dose of oridonin (137.4 $\mu mol\,L^{-1}$) for 12 h, necrotic nuclei with fluorescence emission were observed (Fig. 6c).

Moreover, treatment with oridonin (34.3 $\mu mol\,L^{-1}$) for 12 and 24 h induced DNA fragmentation, which is another apoptotic hallmark (Fig. 7), whereas in treatment with 137.4 $\mu mol\,L^{-1}$ oridonin, for 12 h, smear-like DNA degradation characteristic of necrosis was observed in lane E of Fig. 7. Together, these results showed that a low dose of oridonin apparently induced A375-S2 cell apoptosis and that a high dose of oridonin induced cells necrosis.

It is well known that the most death signals are passed through two distinct pathways: apoptosis and necrosis. Fas-mediated cell death occurs not only by apoptosis but also by necrosis, depending on the cellular context. Fas-induced necrosis requires the adaptor protein

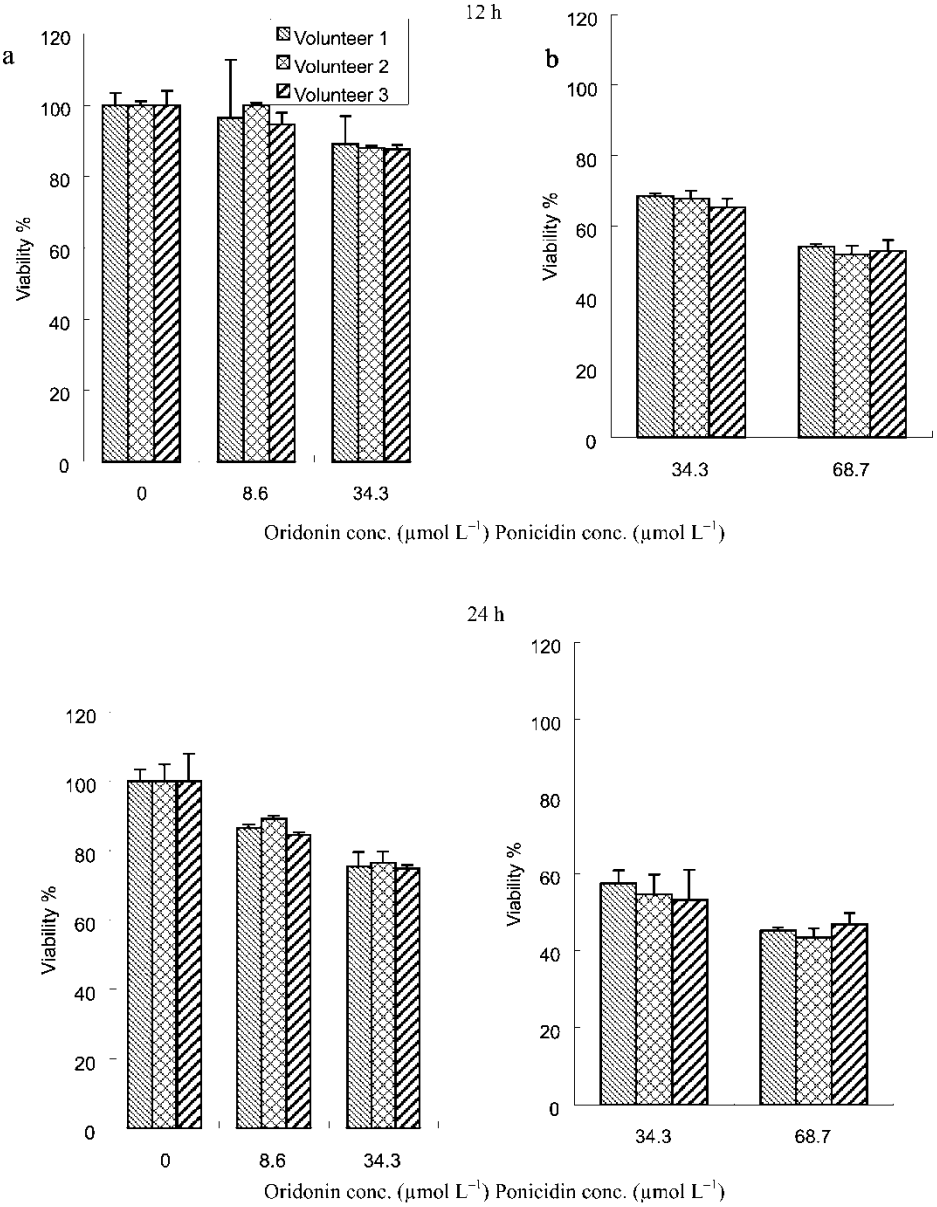

FIGURE 2 Cytotoxic effects of (a) oridonin and (b) ponicidin on human PBMC. Cells treated with various doses of drugs for 12 or 24 h were evaluated by MTT assay. Data are expressed as the mean ± SD of two independent experiments.

FADD and the Fas-interacting serine/threonin kinase receptor-interacting protein (RIP), whereas caspase-8 seems to be dispensable [13]. It has been reported that, at low doses, fostriecin induced HL-60 cell apoptosis, but at high doses it caused necrosis [14].

In our present study, various doses of oridonin not only caused typical apoptosis, but also induced A375-S2 cell necrosis. Meanwhile, the inhibitory activity of oridonin on PBMC might be attributable to the necrotic mechanism. Thus, it is probable that some unidentified proteases or protein kinases participate in this mechanism.

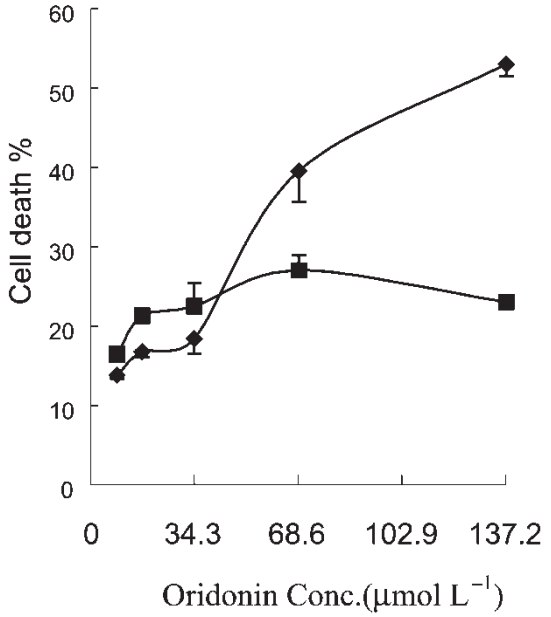

FIGURE 3 Oridonin induced cells LDH release. Cells treated with various doses of oridonin (8.6, 17.2, 34.3, 68.7, 137.4 μmol L^{-1}) for 12 h were measured by LDH activity-based assay. (■: apoptosis, ◆: necrosis). Data shown are means ± SD (bars) of three independent experiments.

Oridonin-induced A375-S2 Apoptosis Activated Caspase-3 and Caspase-8

Caspases are a family of cysteine proteases that play key roles in promoting the degradative changes associated with apoptosis. Especially, the activation of downstream caspase-3 is important in the induction of apoptosis by various stimuli [15]. For further assessment of the participation of caspase-like proteases in A375-S2 cell apoptosis, four caspase inhibitors, z-VAD-fmk, Ac-YVAD-cmk, z-DEVD-fmk and z-IETD-fmk were applied. Caspase inhibitors, z-VAD-fmk and z-DEVD-fmk effectively inhibited oridonin-induced

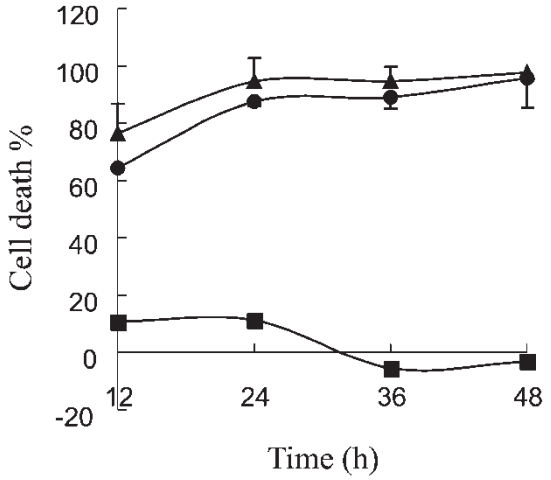

FIGURE 4 Time courses of the induction of cell death by oridonin. A375-S2 cells were treated with oridonin at various doses for 12, 24, 36 and 48 h (●: 34.3, ▲: 68.7 μmol L^{-1}, ■: 0.05% DMSO).

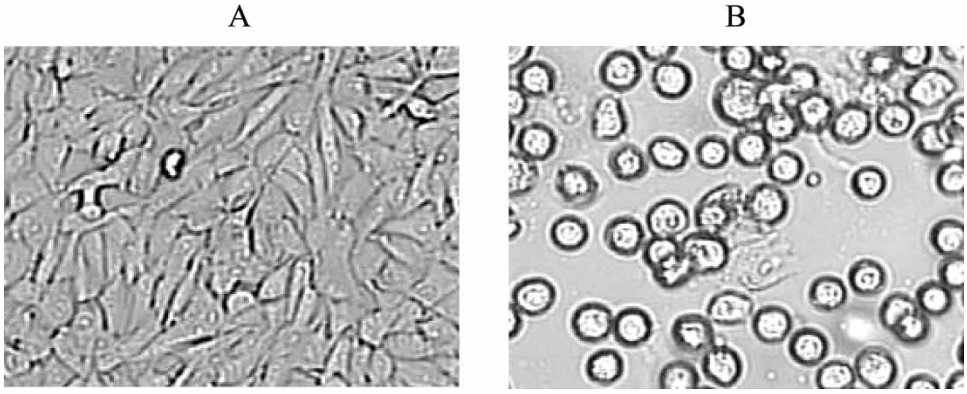

FIGURE 5 Morphological changes of A375-S2 cells treated with oridonin. Cellular morphology was observed at 0 h (a), 12 h (b), in the presence or absence of oridonin (34.3 μmol L^{-1}) with $\times$ 200 magnification.

cell death at a dose of 10 μmol L^{-1} (Fig. 8). But Ac-YVAD-cmk and z-IETD-fmk did not. These data suggest that the process of oridonin-induced apoptosis requires at least the activation of caspase-3 protease. Next, we measured caspase-8 and caspase-3 activities to verify whether they were activated during oridonin-induced A375-S2 cells apoptosis. When A375-S2 cells were treated with oridonin (34.3 μmol L^{-1}) for 12 and 36 h, oridonin increased caspase-3 activity to about six times the control value at 12 h incubation, and was followed by a slow decline. z-DEVD-fmk (10 μmol L^{-1}) effectively decreased oridonin-induced caspase-3 activity. However, caspase-8 activity did not significantly increase *versus* the control group (Table II). Caspase family members are divided into two classes based on the lengths of their N-terminal prodomains. Caspase-8 and caspase-1 each have a long prodomain, which appears to be involved in targeting and regulating activation of the apoptotic pathway. Caspase-3 has a short prodomain localized near the nuclei, and cleaves substrates at the downstream end of cascade [16]. Our results showed that upstream caspase-1 and caspase-8 were not activated in oridonin-induced apoptosis. Therefore, other caspase(s) or apoptosis-associated protein(s) upstream of caspase-3 might be involved in this oridonin-induced A375-S2 apoptosis.

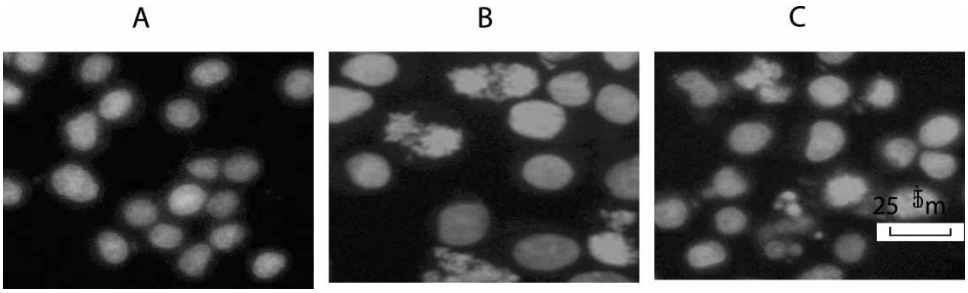

FIGURE 6 Morphological changes of cell nuclei. A375-S2 cells were incubated in the medium alone (a) or the medium containing oridonin: (b) 34.3 or (c) 137.4 μmol L^{-1}, for12 h; the cells were then stained with Hoechst 33258 with $\times$ 200 magnification.

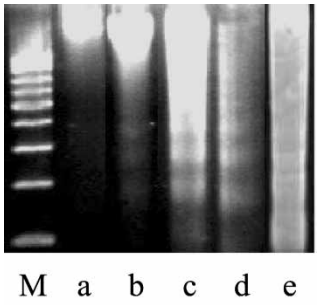

FIGURE 7 Oridonin-induced DNA fragmentation of A375-S2 cells. The cells (1×10^6 cells) were cultured in the absence or presence of oridonin (34.3 μmol L^{-1}) for 24 h (a: control, b: 6, c: 12, d: 24 h) or 137.4 μmol L^{-1} oridonin for 12 h (e).

Bax and Cytochrome *c* are Involved in Oridonin-induced Apoptosis

Apoptosis is an evolutionarily conserved cell suicide process executed by caspase and regulated by the Bcl-2 protein family. Since Apaf-1 requires cytochrome *c* to activate caspase-9, and Bcl-2 prevents mitochondrial cytochrome *c* release [17], post-mitochondrial caspase-9 and activator Apaf-1 are thought to be essential. In view of the roles of Bcl-2 families in the apoptotic pathway, Western blot analysis was performed to observe expression changes of Bcl-2 and Bax protein during oridonin-induced A375-S2 cell apoptosis. As shown in Fig. 9, at 12 h after oridonin treatment the expression of Bax protein and the release of cytochrome *c* began to increase, but Bcl-2 expression had not changed, and, simultaneously, caspase-9 was activated (Table II). Indeed, the Bcl-2 family proteins

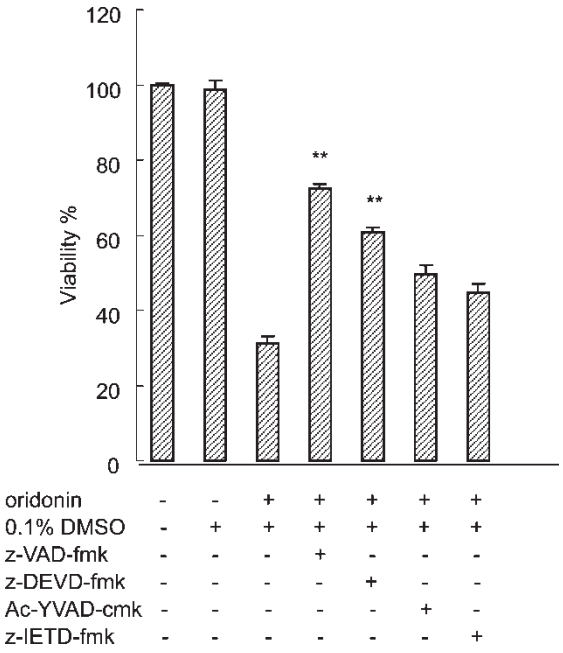

FIGURE 8 Effect of caspase inhibitors on oridonin-induced A375-S2 cell apoptosis. These cells were cultured in the absence or presence of the caspase inhibitors, z-DEVD-fmk (10 μmol L^{-1}) Ac-YVAD-cmk (10 μmol L^{-1}), z-IETD-fmk (20 μmol L^{-1}) or z-VAD-fmk (10 μmol L^{-1}) 60 min prior to the addition of oridonin (34.3 μmol L^{-1}), then incubated for 12 h. $n = 3$ $^{**}p < 0.01$, *vs.* treatment with oridonin (34.3 μmol L^{-1}).

TABLE II Activities of caspase-3, -8 and -9 in oridonin-treated A375-S2 cells

Groups	*Caspase-3* (*unit*)	*Caspase-8* (*unit*)	*Caspase-9* (*unit*)
Time (h)			
0	5.1 ± 0.3	4.0 ± 0.2	3.8 ± 0.1
12	31.2 ± 0.2**	6.5 ± 0.2	13.4 ± 0.5*
24	21.3 ± 0.4**	3.3 ± 0.4	11.2 ± 0.3
12 h + z-IETD-fmk		2.7 ± 0.3	
12 h + z-DEVD-fmk	10.5 ± 0.4		
12 h + Ac-LEHD-CHO			7.8 ± 0.4
36	16.4 ± 0.3*		4.0 ± 0.5

$n = 3\ \bar{x} \pm s$. $^{**}p < 0.01$ or $p^{*} < 0.05$ *vs.* oridonin (34.3 μmol L^{-1}) 0 h.

constitute an important control mechanism in the regulation of apoptosis. Some of them suppress apoptosis, including Bcl-2 and Bcl-X_L and some others promote apoptosis, such as Bax and Bid [18]. Bcl-2 is the prototypic family member and binds to the adapter CED-4 to prevent it from activating caspase CED-3 [19]. However, in this study, oridonin only increased the expression of Bax protein without affecting Bcl-2, indicating that the Bax gene might function as a tumor suppressor, independent of p53 [20]. Because Apaf-1 does not bind to Bcl-2 and requires cytochrome *c* to activate caspase-9, the activation of p53 translocated Bax to the mitochondria and the translocation of Bax to the mitochondria released cytochrome *c*. It was shown that Bax activated caspase-3 by promoting cytochrome *c* release from mitochondria [21]. Cytochrome *c* binds Apaf-1, and then activated caspase-9, which triggered caspase-3 activation in the presence of dATP [22]. Our data showed that an increased Bax expression by oridonin resulted in cytochrome *c* release, and then activated caspase-9 and caspase-3, inducing A375-S2 cell apoptosis.

EXPERIMENTAL

General Experimental Procedures

Thin-layer chromatography (TLC) was performed on silica gel GF 254 plates or silica gel G plates (0.25 mm thick, Qingdao Haiyang Chemical Group Co., China) and the spots were

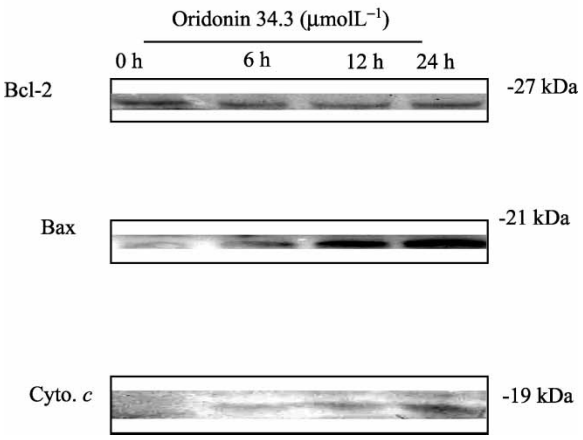

FIGURE 9 Expression of Bcl-2, Bax and cytochrome *c* in oridonin-treated HeLa cells. The cells were treated oridonin (34.3 μmol L^{-1}) for 0, 6, 12 and 24 h. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and the protein bands were detected by Western blot analysis.

detected under UV light (365 nm) or by the use of 10% aqueous sulfuric acid reagent. ^{1}H and ^{13}C NMR spectra were taken on a Bruker ARX-300 NMR spectrometer, using TMS as an internal standard.

Plant Material

The dried, aerial parts of *R. rubescens* were collected in Ji-Yuan (Henan, China) in 2000. The original plant was identified by Professor Q.-S. Sun of Shengyang Pharmaceutical University, China and a voucher of the plant material (2001310) is deposited in the Department of Phytochemistry, Shenyang Pharmaceutical University. All the chemicals used were analytical grade reagents.

Isolation and Purification of Active Compounds

Air-dried and powdered aerial parts of *R. rubescens* (1 kg) were soaked with 95% ethanol (10 L) at room temperature for 12 days, as previous reports described [23]. The ethanol extract was then filtered and concentrated with a rotary evaporator to remove ethanol, leaving a residue of about 3 L. The residue was extracted with light petroleum repeating 3 × (3 L at a time), the aqueous fraction was then concentrated under reduced pressure, to obtain a residue (29.0 g). Furthermore, the extract was subjected to silica gel column chromatography (6 cm id × 80 cm) with a light petroleum–acetone gradient (10:0 → 9:1 → 8:2 → 7:3 → 6:4 → 3:1 → 2:1 → 1:1 → 1:10). One portion of the light petroleum–acetone (3:1) elute, after being recrystallized with acetone, gave 0.03 g of ponicidin. The light petroleum–acetone (2:1) elute, gave 0.1 g of oridonin. The structures of the diterpenoids were assigned by comparing the chemical and spectral data (^{1}H, ^{13}C NMR) with those reported in the literature. The purity of each compound was tested by HPLC apparatus equipped with an ultraviolet detector and a Quest 100 RP-18C column (4 mm id × 200 mm). The mobile phase was composed of methanol–H_2O (v/v 65:3), the detector wavelength was 238 nm, the column was at room temperature, and the flow rate was 0.8 ml min^{-1}; t_R of oridonin: 7.0 min, t_R of ponicidin: 7.4 min. The purity of these two compounds was shown to be more than 99.0%. Their formula and molecular weights are $C_{20}H_{28}O_6$, 364.42 (oridonin) and $C_{20}H_{26}O_6$, 362.17 (ponicidin).

Biological Materials

Cell Cultures

Human melanoma A375-S2, human cervical cancer HeLa, human breast adenocarcinoma MCF-7 and murine fibrosarcoma L929 cells were obtained from American Type Cell Culture (ATCC) (Rockville, MD, USA). Human PBMC were obtained from healthy adult volunteers.

RPMI-1640, fetal bovine serum (FBS), antibiotics and L-glutamine were purchased from GIBCO (Grand Island, NY). Pan-caspase inhibitor, z-Val-Ala-Asp (OMe)-FMK (z-VAD-fmk) and caspase-8 inhibitor, z-Ile-Glu (OMe)-Thr-Asp (OMe)-FMK (z-IETD-fmk), were from Enzyme Systems (CA, USA). Caspase-1 inhibitor, Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk) was obtained from Bachem (Bubendorf, Switzerland). Caspase-3 inhibitor, Z-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk) and Caspase-9 inhibitor, Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Leu-Glu-His-Asp-CHO (Ac-LEHD-CHO), were from Calbiochem (CA, USA). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was obtained from Sigma (MO, USA).

In Vitro Evaluation of Antiproliferation Activity

Drug Solutions

Oridonin and ponicidin were dissolved in DMSO to make stock solutions, then diluted in cell culture medium at different concentrations and immediately used. In all the assays, the final concentrations of DMSO in the culture medium were below 0.05%.

Cell Culture

The cells were cultured in RPMI-1640 supplemented with 10% FBS, streptomycin ($100\,mg\,L^{-1}$), penicillin ($100\,IU\,mL^{-1}$), and 0.03% L-glutamine and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

MTT Assays

The cytotoxic effect of these two diterpenoids on tumor cells was assayed by MTT as described previously [24]. The cells were dispensed in 96-well flat bottom micotiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells $well^{-1}$. After 10 h incubation they were treated with various concentrations of the test samples, followed by 12 h cell culture. Four hours before the end of incubation, MTT solution ($20\,\mu L$, $5.0\,mg\,mL^{-1}$) was added to each well. The resulting crystals were dissolved in DMSO. Absorbance was measured by an ELISA reader (TECAN SPECTRA, Australia). The cytotoxic effect was expressed as a relative percentage of inhibition calculated as follows:

$$\text{Relative\% inhibition} = [(\text{control absorbance} - \text{experimental absorbance})/(\text{control absorbance} - \text{blank absorbance})] \times 100$$

Separation of Human PBMC

Blood was obtained from three healthy adult volunteers; PBMC were separated by density centrifugation in the cell separation solution (Shanghai Biological Reagent Factory, China) and suspended in RPMI-1640 medium containing 2% heat-inactivated human AB serum. PBMC 1.5×10^4 cells $well^{-1}$ were seeded on the 96-well culture plates and, after 5 h incubation, they were treated with various doses of oridonin and ponicidin. At 12 and 24 h the cell viability was measured by MTT assay.

Nuclear Damage observed by Hoechst 33258 Staining

Apoptotic nuclear morphology was assessed using Hoechst 33258 as described previously [25]. Cells were fixed with 3.7% paraformaldelyde for 30 min at room temperature, then washed and stained with Hoechst 33258 ($167\,\mu mol\,L^{-1}$) for 30 min at 37°C. The cells were then washed and resuspended in PBS for morphological observation using a fluorescence microscope (Leica, Wetzlar, Germany).

LDH Activity-based Cytotoxicity Assays

Lactate dehydrosenase (LDH) activity was assessed using a standardized kinetic determination (Zhongsheng LDH kit, Beijing, China). It was measured in both floating

dead cells and viable adherent cells. Floating cells were collected from the culture medium by centrifugation (240*g*) at 4°C for 5 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp) [26]. The LDH released in the culture supernatant (extracellular LDH, or LDHe) was used as an index of necrotic death, and the LDH present in the adherent viable cells as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\text{Apoptosis}\% = \text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

$$\text{Necrosis}\% = \text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

DNA Extraction and Detection of DNA Fragments

A375-S2 cells (1×10^6 cells) were collected by centrifugation at 150*g* for 5 min and washed once with Ca^{2+}- and Mg^{2+}-free phosphate buffered-saline (PBS). The cell pellet was suspended in 100 μL of cell lysis buffer at pH 8.4 (Tris-HCl 10 mmol L^{-1} pH 7.4, EDTA 10 mmol L^{-1} pH 8.0, Triton X-100 0.5%) and kept at 4°C for 30 min. The lysate was centrifuged at 7200*g* for 20 min and the supernatant then incubated with 2 μL of RNase A (20 μg μL^{-1}) at 37°C for 60 min, then incubated with 2 μL proteinase K (20 μg μL^{-1}) at 37°C for 60 min. The supernatant was again mixed with NaCl (0.5 mol L^{-1}) 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^{-1} pH 7.4 and EDTA 1 mmol L^{-1} pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 40 min and stained with ethidium bromide (0.1 mg L^{-1}).

Assay of Caspase Activity

A375-S2 cells (5×10^5 cells) were incubated with or without oridonin (34.3 μmol L^{-1}) for 12, 24 and 36 h. The cells (5×10^5 cells) were also incubated with or without oridonin (34.3 μmol L^{-1}) in the presence of z-IETD-fmk (10 μmol L^{-1}), Ac-LEHD-CHO (10 μmol L^{-1}) or z-DEVD-fmk (10 μmol L^{-1}) for 1 h, followed by addition of oridonin (34.3 μmol L^{-1}) and incubation for 12 h. The activities of caspase-8, -9 and -3 were measured by means of the Apoptosis Detection Kit (Santa Cruz. Biotec, CA, USA).

Western Blot Analysis

A375-S2 cells were treated with oridonin (34.3 μmol L^{-1}) for 0, 6, 12 and 24 h. Both adherent and floating cells were collected. Then Western blot analysis was performed as previously described [27], with some modification. Briefly, the cell pellets were resuspended in lysis buffer, including Hepes (50 mmol L^{-1} pH 7.4), Triton-X 100 1%, sodium orthovanadate (2 mmol L^{-1}), sodium fluoride (100 mmol L^{-1}), edetic acid (1 mmol L^{-1}), PMSF (1 mmol L^{-1}), aprotinin (Sigma, MO, USA; 10 μg mL^{-1}), leupeptin (Sigma, MO, USA; 10 μg mL^{-1}), and lysed in 4°C for 60 min. After 13,000*g* centrifugation for 15 min, the protein content of supernatant was 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. Bcl-2 was detected using monoclonal antibody Bcl-2 (rabbit IgG, Oncogene), Bax (rabbit IgG, Oncogene) and cytochrome *c* (rabbit IgG, Oncogene) were 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

Data are expressed as mean ± SD. The IC_{50} values were calculated by regression analysis of the concentration response data. Statistical comparisons were made by student's *t*-test; $p < 0.05$ was considered significant.

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