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Caspase Inhibition Augmented Oridonin-Induced Cell Death in Murine Fibrosarcoma L929 by Enhancing Reactive Oxygen Species Generation

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Abstract. Oridonin, a diterpenoid isolated from *Rabdosia rubescences*, has been reported to have antitumor effects. In this study, the growth-inhibitory activity of oridonin for L929 cells was exerted in a time-and dose-dependent manner. After treatment with oridonin for 24 h, L929 cells underwent both apoptosis and necrosis as measured by an lactate dehydrogenase (LDH) activity-based assay. A rapid generation of reactive oxygen species (ROS) was triggered by oridonin, and subsequently up-regulation of phospho-p53 (ser 15) expression and an increased expression ratio of Bax/Bcl-2 was observed. Furthermore, there was a significant fall in mitochondrial membrane potential (MMP) and increase in caspase-3 activity after exposure to oridonin for 24 h. Surprisingly, the pan-caspase inhibitor z-VAD-fmk and caspase3 inhibitor z-DEVD-fmk rendered L929 cells more sensitive to oridonin, rather than preventing oridonin-induced cell death. Oridonin and z-VAD-fmk co-treatment not only resulted in an even higher ROS production, but also made a more significant reduction in the MMP. Pretreatment of ROS scavenger *N*-acetylcysteine (NAC) led to a complete inhibition of oridonin-induced cell death, intracellular ROS generation, and MMP collapse. NAC treatment also reversed the potentiation of cell death by the pan-caspase inhibitor z-VAD-fmk. Taken together, these observations showed that oridonin-induced cell death in L929 cells involved intracellular ROS generation, activation of phospho-p53 (ser 15), and up-regulation of the Bax/Bcl-2 ratio; and the augmented cell death by z-VAD-fmk was dependent on an increased ROS production.

Keywords: oridonin, reactive oxygen species (ROS), Bcl-2, L929, apoptosis

Introduction

Apoptosis is an active form of cell death that plays an important role in development and survival by eliminating damaged or other unneeded cells (1). Impaired regulation of apoptosis leads to a variety of diseases such as AIDS, cancer, and neurodegenerative disorders (2, 3). A large number of genes and their products control the progression of apoptosis. The Bcl-2 protein family, a group of apoptosis regulatory genes, has attracted particular attention. In this family, Bcl-2 and Bcl-xL are antiapoptotic, whereas Bax, Bcl-xS, Bad, Bak, and Bik are proapoptotic. Apoptosis-inducing signals cause translocation of proapoptotic proteins of the Bcl-2 family from the cytoplasm to the outer mitochondrial membrane and facilitate the release of some components including cytochrome *c* and apoptosis inducing factor (AIF) from mitochondria into the cytosol, resulting in caspase-dependent or caspase-independent apoptosis (4, 5). p53, another important apoptosis regulatory factor, can inhibit cell division and facilitate DNA repair or induce apoptosis by increasing the ratio of Bax/Bcl-2 (6 – 8).

Various natural products exert their antitumor effects by induction of cancer cell apoptosis (9). Oridonin, a diterpenoid isolated from the plant *Rabdosia rubescens*, has been used in traditional Chinese medicine for treatment of cancer, especially esophageal carcinoma (10). It has various biological, pharmaceutical, and physiological functions such as anti-cancer activity,

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anti-bacteria, and others (11, 12).

It is well known that L929 cells are significantly sensitive to tumor necrosis factor-α (TNFα). Several studies reported that caspase inhibitors augmented TNFα-induced cell death, and this was related to the generation of intracellular reactive oxygen species (ROS) (13, 14). Our previous studies show that oridonin was able to induce apoptosis and necrosis in L929 cells, and caspase inhibition also rendered the cells more sensitive to death (15, 16). Therefore, the focus of this study is to investigate the roles of ROS in oridonin-induced L929 cell death.

Materials and Methods

Reagents

Oridonin was obtained from the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). The structure of oridonin was assigned by comparing the chemical and spectral data (^{1}H-, ^{13}C-NMR) with those reported in the literature (17, 18). The purity of the oridonin was measured by HPLC and determined to be 99.4%. Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution.

The pan-caspase inhibitor (z-VAD-fmk) and caspase-3 inhibitor (z-DEVD-fmk) were purchased from Enzyme Systems (Livermore, CA, USA). *N*-Acetylcysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), 3,3-diaminobenzidine tetrahydrochloride (DAB), and rhodamine 123 (Rh-123) were purchased from Sigma (St. Louis, MO, USA). Murine polyclonal antibodies against p53 and cytochrome *c*; rabbit polyclonal antibodies against Bax, Bcl-2, and phospho-p53 (ser 15); and horseradish peroxidase (HRP)-conjugated secondary antibody (goat-anti-rabbit) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase-3 activity assay kit was purchased from Beyotime Institute of Biotechnology (Jiang Su, China). All of the other chemicals used were of the highest and purest grade.

Cell culture

The murine fibrosarcoma cells L929 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% FBS (Shengma Yuanheng, Beijing, China), 100 mg/l streptomycin, 100 IU/ml penicillin, and 0.03% L-glutamine (Hyclone, Logan, UT, USA) and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

Cytotoxity assay

L929 cells were incubated in 96-cell plates (NUNC, Roskide, Denmark) at a density of 1×10^5 cells/ml. The cells were pretreated with z-VAD-fmk, z-DEVD-fmk, or NAC for 1 h and then treated with oridonin for different time periods. Cell growth was measured with a plate reader (TECAN SPECTRA, Wetzlar, Germany) by MTT. The percentage of cell death was calculated as follows:

$$\text{Cell death (\%)} = [A_{570}\,(\text{control}) - A_{570}\,(\text{oridonin})] / A_{570}\,(\text{control}) \times 100$$

Observation of morphological changes

L929 cells in RPMI-1640 containing 10% FBS were seeded into 6-well culture plates at a density of 1×10^5 cells/ml and cultured for 24 h; and z-VAD-fmk (20 μM), z-DEVD-fmk (20 μM), or NAC (5 mM) was added 1 h before oridonin treatment. The cellular morphological changes were observed using phase contrast microscopy (Leica, Nussloch, Germany) at 24 h.

LDH activity-based cytotoxicity assays

Lactate dehydrogenase (LDH) activity was assessed using a standardized kinetic determination kit (Zhongsheng LDH kit; Zhongsheng, Beijing, China). LDH activity was measured in both floating dead cells and viable adherent cells. The floating dead cells were collected from culture medium by centrifugation ($240 \times g$) at 4°C for 5 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp). The LDH released in the culture supernate [designated as extra cellular LDH (LDHe)] was used as an index of necrotic death, and the LDH present in the adherent viable cells was designated as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

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

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

Determination of intracellular ROS production

ROS generation was measured after staining the cells with dichlorofluorescein-diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) This dye is a stable nonpolar compound that diffuses readily into the cells and yields DCFH. Intracellular H_2O_2 or OH in the presence of peroxidase changes DCHF to the highly fluorescent compound DCF. Thus, the fluorescent intensity is proportional to the peroxides activity in the cells. Following exposure to oridonin for 2 h, L929 cells (1×10^6 cells) were harvested and washed with ice-cold PBS; 1 ml of PBS containing 20 μM DCFH-DA was added to the cells and then the cells were incubated for

15 min at 37°C. The fluorescence emission from DCF was analyzed by FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). The results were analyzed with Cell Quest software (Becton Dickinson).

Determination of mitochondrial membrane potential (MMP)

To determine the change of MMP in L929 cells, flow cytometry was applied using Rh-123 staining (19). L929 cells (1×10^6 cells) were pretreated with z-VAD-fmk, z-DEVD-fmk, or NAC for 1 h and then treated with oridonin for 24 h. The cells were harvested and washed with ice-cold PBS twice by centrifugation at $1,000 \times g$ for 5 min, and then 1 ml PBS contained 10 μg/ml Rh-123 was added to the cells. The tubes were vortexed gently and incubated at 37°C in the dark for 15 min. Flow cytometric analysis was carried out by FACScan. The results were analyzed by using Cell Quest software.

Western blot analysis

L929 cells were cultured for different time periods. Both adherent and floating cells were collected, and then Western blot analysis was performed as follows. Briefly, the cell pellets were resuspended in lysis buffer consisting of 50 mM Hepes (pH 7.4), 1% Triton-X 100, 2 mM sodium orthovanada, 100 mM sodium fluoride, 1 mM edetic acid, 1 mM PMSF, 10 mg/L aprotinnin (Sigma), and 10 mg/L leupeptin (Sigma) and lysed at 4°C for 60 min. After $13,000 \times g$ centrifugation for 15 min, the protein contents of the supernants were determined by a protein assay reagent (Bio-Rad, Hercules, CA, USA). The protein lysates were separated by electrophoresis in a 12% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. Each membrane was blocked with 5% skim milk and then incubated with the indicated primary antibodies against p53, phospho-p53, Bax, Bcl-2, cytochrome *c*, and β-actin, followed by addition of HRP-conjugated secondary antibody and DAB as the HRP substrate.

Statistical analyses of the data

All data represent at least three independent experiments and are expressed as the mean ± S.D. Statistical comparisons were made by Student's *t*-test, *P*-values of less than 0.05 were considered to represent a statistically significant difference.

Results

Cytotoxic effect of oridonin on L929 cells

Oridonin-induced L929 cell death was time- and concentration-dependent. Oridonin at 30 – 90 μM exerted a potent inhibitory effect on L929 cell growth. By 24 h after treatment with 65 μM oridonin, cell death rate reached to almost 50%. The IC_{50} for 24-h oridonin treatment was 65.8 μM. Therefore, 24-h incubation with oridonin seemed to be sufficient for half induction of cell death (Fig. 1).

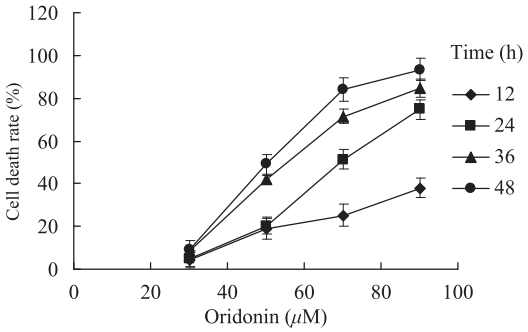


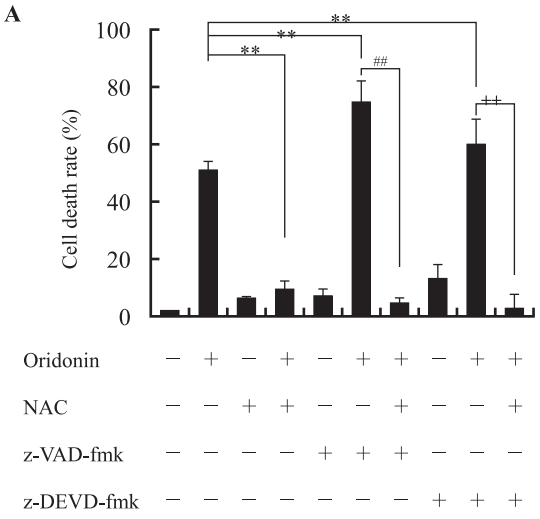
Fig. 1. Time and dose curves of cell death by oridonin treatment. L929 cells were treated with oridonin at various doses for 12, 24, 36, and 48 h. n = 3, mean ± S.D.

Caspase inhibition augmented oridonin-induced L929 cell death

Since it is well known that caspase family members play an executive role in the cell apoptosis process, the effect of caspase inhibitors on oridonin-induced L929 cell death was examined. Unexpectedly, the pan-caspase inhibitor z-VAD-fmk and caspase-3 inhibitor z-DEVD-fmk both rendered the cells more sensitive to death, instead of preventing oridonin-induced apoptosis. At the same time, the ROS scavenger NAC not only completely blocked oridonin-alone–induced L929 cell death, but also reversed the cell death induced by combined treatment of oridonin and caspase inhibitors (Fig. 2). This indicated that oridonin-induced L929 cell death depended on intracellular ROS generation.

Oridonin induced L929 cell death by affecting the balance between apoptosis and necrosis

To determine whether oridonin-induced cell death in L929 was caused by apoptosis, we examined the morphologic changes of the cells. When the cells were incubated with 65 μM oridonin for 24 h, marked changes were observed as compared with the control group (Fig. 3). By 24 h, the majority of the L929 cells had become round, with shrunken nuclei and membrane blebbing (Fig. 3B). Untreated cells did not show these apoptotic characteristics (Fig. 3A). Meanwhile, the z-VAD-fmk–pretreated group showed more marked apoptotic changes (Fig. 3C). However, NAC pretreatment prevented the morphologic changes induced by oridonin as well as by oridonin and z-VAD-fmk co-treatment (Fig. 3: D and E). To further characterize



B

	Oridonin			z-VAD-fmk
	12	18	24	24 (h)
Activities (fold)	5.02±1.8	6.22±1.0	7.65±0.7	3.2±1.4

Fig. 2. Effects of caspase inhibitors and NAC on oridonin-induced L929 cell death. The cells were pretreated with z-VAD-fmk at 20 μM (pan-caspase), z-DEVD-fmk at 20 μM (caspase-3), or NAC at 5 mM for 1 h, then incubated with 65 μM oridonin for 24 h and the inhibitory ratio was measured by MTT assay (A); Caspase-3 activities were assayed (B). n = 3, mean ± S.D. $^{**}P<0.01$ *vs* oridonin group, $^{\#\#}P<0.01$ *vs* oridonin + z-VAD-fmk group, $^{++}P<0.01$ *vs* oridonin + z-DEVD-fmk group.

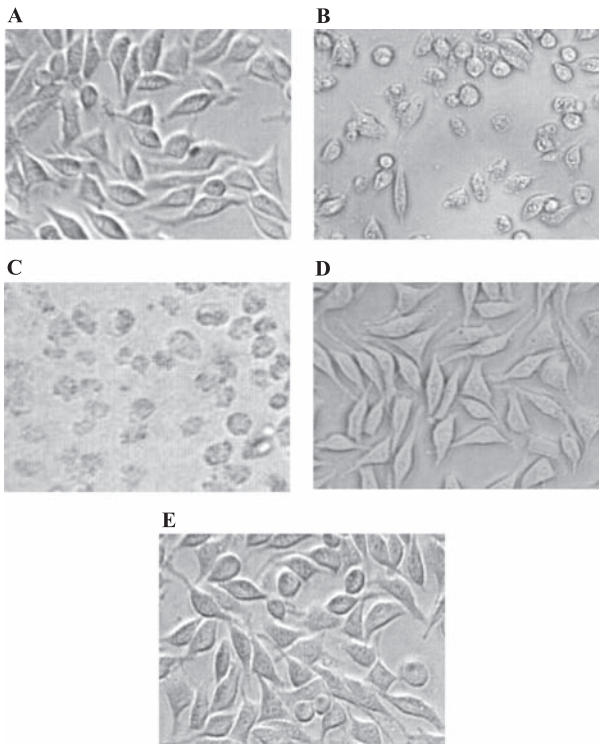


Fig. 3. Oridonin-induced morphologic changes in L929 cells. The cellular changes were observed after the cells were cultured in the medium for 24 h (A); 65 μM oridonin for 24 h (B); 65 μM oridonin and 20 μM z-VAD-fmk for 24 h (C); 65 μM oridonin and 5 mM NAC for 24 h (D); 65 μM oridonin, 20 μM z-VAD-fmk, and 5 mM NAC for 24 h (E). (×200 magnification)

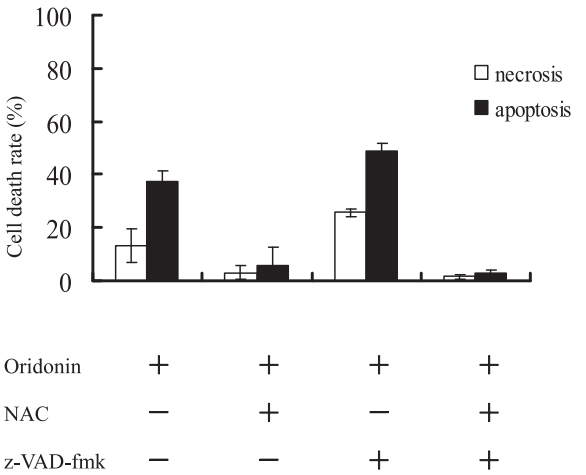


Fig. 4. Characterization of cell death by LDH-based assay. L929 cells were pretreated with 20 μM z-VAD-fmk or 5 mM NAC for 1 h and then incubated with 65 μM oridonin for 24 h. The cell death rate was measured by LDH-based assay. n = 3, mean ± S.D.

oridonin-induced L929 cell death, the ratio of LDH released from viable cells, floating dead cells, and the culture medium were compared (Fig. 4). After incubation with oridonin for 24 h, the majority of the cells underwent apoptosis. However, a small number of necrotic cells was still observed. The pan-caspase inhibitor z-VAD-fmk augmented both the ratio of apoptosis and necrosis in oridonin-treated L929 cells. Consistent with the cytotoxicity assay, NAC also played a role of protector to prevent cell death.

Oridonin-induced increase in ROS generation was enhanced by pan-caspase inhibitor z-VAD-fmk

To determine the involvement of ROS during oridonin-induced cell death in L929 cells, we monitored ROS generation with flow-cytometry. As compared to the control group, ROS generation significantly increased after exposure to oridonin for 2 h (Fig. 5: A and B). The pan-caspase inhibitor z-VAD-fmk effectively augmented oridonin-induced intracellular ROS generation (Fig. 5C). As shown in Fig. 5: D and E, NAC pretreat-

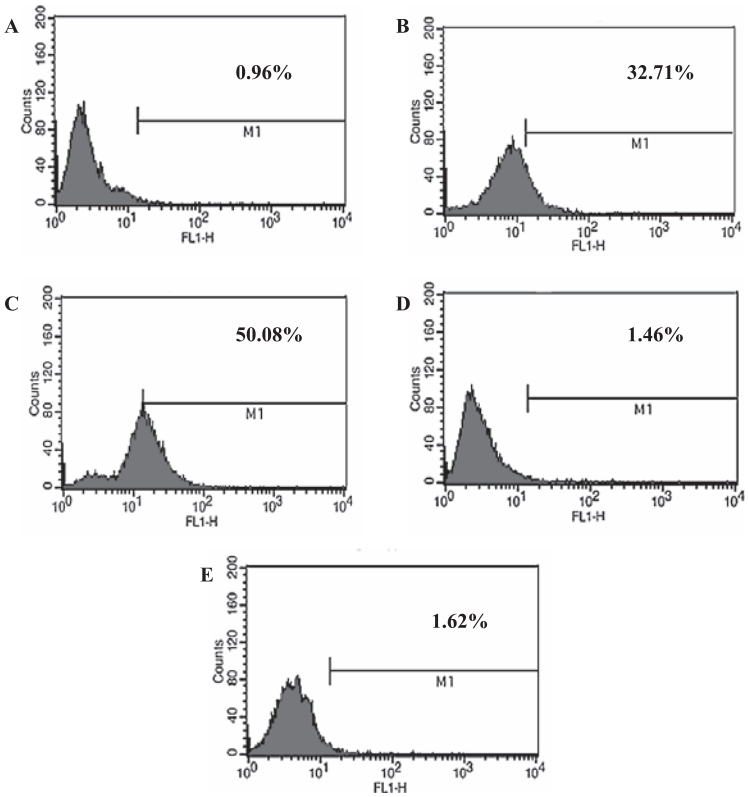


Fig. 5. Flow cytometric analysis of ROS generation in oridonin-treated L929 cells. A: medium control; B: the cells were cultured with 65 μM oridonin for 2 h; C: the cells were pretreated 20 μM z-VAD-fmk for 1 h and then cultured with 65 μM oridonin for 2 h; D: the cells were pretreated 5 mM NAC for 1 h and then incubated with 65 μM oridonin for 2 h; E: the cells were pretreated 5 mM NAC and 20 μM z-VAD-fmk for 1 h and then cultured with 65 μM oridonin for 2 h.

ment totally blocked ROS generation induced by oridonin as well as that by oridonin and z-VAD-fmk. A rapid production of ROS was capable of triggering not only apoptosis but also necrosis.

Oridonin triggered a marked loss of MMP in L929 cells

As mitochondria constitute the main source of ROS generation, we then tried to characterize the relationship between ROS production and changes in MMP. MMP was detected with Rho-123 staining by flow cytometric analysis. Exposure of L929 cells to oridonin for 24 h caused a marked loss of MMP compared with the control group, whereas the collapse of MMP was completely reversed by NAC pretreatment group, and caspase inhibition produced a progressive loss of MMP (Fig. 6). These results suggested the oridonin was capable of inducing mitochondrial dysfunction and that MMP loss might be affected indirectly by ROS generation.

Involvement of Bcl-2, Bax, and caspase-3 in oridonin-induced cell death

Since the Bcl-2 family members play a critical role in cell apoptosis (4), Western blot analysis was carried out to assess the expression of the Bcl-2 family protein Bax and Bcl-2. After treatment with oridonin for different time periods, the expression of Bax was up-regulated, whereas the expression of Bcl-2 was down-regulated (Fig. 7A). In addition, the 32-kDa band of procaspase-3 was degraded after oridonin-treatment, indicating the activation of the caspase-3 (Fig. 7B).

In order to evaluate the relationship between Bcl-2 family members and ROS, the cells were pretreated with NAC and then incubated with oridonin for 24 h. As shown in Fig. 8A, NAC pretreatment significantly reversed the changes of Bax and Bcl-2 expression compared to oridonin alone treatment. However, the pan-caspase inhibitor z-VAD-fmk increased the expression of Bax slightly. The expression of Bax and Bcl-2 might be indirectly affected by ROS generation, and ROS was indeed responsible for the oridonin-induced cell death.

Contribution of p53 in oridonin-induced cell death

As Bax expression was increased, attention was drawn to the tumor suppressor p53 because of the existence of the p53-binding element in the Bax gene-promoter region (7). Western blot results showed that the expression of phospho-p53 (ser 15) was significantly upregulated after incubation with oridonin for 24 h, whereas the expression of p53 did not show obvious change. However, the expression of phospho-p53 was completely blocked by NAC pretreatment. Interestingly,

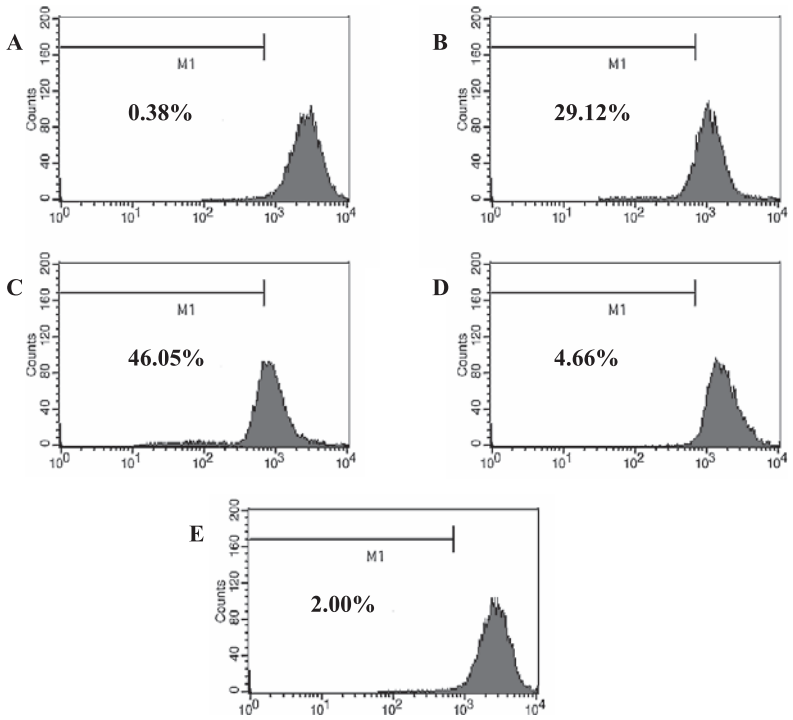


Fig. 6. Analysis of MMP loss during oridonin treatment. A: medium control; B: The cells were incubated with 65 μM oridonin for 24 h; C: The cells were pretreated with 5 mM NAC for 1 h and then incubated with 65 μM oridonin for 24 h; D: the cells were pretreated with 20 μM z-VAD-fmk for 1 h and then incubated with with 65 μM oridonin for 24 h; E: The cells were pretreated with 5 mM NAC and 20 μM z-VAD-fmk for 1 h and then incubated with 65 μM oridonin for 24 h; The cells were collected and washed with PBS, followed by incubation at room temperature with PBS containing 1 μg/ml rhodamine 123 for 30 min.

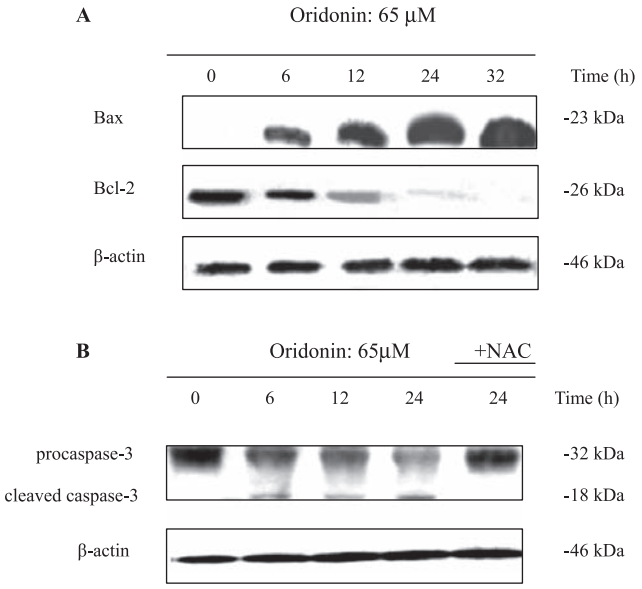


Fig. 7. Western blot analysis of Bax and Bcl-2 (A) and caspase-3 protein (B) expression. The cells were treated with 65 μM oridonin for various time periods. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and the protein expressions were detected by Western blotting. β-Actin was used as an equal loading control.

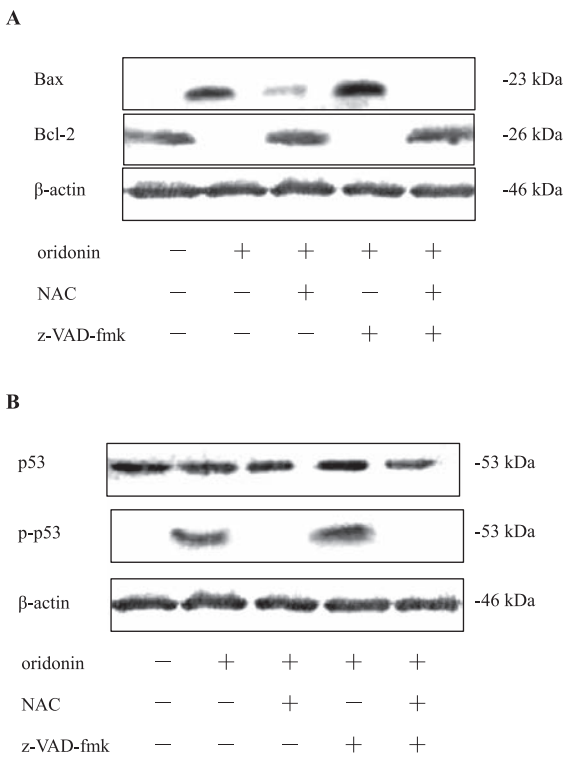


Fig. 8. Expressions of Bax, Bcl-2, and p53 in L929 cells. The cells were pretreated with 5 mM NAC and 20 μM z-VAD-fmk for 1 h and then cultured with 65 μM oridonin for 24 h. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and the protein expressions were detected by Western blotting. β-Actin was used as an equal loading control.

both p53 and phospho-p53 protein expression were not affected by the pan-caspase inhibitor (Fig. 8B). Taken together, excessive ROS generation may cause DNA damage, p53 activation, and upregulation of Bax/Bcl-2 ratio.

Discussion

It is well known that apoptosis and necrosis are two forms of cell death, with distinct morphological and biochemical features. In this study, we demonstrated that oridonin induced apoptosis and necrosis in L929 cells based on changes of cellular morphology and LDH assay. However both the pan-caspase inhibitor z-VAD-fmk and caspase-3 inhibitor z-DEVD-fmk rendered the cells even more sensitive to oridonin. The ratios of apoptosis and necrosis were both augmented by caspase inhibitors. This result was different from other studies, which showed that caspase inhibition can switch the mode of death from apoptosis to necrosis in cyanide-treated L929 cells (20).

Groossens and his colleagues reported that caspase inhibitors augmented TNFα-induced cell death in L929. This sensitization may have a connection with the production of ROS (14). Furthermore, other studies have demonstrated that intracellular ROS generation is intimately associated with apoptosis and necrosis (21, 22). In this study, a rapid and significant ROS generation after exposure to oridonin for 2 h was observed; furthermore, ROS production was augmented by pan-caspase inhibitor. Consistent with these results, apoptotic cell death was also augmented in the presence of the pan-caspase inhibitor z-VAD-fmk. However, administration of the ROS scavenger NAC resulted in a complete inhibition of ROS generation as well as cell death. Therefore, ROS generation was capable of triggering oridonin-induced apoptosis and necrosis in L929 cells.

Apoptosis is a tightly regulated process, which involves changes in expression of distinct genes and pathways related to up-regulation of pro-apoptotic (Bax, Bid) or down-regulation of anti-apoptotic (Bcl-2) proteins. It was well documented that increased ROS generation caused mitochondrial dysfunction and down-regulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL (23). In this study, the expression of Bax protein increased in response to oridonin treatment, while the expression of Bcl-2 gradually decreased with time. In addition, oridonin induced ROS generation in the early stages, but the loss of MMP was observed to persist after exposure to oridonin for 24 h. However, pretreatment with NAC not only reversed the expression of Bax and Bcl-2, but also resulted in the complete inhibition of oridonin-induced MMP collapse. These results indicate that a rapid ROS generation took place in the early stages after stimulation, and that was capable of functioning as an initial mediator in the apoptotic pathway.

It is well known the expression of Bax is regulated by p53 tumor suppressor, p53 mediates cell cycle arrest and apoptosis in response to DNA damage, and p53 mutation is closely associated with malignant tumor (8). Other studies also demonstrated that excessive ROS generation was responsible for activation of the p53 (24). P53 has a short half-life, and p53 phosphorylation at multiple sites is the main post-translational modification. In this study, phosphor-p53 (ser 15) was activated after treatment with oridonin for 24 h, which was positively correlated with the generation of ROS and up-regulation of Bax expression. Moreover, p53 phosphorylation (ser 15) was enhanced by pan-caspase inhibitor. Excessive ROS production in oridonin-treated L929 cells may cause serious damage to DNA, then induction of p53 phosphorylation (ser 15), up-regulation of Bax expression, and resulting in MMP collapse. Mitochondria may release some proapoptotic molecules including cytochrome *c* and AIF, which caused caspase-dependant and caspase-independent apoptosis, respectively. In this system, it is possible that caspase-independent apoptosis became a primary form of cell death when caspase-dependant apoptosis was blocked by the pan-caspase inhibitor z-VAD-fmk.

Proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) by caspase is a hallmark of apoptosis, but whether it is indispensable in the process of apoptosis remains unclear. Some researchers reported that over-activating PARP by the pan-caspase inhibitor z-VAD-fmk could promote ROS generation in TNFα-treated L929 cells because of depletion of NAD^+ and ATP. Furthermore, the PARP inhibitor 3-aminobenzamide (3-AB) prevented ROS production as well as cell death induced by TNFα (25). However, our previous studies suggested that PARP cleavage was required for oridonin-induced cell death in L929 (15 – 17), which seemed to be somewhat controversial. It is possible that the Ca^{2+}-dependent proteinase calpian takes the place of caspase-3 after co-treatment with oridonin and z-VAD-fmk (26). Further studies are need to elucidate the relationship between oridonin and PARP.

In conclusion, oridonin induced a rapid and significant ROS generation in L929 cells, subsequently triggering p53- and Bax-mediated cell death. Pretreatment with caspase inhibitors led to an even higher ROS production, which in turn caused a more pronounced mitochondrial dysfunction. As a result, the pathway(s) of cell death might be switched from caspase-dependent to caspase-independent apoptosis.

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