A Comparison of the Signal Pathways between the TNFα- and Oridonin-induced Murine L929 Fibrosarcoma Cell Death

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Oridonin, an active component isolated from *Rabdosia rubescences*, has been reported to have antitumor effects. In this study, we compared the signal transduction pathways between TNFα- and oridonin-induced L929 cell death. Oridonin and TNFα initiated apoptotic morphologic changes, but DNA fragmentation was found in TNFα-treated L929 cells but not in oridonin-treated ones. The pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk) and caspase-3 inhibitor (z-DEVD-fmk) augmented oridonin- and TNFα-induced cell death. However, the caspase-9 inhibitor (z-LEHD-fmk) only increased oridonin-induced L929 cell death. Moreover, poly (ADP-ribose) polymerase (PARP) was cleaved in oridonin-treated L929 cells but not in the TNFα-treated groups, and the caspase-3 inhibitor (z-DEVD-fmk) failed to inhibit PARP cleavage. These results showed that only oridonin-induced L929 cell death required PARP degradation in a caspase-3 independent manner. In addition, oridonin increased the ratio of Bax/Bcl-2 protein expression, but TNFα did not. TNFα induced p38 and ERK activation, whereas oridonin triggered only ERK activation. We also investigated the effect of oridonin on intracellular TNFα expression, and found that oridonin augmented endogenous pro-TNFα expression and its upstream protein IκB phosphorylation. These results indicated that although oridonin promoted endogenous pro-TNFα expression, a great difference existed between the signal pathways through which TNFα- and oridonin-induced cell death.

Key words: oridonin, caspase, Bax/Bcl-2, MAPK, IκB

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inflammatory, anti-bacterial and anti-tumor effects [7-8]. Our previous study showed that oridonin had cytotoxic effects on L929 cells, and that caspase inhibitors also did not protect against oridonin-induced L929 cell death, but instead made the L929 cells more sensitive to oridonin. Therefore, we compared the signal pathways between the TNFα- and oridonin-induced murine L929 fibrosarcoma cell death.

It is well known that the caspase family, mitochondria, and MAPK family signal transduction pathways play an important role in regulating apoptosis. In this study, we further investigated the signal transduction pathways mentioned above to analyze the relationship between TNFα- and oridonin-induced L929 cell death. The results showed that oridonin was different from TNFα in many aspects of induction of L929 cell death though oridonin-promoting endogenous pro-TNFα expression.

Materials and Methods

Regents. Oridonin was obtained from the Beijing Institute of Biological Products (Beijing, China). The structure of oridonin was assigned by comparing the chemical and spectral data (1H-NMR, 13C-NMR) with those reported in the literature. The purity of the oridonin was measured by HPLC and determined to be 99.2%, and oridonin was resolved in the medium. Human TNFα was prepared from PMAL-C3-TNFα/JM109 (E. coli.) in our laboratory. Pan-caspase inhibitor (z-VAD-fmk), caspase-3 inhibitor (z-DEVDFmk), caspase-9 inhibitor (z-LEHD-fmk) and caspase-8 inhibitor (z-IETD-fmk) were purchased from Enzyme Systems (Livermore, CA, USA). ERK inhibitor (PD98059), p38 inhibitor (SB203580), and JNK inhibitor (SP600125) were obtained from Calbiochem (La Jolla, CA, USA). Polyclonal antibodies against IκB phospho- IκB, β-actin, and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-2 and Bax were from Oncogene Research Products (Boston, MA, USA).

Cell Culture. The murine fibrosarcoma cells (L929#CRL-2148) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium ( Gibco, NYC, NY, USA), supplemented with 10% FBS (Shengma Yuanheng, Beijing, China), 100 mg/l streptomycin, 100 IU/ml penicillin, and 0.03% L-glutamine and maintained at 37 °C with 5% CO₂ in a humidified atmosphere.

Cytotoxicity Assay. The L929 cells were incubated at 1 × 10⁵ cells/well in 96-well plates (NUNC, Roskilde, Denmark). The cells were incubated with z-VAD-fmk, z-IETD-fmk, z-DEVDFmk, z-LEHD-fmk, cyclosporine A, PD98059, SB203580, or SP600125 at given concentrations for 1 h, and then treated with TNFα for different time periods. Cell growth was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The percentage of cell growth inhibition was calculated as follows:

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\text{Cell death (%) =} \frac{A_{570}(\text{control}) - A_{570}(\text{oridonin})}{A_{570}(\text{control})} \times 100
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Observation of Morphological Changes. The L929 cells were divided into 2 groups and placed on culture plates and cultured for 18 h. One group was treated with the control medium and the other group was treated with TNFα (20 ng/ml) and the cellular morphology was observed using phase contrast microscopy (Leica, Wetzlar, Germany).

Nuclear Damage Observed by Hoechst 33258 Staining. Apoptotic nuclear morphology was assessed using Hoechst 33258. The cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature and then washed and stained with 167 µmol/l Hoechst 33258 at 37 °C for 30 min. The cells were washed and resuspended in PBS for morphologic observation using fluorescence microscopy (Leica, Wetzlar, Germany).

DNA fragmentation assay. The L929 cells (1 × 10⁶) were collected by centrifugation at 150 × g for 5 min, and washed with PBS. The cells were pelleted and suspended in 10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0), and 0.5% Triton X-100 and kept at 4 °C for 10 min. The supernatant was incubated with 20 mg/ml RNase A (2 µl) and 20 mg/ml proteinase K (2 µl) at 37 °C for 1 h, then kept in 0.5 M NaCl (20 µl) and isopropanol (120 µl) at −20 °C overnight, and then centrifuged at 15,000 × g for 15 min. DNA was dissolved in TE buffer [10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0)] and subjected to 2% agarose gel electrophoresis at 50 V for 40 min and stained with ethidium bromide.

LDH Activity-Based Cytotoxicity Assays. The LDH (lactate dehydrogenase) activity was assessed using a standardized kinetic determination (Zhongsheng LDH kit, Beijing, China). The LDH activity 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 obtained from the pellets was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (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 percentages of apoptotic and necrotic cell death were calculated as follows:

Apoptosis (%) = LDHp/(LDHp + LDHi + LDHe) × 100
Necrosis (%) = LDHe/(LDHp + LDHi + LDHe) × 100

Western Blot Analysis. The L929 cells were treated with TNFα (20 ng/ml) for different time periods. Both adherent and floating cells were collected, then Western-blots analysis was performed as previously described, with some modification. Briefly, the cell pellets were resuspended in lysis buffer consisting of 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 and leupeptin (Sigma) 10 mg/l and lysed at 4 °C for 60 min. After 13,000 × g centrifugation for 15 min, the protein content of the supernatant was determined by a protein assay reagent (Bio-Rad, Hercules, CA, USA). The protein lysates were separated by electrophoresis in 12% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. Proteins were detected using polyclonal antibody and visualized 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 ± S.D. Statistical comparisons were made by student’s t-test. p < 0.05 was considered significant.

Results

Cytotoxic Effects of TNFα and oridonin on L929 cells. To investigate the cytotoxic effects of oridonin and TNFα against L929 cells, the L929 cells were cultured with oridonin or TNFα at various doses for 24 h. We found that both oridonin and TNFα induced L929 cell death in a dose-dependent manner. Oridonin 25 µM or TNFα 20 ng/ml caused almost 50% of the L929 cell deaths (Figs. 1A, B).

Characterization of TNFα- and oridonin-induced L929 cell death. To characterize the oridonin- and TNFα-induced L929 cell death, we examined the morphologic changes and DNA fragmentation. When L929 cells were cultured with oridonin 25 µM or 20 ng/ml TNFα for 18 h, marked morphologic changes were observed as compared with the medium control group. The oridonin- or TNFα-treated L929 cells underwent contraction and became round in shape, with shrunken nuclei (Figs. 2C, J, E, H), but the untreated cells did not show these apoptotic changes (Fig. 2A). Morphologic changes were further confirmed by Hoechst 33258 staining of the cell nuclei. In the control group, the nuclei of the L929 cells were round and homogeneously stained (Fig. 2B), but the 25 µM oridonin- or 20 ng/ml TNFα-treated cells showed marked nuclear fragmentation (Figs. 2D, F).

DNA regular fragmentation is another hallmark of typical apoptosis [9]. In our study, DNA ladders were observed in TNFα-treated but not in oridonin-treated L929 cells by agarose electrophoresis (Fig. 3).

In order to further investigate whether TNFα and oridonin also initiated necrosis in L929 cells, the rates of lactate dehydrogenase (LDH) released from viable cells, floating dead cells, and the culture medium were measured. In the presence of oridonin 5–50 µM, the number

Fig. 1  Cytotoxic effects of oridonin or TNFα on L929 cells. The cells were treated with various doses of oridonin (A) or TNFα (B) for 24 h. The cell death rate was measured by MTT assay. n = 3, Mean ± S.D.
of necrotic cells increased from 5.5% to 32.3% (Fig. 4A). Moreover, in the presence of TNFα 10–400 ng/ml, the number of necrotic cells increased from 8.8% to 37.4% (Fig. 4B). Therefore, we concluded that both TNFα and oridonin induced cell death through the balance between apoptosis and necrosis.

The caspases protected against TNFα- and oridonin-induced cell death. It is well known that caspases are required in apoptosis. Some researchers have also reported that caspase inhibitors render L929 cells more sensitive to TNFα [10, 11]. We further compared the function of caspase inhibitors in TNFα-
and oridonin-induced L929 cell death. In the TNFα- and oridonin-treated L929 cells, the inhibitor of caspase-3, z-DEVD-fmk, the inhibitor of caspase-8, z-IETD-fmk, and the pan-caspase inhibitor, z-VAD-fmk, augmented the cell death to a different degree. The caspase-9 inhibitor, z-LEHD-fmk, had almost no effect on the TNFα-induced L929 cell death, but it made the cells even more sensitive to oridonin. In the 25 μM oridonin-treated L929 cells, the caspase-9 inhibitor, z-LEHD-fmk, augmented the cell death ratio from 42% (cultured with oridonin alone) to 69% at 24 h (Figs. 5A, B). In the LDH-based assay, we found that the caspase inhibitors increased both apoptosis and necrosis in the oridonin-treated L929 cells (Fig. 5 C). The results suggest that the caspases play protective roles against TNFα- or oridonin-induced L929 cell death, but caspase-9 was only effective on oridonin-induced L929 cell death.

**Bcl-2/Bax involvement in oridonin-induced but not in TNFα-induced L929 cell death.** It is reported that the Bcl-2 family of proteins plays a role in either the inhibition or promotion of apoptotic cell death [12]. We also examined the expression of the Bcl-2 family protein, Bcl-2 and Bax, in oridonin- and TNFα-treated L929 cells by Western-blot analysis. The results showed that the treatment of L929 cells with 25 μM oridonin decreased the Bcl-2 expression and increased the Bax expression, but in the TNFα-treated L929 cells, the Bax and Bcl-2 expressions did not change with the time period (Fig. 6), indicating that only the Bcl-2/Bax ratio was involved in the oridonin-induced
L929 cell death.

MAPK modulation in TNFα- and oridonin-induced L929 cell death. MAPK signaling pathways have been shown to be implicated in cell growth and cell death [13]. To compare the regulation of the MAPK cascades in TNFα- and oridonin-induced cell death, specific inhibitors for p38 (SB203580), JNK (SP600125) and ERK (PD98059), were applied to evaluate the function of MAPK in both the TNFα- and oridonin-induced L929 cell death. After incubation with 20 ng/ml TNFα or 25 μM oridonin for various periods of time, 5 μM SB 203580 and PD98059 markedly reduced TNFα-induced cell death, but the PD98059 inhibitory function declined with the time period. However, in the oridonin-treated L929 cells, only PD98059 effectively reversed the cell death (Fig. 7). The results showed that in the TNFα- and oridonin-treated L929 cells, ERK had the same functions of mediating the cell death, but p38 merely regulated the TNFα-induced L929 cell death.

PARP regulation in both the TNFα- and oridonin-induced L929 cell death. To investigate the role of PARP, a downstream regulator, the PARP inhibitor (DPQ) was applied to access the PARP
Figure 6: Western-blot analysis of Bax and Bcl-2 protein expression in oridonin- and TNF-α-treated L929 cells. The cells were treated with 25 μM oridonin (A) or 20 ng/ml TNF-α (B) for various time periods. The cell lysates were separated by 12% SDS-PAGE electrophoresis and the protein expression was detected by Western-blot analysis.

Figure 7: Effects of MAPK inhibitors on oridonin- and TNF-α-treated L929 cell death. The cells were treated with 25 μM oridonin (A) or 20 ng/ml TNF-α (B) and MAPK inhibitors including 5 μM SP600125 (JNK), 5 μM SB203580 (p38) or 5 μM PD98059 (ERK) for various time periods. Cell death was measured by MTT assay. n = 3, Mean ± S.D., **p < 0.01 vs TNF-α or oridonin group.

Figure 8: Effects of PARP inhibitor DPQ on oridonin- and TNF-α-induced L929 cell death. The cells were treated with 25 μM oridonin or 20 ng/ml TNF-α in the presence of the PARP inhibitor DPQ with various doses for 18 h. Cell death was measured by the MTT assay. n = 3, Mean ± S.D., **p < 0.01 vs oridonin (alone) group.
function in both the oridonin- and TNFα-induced L929 cell death. It was found that 20 μM DPQ reduced the oridonin-induced cell death from 60.1 to 41.2% at 18 h; however, DPQ almost had no inhibitory effect on the TNFα-induced L929 cell death (Fig. 8). In order to further confirm the PARP participation in the oridonin-induced cell death, we examined the PARP protein expression by Western-blot analysis. The results showed that after 18 h treatment with 25 μM oridonin, 116 KDa PARP protein was cleaved to the 85 KDa fragment, and that the caspase-3 inhibitor, z-DEVD-fmk, failed to inhibit the PARP cleavage (Fig. 9), but in the 20 ng/ml TNFα-treated group, we did not detect the PARP degradation, indicating that PARP was required for the oridonin-induced L929 cell death but not for the TNFα-induced cell death in a caspase-3 independent manner.

**Oridonin up-regulated intracellular pro-TNFα expression in L929 cells.** We also examined the effect of oridonin on the intracellular pro-TNFα expression by Western-blot analysis. Exposure of the L929 cells to 25 μM oridonin for 14 h initiated the pro-TNFα expression. In order to further confirm this, we also analyzed the IκB expression and IκB phosphorylation in oridonin-treated L929 cells. The NF-κB transcription factor plays an essential role in the expression of proinflammatory cytokines such as TNFα and IL-1. NF-κB activity is controlled by the inhibitory IκB protein, which masks the nuclear localization sequence in the Rel homology domain of NF-κB, thereby sequestering NF-κB in a latent state in the cytoplasm [14-16]. The results showed that treatment with 25 μM oridonin for 12 h reduced the IκB expression and increased IκB

![Western-blot analysis of PARP protein expression in oridonin- and TNFα-treated L929 cells.](image)

**Fig. 9** Western-blot analysis of PARP protein expression in oridonin- and TNFα-treated L929 cells. The cells were treated with 25 μM oridonin (A) or 20 ng/ml TNFα (B) for various time periods. The cell lysates were separated by 12% SDS-PAGE electrophoresis, and the protein expression was detected by Western-blot analysis.

![Western-blot analysis of endogenous pro-TNFα protein expression in oridonin-treated L929 cells.](image)

**Fig. 10** Western-blot analysis of endogenous pro-TNFα protein expression in oridonin-treated L929 cells. The cells were treated with 25 μM oridonin for various time periods. The cell lysates were separated by 12% SDS-PAGE electrophoresis, and the protein expression was detected by Western-blot analysis.
phosphorylation (Fig. 10). Thus, these results suggested that oridonin up-regulated the TNFα expression through activation of the transcription factor NF-κB.

Discussion

In this study we compared the signal pathways between the TNFα- and oridonin-induced murine L929 fibrosarcoma cell death, and the results showed that differences exist between the oridonin- and TNFα-induced cell death signal pathways. Although we observed the apoptotic changes of cellular morphology in both the oridonin- and TNFα-treated groups, DNA fragmentation did not occur in the oridonin-treated group, but did in the TNFα-treated group. DNA fragmentation, a hallmark of apoptosis, is the internucleosomal DNA degradation result, which a Ca2+, Mg2+-dependent deoxyribonuclease I (DNase I) accounts for. In addition, several isoforms of DNase I exist, and some of them are unable to degrade the DNA into the apoptotic ladder, but cause a fuzzy pattern of digested nuclear DNA when apoptosis occurs [17]. Based on the results of our experiment, we speculated that unlike TNFα, oridonin might initiate the activation of the isoform(s) of DNase I, and this kind of DNase I digested the DNA into smaller-sized fragments.

Caspases are a family of cysteases, which cleave protein substrates after their Asp residues. Caspases appear to be involved in regulating the activation of apoptotic signal transmission, and are divided into 2 classes based on the lengths of their N-terminal prodomains, including upstream caspases such as caspase-2, -8, and -10, and downstream caspases such as caspase-3, -6, and -7 [18]. In addition, previous reports have demonstrated that the caspase inhibitors augment TNFα-induced cell death in L929 cells and in RAW264.7 macrophages [19]. In L929 cells, it has been inferred that caspases act as a negative regulator of reactive oxygen intermediate (ROI) [6]. In this study, the inhibitor of caspase-3, z-DEVD-fmk, the inhibitor of caspase-8, z-IETD-fmk, and the pan-caspase inhibitor, z-VAD-fmk augmented the cell death in both the TNFα- and oridonin-treated L929 cells; however, the caspase-9 inhibitor, z-LEHD-fmk, only made the L929 cells more sensitive to oridonin, indicating that caspase-9 was only activated in oridonin-induced L929 cell death. Bcl-2 family members are characterized by the fact that they contain at least one of four Bcl-2 homology domains (BH1-BH4). Some of these proteins, such as Bax, function to promote apoptosis, whereas others like Bcl-2 inhibit apoptosis [12]. In this study, only oridonin up-regulated the Bax expression and down-regulated the Bcl-2 expression in L929 cells. In mitochondria, when apoptosis occurred, cytochrome c is released into the cytoplasm, and then cytochrome c, Apaf-1 and pro-caspase-9 form a complex. Activated caspase-9 cleaved the caspase-3 proenzyme [20]. PARP is a nuclear enzyme that responds to DNA damage, facilitates DNA repair, and promotes cell survival [21]. When apoptosis occurs, it is cleaved to an 85-KDa fragment by caspase-3 [22]. In this study, the PARP inhibitor DPQ reduced oridonin-induced L929 cell death, but had no inhibitory effect on TNFα-induced L929 cell death. In addition, an 85-KDa fragment was also observed after 12 h treatment with oridonin, and the caspase-3 inhibitor failed to suppress PARP degradation, indicating that PARP was involved in oridonin-induced L929 cell death but not in TNFα-induced L929 cell death. Together, all these results suggested that unlike in TNFα-induced cell death, oridonin-induced L929 cell death required the up-regulation of Bax, caspase-9 activation and PARP cleavage, and that some protease(s) different from caspase-3 might be responsible for the degradation of PARP.

The MAPK family members, including extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, play important roles in apoptosis and necrosis. The ERK pathway is predominantly activated by mitogens through a Ras-dependent mechanism, which is required for cell proliferation and differentiation; however, JNK and p38 are activated by pro-inflammatory cytokines and various environmental stresses [23, 24]. In this study, the results showed that in both the TNFα- and oridonin-treated L929 groups, ERK had the same functions of mediating cell death, but p38 only mediated TNFα-induced L929 cell death. Activation of the distinct MAPK subtype cascade depends on the types of cells and the stimuli, and the functional role of each MAPK subtype may be different according to the cell types [13]. Therefore, ERK was activated as the same functional pathway in both the TNFα- and oridonin-induced L929 cell death; however, the p38 signal cascade might be activated as different signal pathways involved in the TNFα-treated L929 cells.

TNFα is a pleiotropic cytokine which is involved in inflammatory and immune responses. The nuclear factor
NF-κB plays a crucial role in pro-TNFα gene expression mediated by agents such as TNFα and PMA. IκB is phosphorylated and targeted to the 26S proteasome complex, resulting in the release and nuclear translocation of NF-κB [14–16]. We investigated the effect of oridonin on TNFα expression, and the results showed that oridonin increased pro-TNFα expression and IκB phosphorylation, and reduced IκB expression, indicating that oridonin up-regulated TNFα expression through activating the transcription factor NF-κB. Endogeneous pro-TNFα might be c leaked and secreted into medium, then TNFα might be bound to its receptors and promote oridonin-induced cell death. However, in our study, oridonin induced L929 cell death in very different ways from TNFα, so we speculated that the pro-TNFα was not c leaked or secreted into the medium.

In summary, we compared the signal pathways between the TNFα and oridonin-induced murine L929 fibrosarcoma cell deaths and found that oridonin increased the endogeneous pro-TNFα expression. However, there was a considerable difference between the TNFα- and oridonin-induced L929 cell death signal pathways. Moreover, oridonin is a diterpenoid compound and easily enters the cytoplasm, so target proteins may exist for oridonin to bind with in the upstream of the cell death signaling pathway.

References