A novel combination of oridonin and valproic acid in enhancement of apoptosis induction of HL-60 leukemia cells

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Abstract. Oridonin, obtained from the traditional Chinese herbal medicine rabdosia rubescens, exerts potent antitumor activities in cancer cells. Valproic acid (VPA), as a potent histone deacetylase inhibitor (HDACI), also plays an important role in inhibition of proliferation of tumor cells. However, there are no reports so far on the cooperation between oridonin and VPA for anti-leukemic effect. Therefore, in the present study, we undertook experiments to determine whether lower concentration of oridonin in conjunction with lower concentration of VPA would produce even more encouraging synergistic effect than each of them alone, and to clarify its molecular mechanism. The results demonstrated that the lower concentration of oridonin in combination with lower concentration of VPA synergistically inhibited the proliferation of HL-60 cells, and induced obvious caspase-dependent apoptosis through activation of the intrinsic apoptosis pathway, which is involved in the downregulation of Bcl-2/Bax ratio, release of cytochrome *c* to cytosol and caspase-9 activation, as well as through the extrinsic apoptosis pathway mediated by Fas/FasL and caspase-8 activation. In addition, MAPK signaling pathway was also involved in apoptosis induced by oridonin plus VPA. Furthermore, the combination treatment *in vivo* remarkably reduced the xenograft tumor size and triggered tumor cell apoptosis. Taken together, the novel combination of oridonin plus VPA exerted synergistic anti-proliferative and apoptosis-inducing effects on human myeloid leukemia cells, and may serve as a potential promising anti-leukemia strategy.

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Introduction

Acute promyelocytic leukemia (APL) accounts for ~5-10% of acute myeloid leukemia (AML) (1). It is identified as a distinct entity among the AML by its consistent chromosomal translocation t(15;17) with corresponding PML-RARα and RARA-PML fusion genes (2). APL accounted for high mortality rates during induction therapy by chemotherapy drugs because of bleeding diathesis in the pre-all transretinoic acid (ATRA) era (3). However, the discovery in the late 1980s of the clinical efficacy of ATRA, which promotes the terminal differentiation of malignant promyelocytes to mature neutrophils, changed the natural history of APL (4,5). Regimens using a combination of ATRA and anthracyclines (such as idarubicin or daunorubicin) have been shown to achieve very high remission rates of up to 90% and prolong event-free survival in patients with APL (6-8). Now it has become the standard treatment for induction and consolidation in APL. The use of arsenic trioxide (ATO) since early 1990s further improved the clinical outcome of refractory or relapsed as well as newly diagnosed APL (9,10). While death during the induction phase from causes such as haemorrhage, differentiation syndrome (DS) and infection poses a significant challenge in early treatment, resistance to therapy is an uncommon cause of induction failure (11). However, so far only ATRA alone, or combined with ATO, was able to induce CR in most of patients with APL, and they do not work on the other types of patients

with AML. Therefore, scientists have begun to explore new agents and strategies to induce differentiation or apoptosis of leukemia cells. The combination of traditional Chinese medicine and Western medicine is possibly an ideal choice to treat patients with AML.

Oridonin is a diterpenoid compound isolated from the Chinese medicinal herb Isodon rubescens, and possesses a variety of biological effects such as anti-inflammatory, antiviral and immunoregulatory functions (12). To date, oridonin has been demonstrated to be an effective anti-tumor agent with significant effects on some malignancies of different pathological types, included acute leukemia (13,14). A recent study showed that oridonin induced potent growth inhibition, cell cycle arrest and apoptosis induction by increasing histone hyperacetylation (H3 and H4) and regulation of p16, p21, p27 and c-myc (15). Another report revealed that oridonin greatly enhanced apoptosis induced by As_2O_3 in hepatocellular carcinoma cells by increasing intracellular reactive oxygen species (ROS) level, decreasing mitochondrial membrane potential (MMP), and relocating Bax and cytochrome *c* (16).

Histone deacetylase (HDAC) inhibitors have emerged recently as promising antineoplastic agents (17). By promoting histone acetylation, HDAC inhibitors permit chromatin to assume a more relaxed state, thereby allowing transcription of genes involved in cell cycle arrest, differentiation and (or) apoptosis. Valproic acid (VPA), as a well-tolerated agent for neurological disorders, has been safely used for >30 years. VPA has been shown to be a histone deacetylase inhibitor which binds to and directly inhibits HDAC (18,19). Accumulating evidence demonstrates that VPA can induce apoptosis or differentiation of leukemia cells either alone or in combination with other anti-leukemic agents (20,21).

Therefore, prompted by the above reports and based on effect of oridonin and VPA on histone acetylation, we determined whether oridonin in conjunction with VPA would produce even more encouraging synergistic effect than each of them alone, which has not been reported so far. The results indicated that combination of oridonin plus VPA could potentially be a promising regimen for treatment of AML.

Materials and methods

Reagents. Oridonin was kindly provided by Dr Xiao Wang (Shandong Academy of Sciences). It was dissolved in DMSO at a stock concentration of 5 mg/ml and was stored at -20˚C. Valproic acid sodium salt (VPA) was from Sigma. Caspase-inhibitor (Z-VAD-fmk), JNK inhibitor (SP600125), p38 inhibitor (SB203580), ERK inhibitor (PD98059) and Hoechst 33342 was obtained from Beyotime Biotechnology, Inc. (Nantong, China). Annexin V fluorescein isothiocyanate (FITC) kit was obtained from BD Biosciences (San Diego, CA, USA). Antibodies for detecting Bax, Bcl-2, cleaved caspase-3, caspase-8, caspase-9, Fas, FasL, ERK, p38, phosphorylated-JNK, phosphorylated-ERK, phosphorylated-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). GAPDH antibody was purchased from Proteintech Group, Inc. (Chicago, IL, USA). JNK antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-labeled IgG anti-mouse and anti-rabbit antibodies were supplied by Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan).

Cell culture. Human acute myeloid leukemia HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum (NCS, Sijiqing Biotechnology Co. Hangzhou, China). Logarithmically growing cells were exposed to the indicated drugs for the indicated time-points.

Cell proliferation assay. Cell proliferation was detected by CCK-8 assay according to the manufacturer's instructions. In brief, cells were plated in 96-well plates at a density of $1x10^4$ cells/well in 100 μl of medium in triplicate and treated with oridonin (3, 6, 9 and 12 μM) or VPA (0.5, 1 and 2 mM) or in combination. Cells exposed to RPMI-1640 medium only were used as control. Following incubation for 24 h, 10 μl of CCK-8 solution was added to each well in the assay plate and incubated for an additional 2 h at 37˚C. Absorbance was measured at 450 nm using a microplate reader (Model 550; Bio-Rad, USA). Each group had triplicate samples. The inhibition rate was calculated as the following formula: inhibition rate (%) = 1 - average absorbance of treated group / average absorbance of control group x 100%. Data were indicated as the means ± SD of triplicate samples. The 50% inhibitory concentration (IC_{50}) was calculated by the software for IC_{50} calculation.

Determination of drug interactions. Drug interaction between oridonin and VPA was assessed by CCK-8. The combination index (CI) was calculated by Chou-TC association index. CI<1, CI=1, and CI>1 indicated synergistic, additive, and antagonistic effects, respectively (22,23).

Morphological detection of apoptosis. HL-60 cells were exposed to 6 μM oridonin and/or 1 mM VPA for 24 h, the cell morphology was observed by light microscopy. For nuclear morphology, cells were washed twice with PBS, stained with Hoechst 33342 (10 μg/ml) for 5 min at room temperature in the dark and subjected to a Nikon Eclipse Ti fluorescence microscope (Nikon, Japan).

Annexin V/PI assay. After treatment with drugs for 24 h, HL-60 cells were harvested, washed with cold PBS twice and re-suspended in 100 μl of 1X binding buffer containing 5 μl Annexin V and 10 μl PI for 15 min at room temperature in the dark. Flow cytometry measurements were made on a Beckman Coulter Epics XL cytometer.

Reverse transcription (RT)-PCR analysis. The total RNA was extracted with TRIzol agent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA concentration was measured by spectrophotometry. RT-PCR assay was performed as previously described (24). The PCR products were electrophoresed in 1.5% agarose gels. The primers were all synthesized by Sangon Co., Ltd. (Shanghai, China). The sequences are listed as Table I.

Western blot analysis. HL-60 cells were washed twice with cold PBS and lysed in extraction buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100,

Table I. Primers for RT-PCR.

Gene	Sequence of primers	Size of products (bp)
β-actin	Sense: 5'-GTGGGGCGCCCCAGGCACCA-3'	540
	Antisense: 5'-CTCCTTAATGTCACGCACGATTTC-3'	
Bax	Sense: 5'-CTGACATGTTTTCTGACGGC -3'	289
	Antisense: 5'-TCAGCCCATCTTCTTCCAGA -3'	
Bcl-2	Sense: 5'-AGGCACCCAGGGTGATGCAA-3'	304
	Antisense: 5'-GTGGAGGAGCTCTTCAGGGA-3'	

0.5% deoxycholate, 0.1% SDS) for 30 min on ice. The lysates were centrifuged at 12,000 x g for 15 min and quantified using Bradford protein assay. Total proteins (50 μg) were separated by SDS-PAGE and electroblotted onto PVDF membranes. The membranes were blocked with 5% milk for 1 h and incubated with antibodies against caspase-8 (1:1,000), caspase-9 (1:1,000), cleaved caspase-3 (1:1,000), Fas (1:1,000), FasL (1:1,000), Bcl-2 (1:1,000), Bax (1:1,000), Cyt-C, JNK (1:1,000), ERK (1:1,000), p38, P-JNK (1:1,000), P-ERK (1:1,000), P-p38 (1:1,000) (Cell Signaling Technology) overnight at 4˚C followed by incubation with HRP-conjugated secondary antibodies (Zhongshan Golden Bridge Biotechnology Co.) for 1 h, visualized using ECL detection system (Millipore, Billerica, USA) and pictured by LAS-4000 mini luminescent image analyzer (Fujifilm, Tokyo, Japan).

Antitumor effect in vivo. All management procedures were approved by the Institutional Animal Care and Use Committee of the Shandong Academy of Medical Science. BALBc nude mice were established by subcutaneous inoculation of $1x10^7$ HL-60 cells as previously described. Then the nude mice were randomly assigned to two groups: control, and oridonin plus VPA group (n=6). The treatments began 10 days later with 15 mg/kg/d oridonin and/or 100 mg/kg/d VPA for 14 days, and the nude mice in control group were treated with the same volume of saline. Tumor volume and weights of the nude mice were measured daily.

TUNEL assay. TUNEL assay was performed to detect *in situ* apoptosis using a TUNEL assay kit (Roche Co., USA). Briefly, fresh cleaned specimens were fixed in 4% formalin and embedded in paraffin, then cut into 3-μm thick sections, affixed to silane-coated slides and stained with TUNEL assay kit (Roche Co.) according to the manufacturer's instructions.

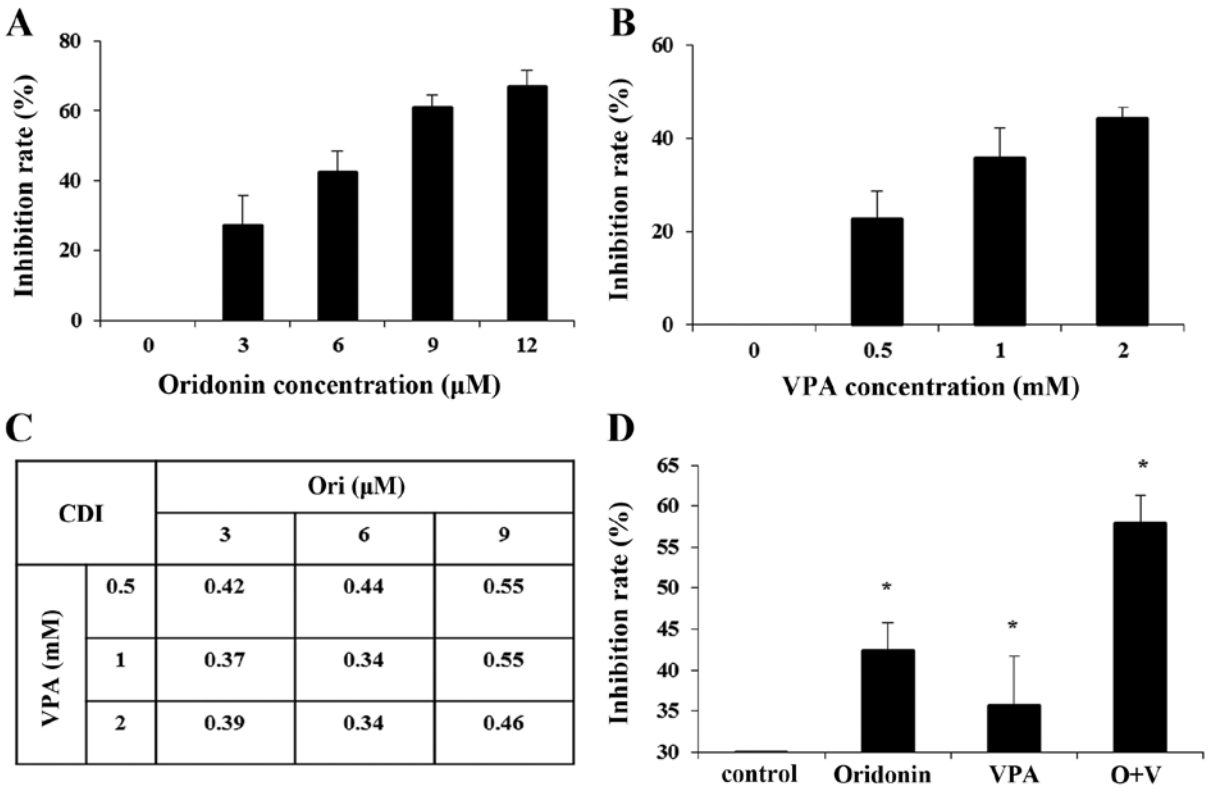
Statistical analysis. Data are presented as the means ± SD from at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS 13.0 (SPSS, Chicago, IL, USA). $P<0.05$ was considered to indicate statistically significant differences.

Results

Combination treatment of oridonin with VPA synergistically inhibited the proliferation of HL-60 cells. First, we detected the effects of oridonin and VPA on the proliferation of HL-60 cells at the indicated concentration scope. As shown in Fig. 1A, oridonin inhibited the proliferation of HL-60 cells in a dose-dependent manner with an IC_{50} of 6.85 μM at 24 h. Similarly, CCK-8 assay showed that VPA inhibited cell growth in a dose-dependent manner with an IC_{50} of 2.59 mM (Fig. 1B). To detect the inhibitory effect of the combination treatment, HL-60 cells were exposed to oridonin (3, 6 and 9 μM) and VPA (0.5, 1 and 2 mM) concurrently for 24 h. The data showed the CI values were 0.34-0.55 (CI<1), which implied a synergistic anti-proliferative effect of the combination group on HL-60 cells (Fig. 1C). When the concentration of oridonin and VPA was 6 and 1 mM, respectively, the CI was the minimum value. Thus, we used oridonin (6 μM) and VPA (1 mM) as the optimal concentration for the forthcoming experiment. After HL-60 cells were treated with 6 μM oridonin plus 1 mM VPA for 24 h, the inhibition rate strikingly increased from 42.34±6.04% for 6 μM oridonin alone, 35.70±6.59% for 1 mM VPA, to 57.94±4.83% ($P<0.01$) for the combination, which confirmed that combination treatment exerted a synergistic inhibitory effect on the proliferation of HL-60 cells (Fig. 1D).

Combination of oridonin plus VPA synergistically induced the apoptosis of HL-60 cells. To determine the effect of oridonin plus VPA on apoptosis in HL-60 cells, morphological changes were observed by light microscopy, and an inverted fluorescence microscope after Hoechst-33342 staining. It was noted that part of the cells treated by oridonin or VPA alone exhibited cell shrinkage, or (and) apoptotic bodies, which are typical morphological characteristics of apoptosis (Fig. 2A). The phenomenon was more evident in cells treated with oridonin and VPA. In parallel, Hoechst-33342 staining results showed that the nuclei of control cells were round and exhibited homogeneous blue fluorescence. While cells treated with oridonin or VPA alone for 24 h appeared with condensed or fragmented nuclei which is characteristic of cell apoptosis. Moreover, the apoptosis events in the combination group were more distinguished than each agent alone (Fig. 2B).

To confirm the enhanced apoptosis induced by combination treatment, Annexin V/PI assay using flow cytometry was performed. As seen in Fig. 3, the total percentage of apoptotic cells was 11.2±2.5% for oridonin, 17.4±2.8% for VPA, but 53.1±4.5% for the combination for 24 h. In the co-treatment for 48 h, the percentage of apoptotic cells increased to 16.3±2.1, 19.6±2.4 and 63.8±6.6%, respectively. These findings suggest



CDI		Ori (μM)		
		3	6	9
VPA (mM)	0.5	0.42	0.44	0.55
	1	0.37	0.34	0.55
	2	0.39	0.34	0.46

Figure 1. Combination of oridonin and VPA synergistically inhibited proliferation of HL-60 cells. (A and B) HL-60 cells were treated with different concentrations of oridonin or VPA for 24 h. Cell proliferation was measured by CCK-8 assay. Values represent the means ± SD in triplicates in three separate experiments. (C) HL-60 cells were treated with oridonin (3, 6 and 9 μM) and VPA (0.5, 1 and 2 mM) concurrently for 24 h. The combination index (CI) was calculated by Chou-TC association index. (D) HL-60 cells were co-exposed to 6 μM oridonin and 1 mM VPA for 24 h, then cell viability was measured by CCK-8 assay. Values represent the means ± SD of three separate experiments.

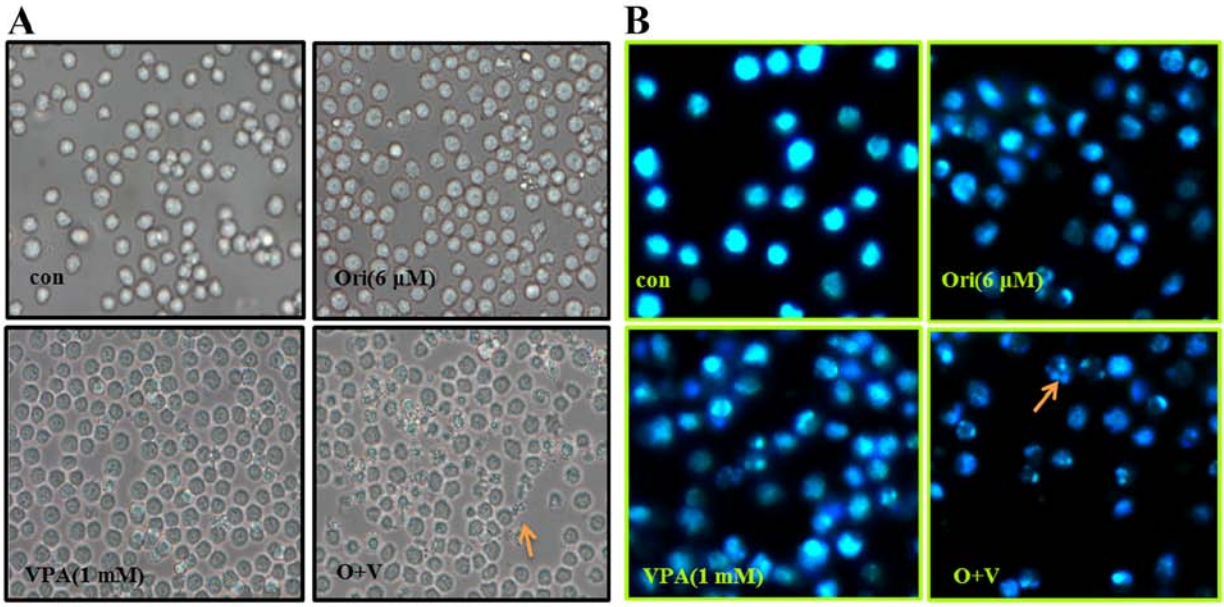


Figure 2. Effect of oridonin and VPA on cell morphology. HL-60 cells were administered with 6 μM oridonin and/or 1 mM VPA for 24 h. (A) The morphological changes of HL-60 cells were examined by light microscopy. Cell shrinkage and formation of apoptotic bodies were considered as apoptotic cells (x200). (B) Nucleolus morphological changes were observed under a fluorescence microscope after Hoechst-33342 staining. Condensed or fragmented nuclei were considered as apoptotic cells (x400). Arrows indicate apoptotic cells.

that combination of oridonin and VPA exerted a synergistic effect on the induction of apoptosis in HL-60 cells.

Activation of caspase is important for combination treatment-triggered cell apoptosis. To clarify the mechanism involved

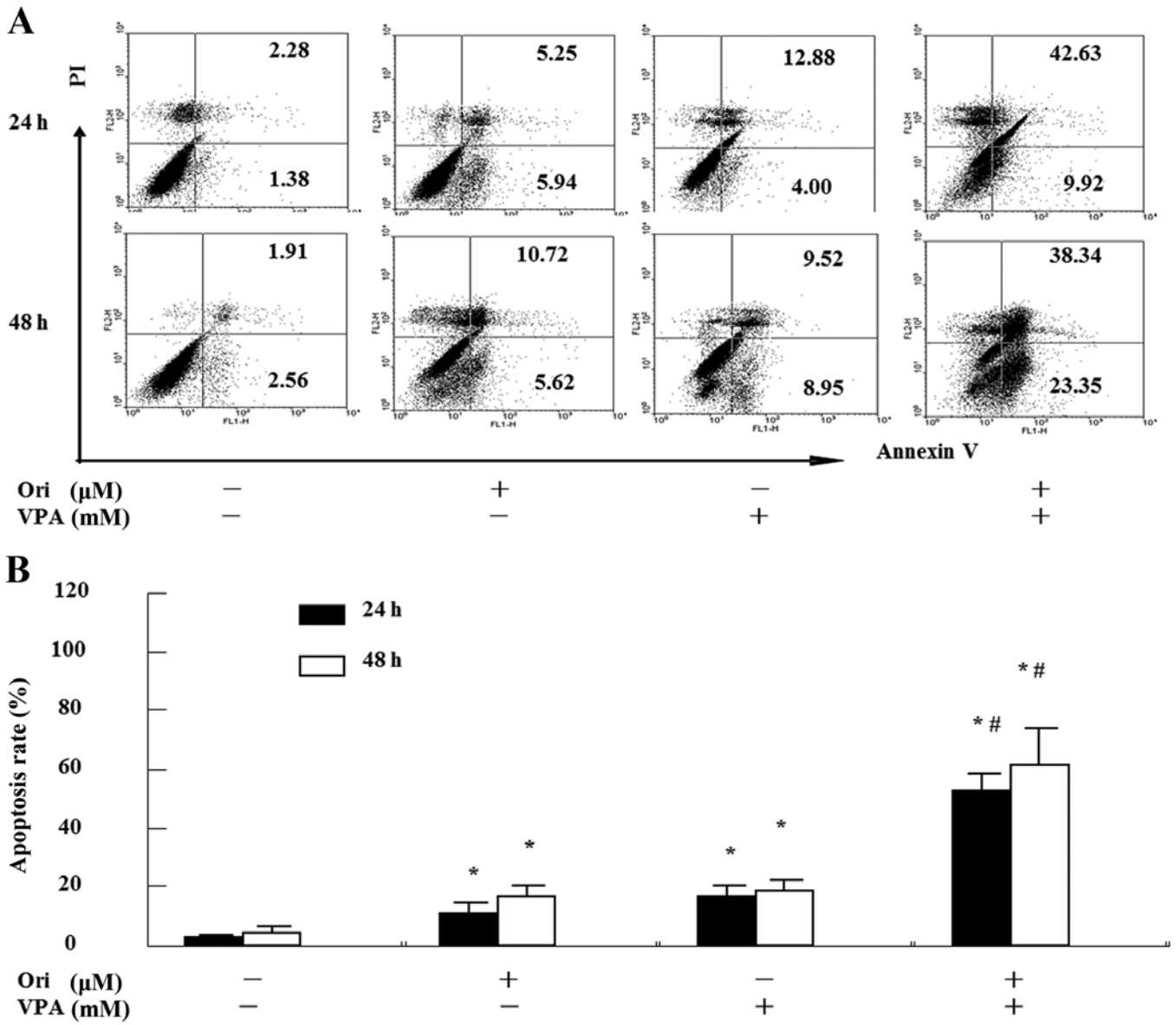


Figure 3. Combination of oridonin and VPA synergistically induced apoptosis of HL-60 cells. (A) HL-60 cells were exposed to 6 μM oridonin and/or 1 mM VPA for 24 h and stained with Annexin V/PI prior to flow cytometry analysis as described in Materials and methods. Cells in the lower right quadrant (Annexin V^+/P^{I-}) correspond to early apoptotic cells, whereas, cells appearing in the right upper quadrant (Annexin V^+/PI^+) correspond to late apoptotic cells. (B) The histogram represents total apoptotic percentages. Values represent the means ± SD of a triplicate experiment. $^*P<0.01$ vs. control, $^\#P<0.01$ combined treatment vs. oridonin or VPA alone.

in apoptosis induced by oridonin and VPA, we detected the protein expression of caspase-8, caspase-9 and cleaved-caspase-3 by western blot analysis. As shown in Fig. 4A, the protein expression of cleaved caspase-3, -8, and -9 were significantly elevated after the combination of oridonin and VPA, more obviously than that of either agent alone. The results implied that these caspase family proteins may be involved in the apoptosis induced by oridonin plus VPA. Furthermore, pretreatment with caspase-inhibitor (Z-VAD-fmk) completely blocked the apoptosis-induced by combination treatment, indicating that the synergistic induction of apoptosis by oridonin and VPA is caspase-dependent (Fig. 4B and C).

Combination of oridonin with VPA triggered the mitochondrial apoptotic pathway in HL-60 cells. Combination treatment induced the activation of caspase-9, suggesting that intrinsic pathway is involved. As confirmation, cytosolic cytochrome *c* was monitored by western blot analysis. As shown in Fig. 5B, an evident increase of cytochrome *c* protein was observed after combined treatment with oridonin and VPA, suggesting that cytochrome *c* was released from mitochondria into cytosol. This result may well account for the activation of caspase-9.

The Bcl-2 family proteins could regulate the release of cytochrome *c* from mitochondria (25). To further confirm that the mitochondrial pathway is involved in oridonin/VPA-induced apoptosis, the expression of pro-apoptotic factor Bax and anti-apoptotic factor Bcl-2 were detected at the transcriptional and post-transcriptional level by RT-PCR and western blot analysis. As shown in Fig. 5, treatment with

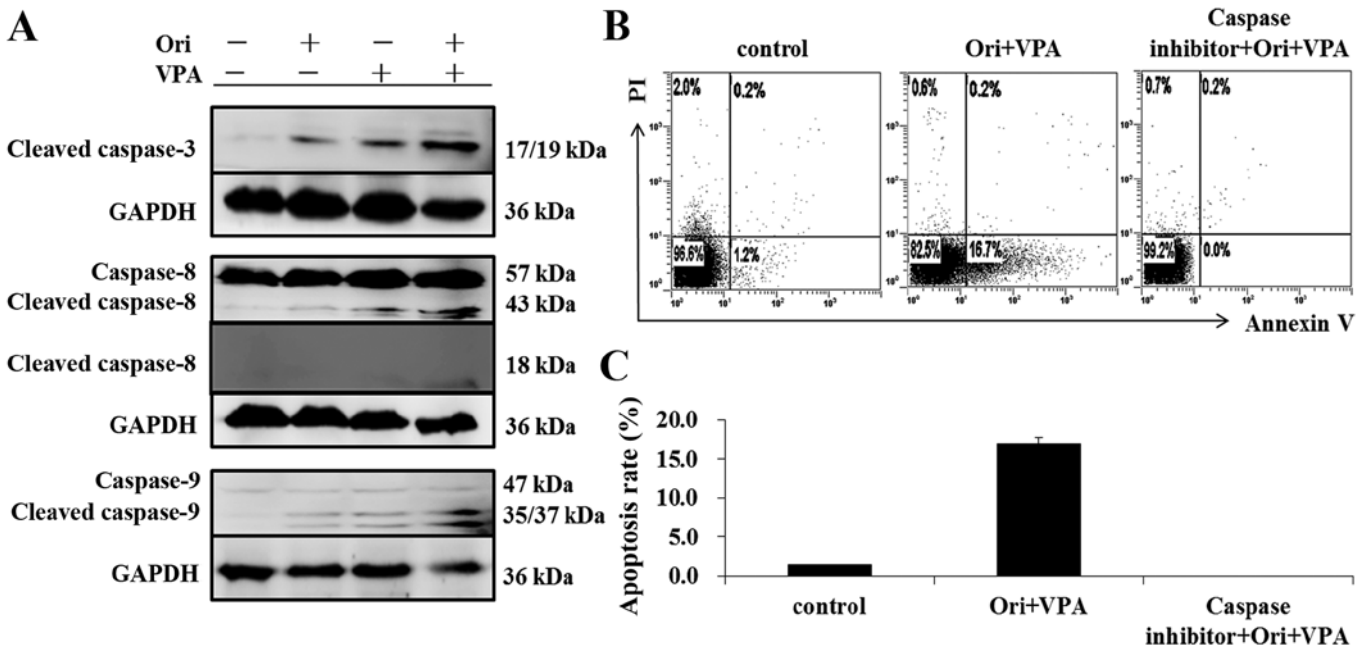


Figure 4. Combination treatment-induced apoptosis was caspase-dependent. (A) Whole-cell lysates treated with oridonin or (and) VPA were assessed for caspase-8, caspase-9 and cleaved caspase-3 expression by western blotting. GAPDH was used as the loading control. Three independent studies yielded equivalent results and representative blots are shown. (B) HL-60 cells were treated with oridonin+VPA in the presence or absence of caspase-inhibitor Z-VAD-fmk for 24 h stained with Annexin V and PI and then analyzed by flow cytometry. (C) The histogram represents total apoptotic percentages.

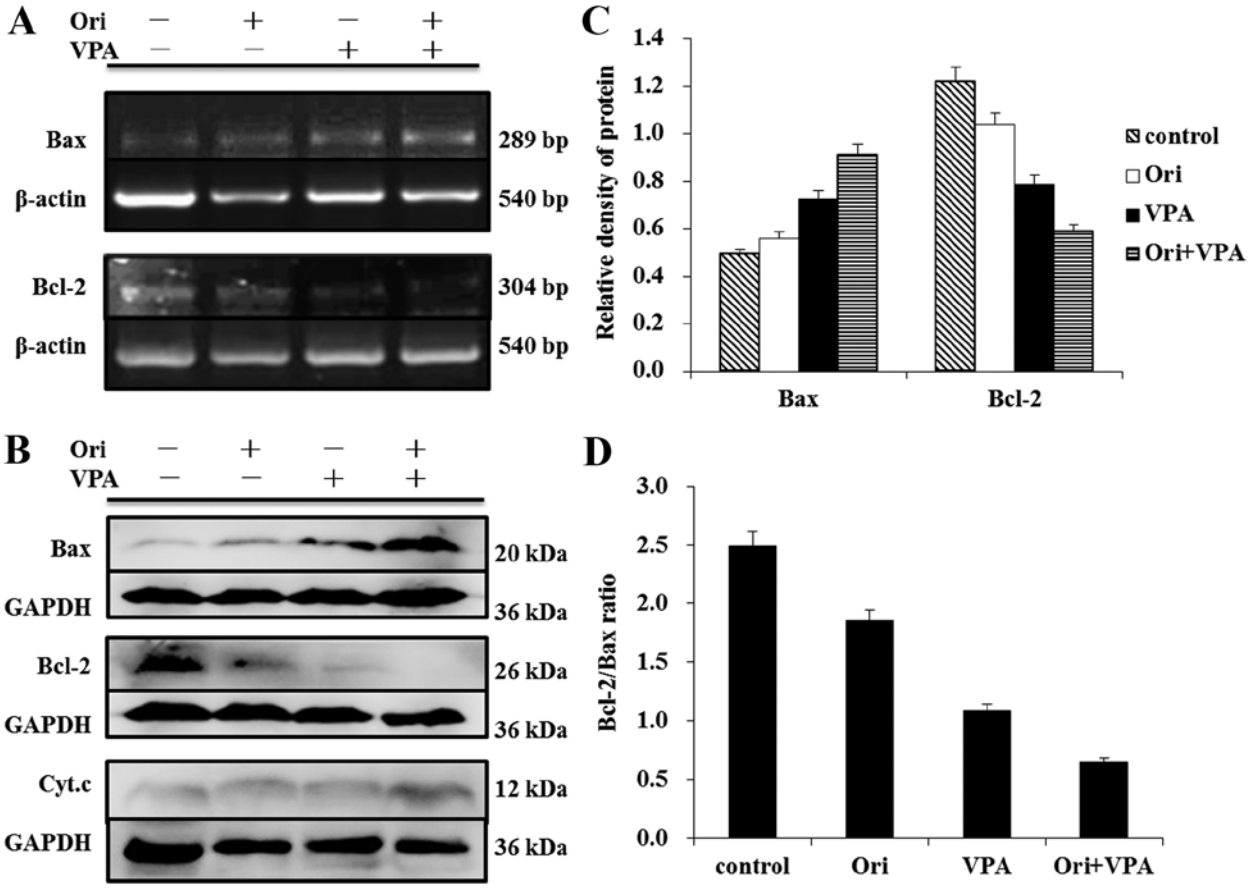


Figure 5. Effect of oridonin and VPA on the expression of the Bcl-2 family genes. (A) Cells were treated with oridonin and VPA alone or in combination for 24 h and the mRNA expression of Bax, and Bcl-2 was then detected by RT-PCR. β-actin was used as the loading control. Each amplification was performed at least three times. (B) The cell lysates were subjected to western blotting to determine protein level of Bax, and Bcl-2. GAPDH was used as the loading control. All the blots were repeated at least three times and one representative blot is presented. (C) The semi-quantative analysis of Bax and Bcl-2 was calculated and presented, respectively. Bars are mean of three different independent experiments ± SD. (D) The histogram represents ratio of values of Bcl-2/Bax.

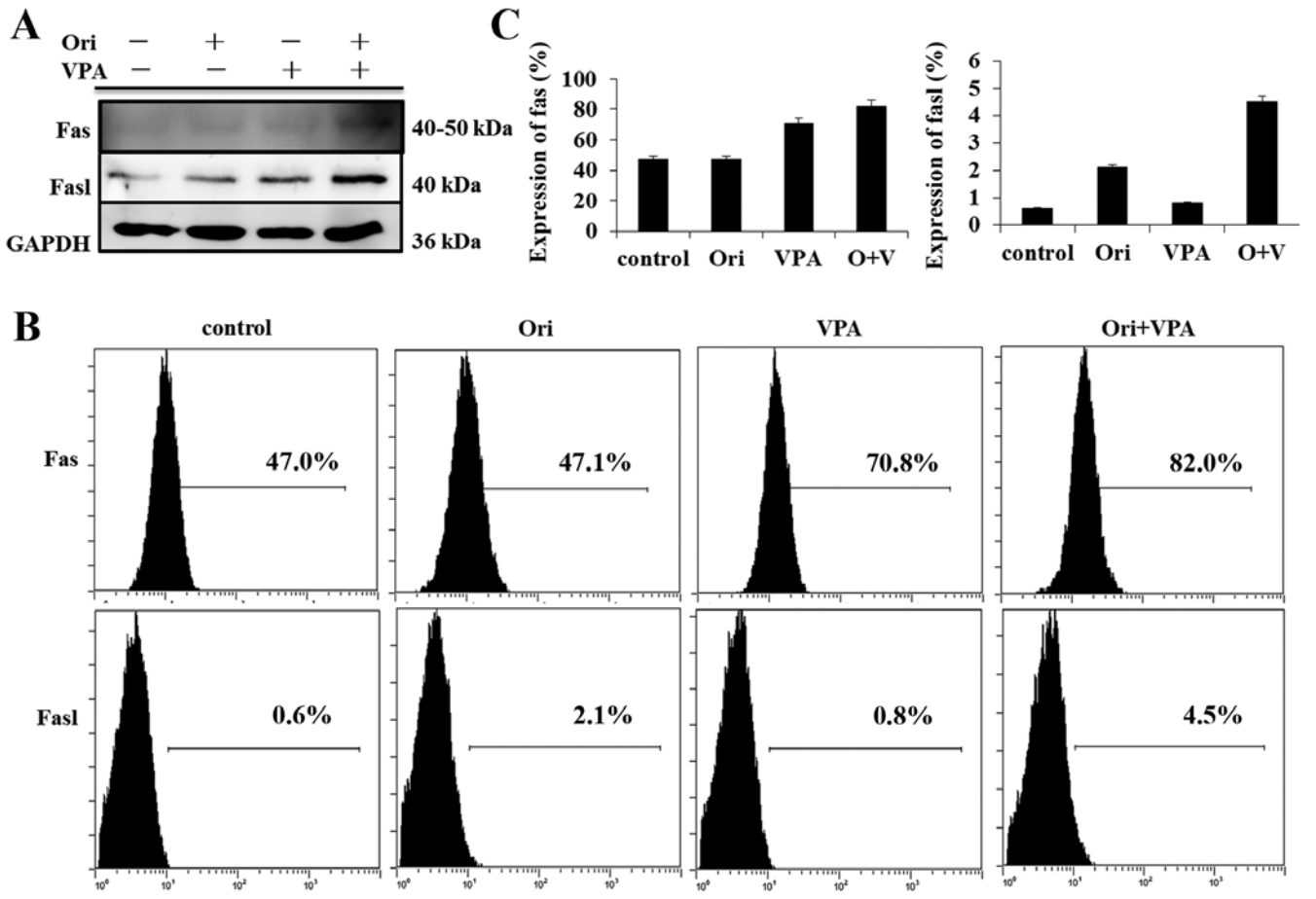


Figure 6. Effect of combined treatment with oridonin and VPA on the expression of the death receptor. (A) HL-60 cells were treated with oridonin and VPA alone or in combination for 24 h. The cell lysates were subjected to western blotting to determine protein level of Fas and FasL. GAPDH was used as the loading control. Three independent studies yielded equivalent results and representative blots are shown. (B) Fas and FasL were analyzed by flow cytometry. (C) The results of Fas and FasL analyzed by flow cytometry were quantified by computer-aid densitometry.

either 6 μM oridonin or 1 mM VPA for 24 h upregulated the expression of Bax at mRNA and protein levels. Moreover, evident augmentation was observed in the combination group as compared with single agent. In contrast, the expression of Bcl-2 decreased more clearly in combination treated cells than in single agent-treated cells (Fig. 5A). Consequently, the ratio of Bcl-2/Bax markedly declined (Fig. 5C). Together, these results indicated that treatment of HL-60 cells with oridonin plus VPA resulted in increased activation of the intrinsic mitochondrial apoptotic pathway.

Combined treatment-induced apoptosis is mediated through the Fas-mediated pathway. To characterize the role of the extrinsic pathway in oridonin plus VPA-induced apoptosis, we detected Fas and FasL protein by western blot analysis. The results showed that exposure to oridonin or VPA alone triggered the Fas and FasL expression and combination treatment led to stronger increase of their expression (Fig. 6B). In parallel, the expression of Fas and FasL was assessed by FCM. The data presented herein suggest that activation of the extrinsic Fas-related pathway plays a major role in the enhanced apoptosis observed in oridonin plus VPA-treated cells.

The MAPK signaling pathway is involved in apoptosis of HL-60 cells induced by oridonin plus VPA. To further elucidate the intracellular mechanisms modulated by oridonin combined with VPA in the apoptosis of HL-60 cells, key proteins involved in MAPK signal pathway were examined by western blot assay. As shown in Figs. 7-9, there were no detectable changes in expression of total ERK, p38, JNK and phosphorylated-JNK protein. In contrast, phosphorylation of ERK was reduced, and phosphorylation of p38 was increased.

To further confirm that the apoptotic effect of combination therapy on AML cells was associated with the MAPK pathway, HL-60 cells were pre-treated with the specific inhibitors SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and PD98059 (ERK inhibitor) respectively, for 30 min to block the three pathways, and then treated with 6 μM oridonin and 1 mM VPA for 24 h. Then, apoptosis analysis and western blot analysis for detecting the expression of cleaved caspase-3 were conducted. As seen in Fig. 7C, PD98059 attenuated levels of phospho-ERK in oridonin/VPA-treated cells, but enhanced apoptosis induced by oridonin/VPA (Fig. 7B), concomitant with elevated expression of cleaved caspase-3 (Fig. 7C). As expected, SB203580 blocked expression of phospho-p38 and protected HL-60 cells from combination-induced apoptosis, accompanied by downregulation of cleaved caspase-3 (Fig. 8B and C). While no change of apoptosis after pre-treatment with SP600125 was observed though JNK signaling pathway blocking (Fig. 9B), neither was there any activity of caspase-3

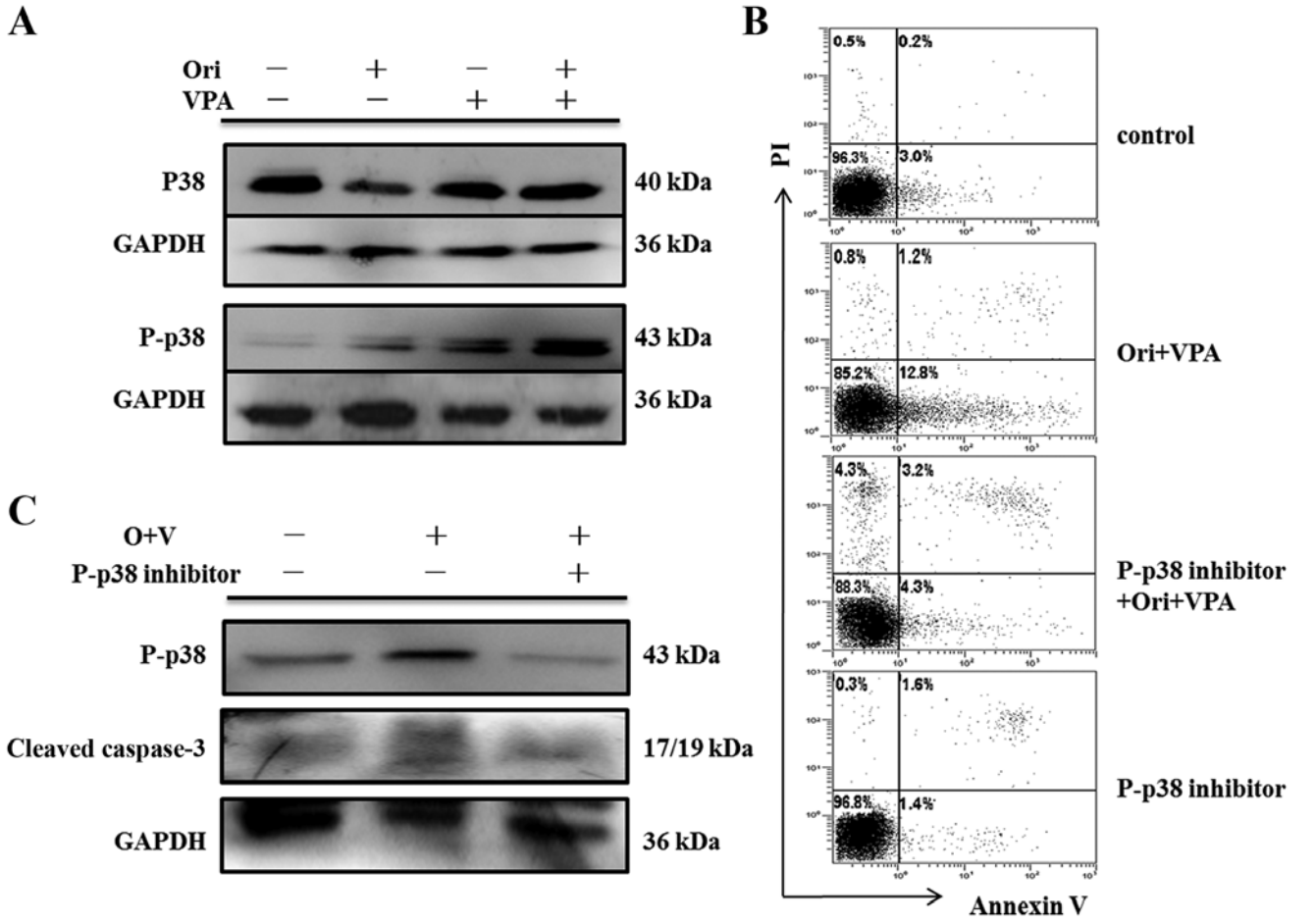


Figure 7. ERK-MAPK signaling pathway is inhibited in oridonin/VPA-induced apoptosis in HL-60 cells. HL-60 cells were treated with oridonin and VPA alone or in combination for 24 h. (A) The cell lysates were subjected to western blotting to determine protein level of ERK and P-ERK. (B) HL-60 cells were incubated for 24 h with oridonin/VPA in the absence or presence of the ERK inhibitor PD98059 (25 mM), after which the percentage of apoptotic cells was determined by Annexin V/PI staining as above. Representative FCM plots of apoptotic cells of three independent experiments are shown. (C) HL-60 cells were incubated for 24 h with oridonin/VPA in the absence or presence of the ERK inhibitor PD98059 (25 mM). Then the expression of P-ERK and cleaved caspase-3 protein was assessed by western blot analysis. GAPDH was used as the loading control. All blots have been repeated at least three times and one representative blot is presented.

(Fig. 9C). These findings strongly indicated that ERK and P38 MAPK may control oridonin/VPA-induced apoptosis as upstream regulators (Figs. 7-9).

Effects of oridonin combined with VPA in vivo. All nude mice were inoculated subcutaneously with HL-60 cells and developed palpable tumor after a mean of 10 days. Then the mice were treated with oridonin and VPA. Tumor volume and tumor weight were measured and are presented as the mean ± SE. As shown in Fig. 10A, compared with the control group, combination treatment significantly reduced the tumors size and the tumor weight ($P<0.05$) (Fig. 10). The results indicated that combined treatment with oridonin and VPA also exerted a significant effect on proliferation inhibition *in vivo*. TUNEL assay revealed that combination of oridonin and VPA could induce apoptosis of leukemic cells, in accord with the results acquired *in vitro* (Fig. 10D).

Discussion

Due to the side effects and frequent acquisition of drug resistance, the clinical outcome for AML remains discouraging despite standard treatment. Therefore, novel therapeutic approaches that act more selectively and more potently are urgently needed. It has been well documented that either oridonin or VPA exerts significant anti-leukemia activity (13,14,20-22). In addition, each of them has been proved to enhance the anticancer effect of other anti-neoplastic agents (13,14,17). Nevertheless, there is no report on the activity and mechanism of the combination of oridonin and VPA in AML cells. In the present study, we demonstrated that the combined exposure of human leukemia HL-60 cells to relatively low dose of oridonin and the histone deacetylase inhibitor VPA exerts a synergistic effect on proliferation inhibition, caspase activation, and apoptosis. Moreover, the study revealed that the combination treatment-induced apoptosis is associated with inhibition of ERK signaling as well as activation of p38 MAPK signaling pathway. Finally, *in vivo* studies demonstrated that tumor growth in a mouse model could be inhibited by the combination therapy.

Classical apoptosis may occur by two major pathways: the intrinsic (mitochondrial-mediated) and the extrinsic (receptor-mediated) pathway (26). The intrinsic pathway is characterized by change of mitochondrial membrane, resulting in the release

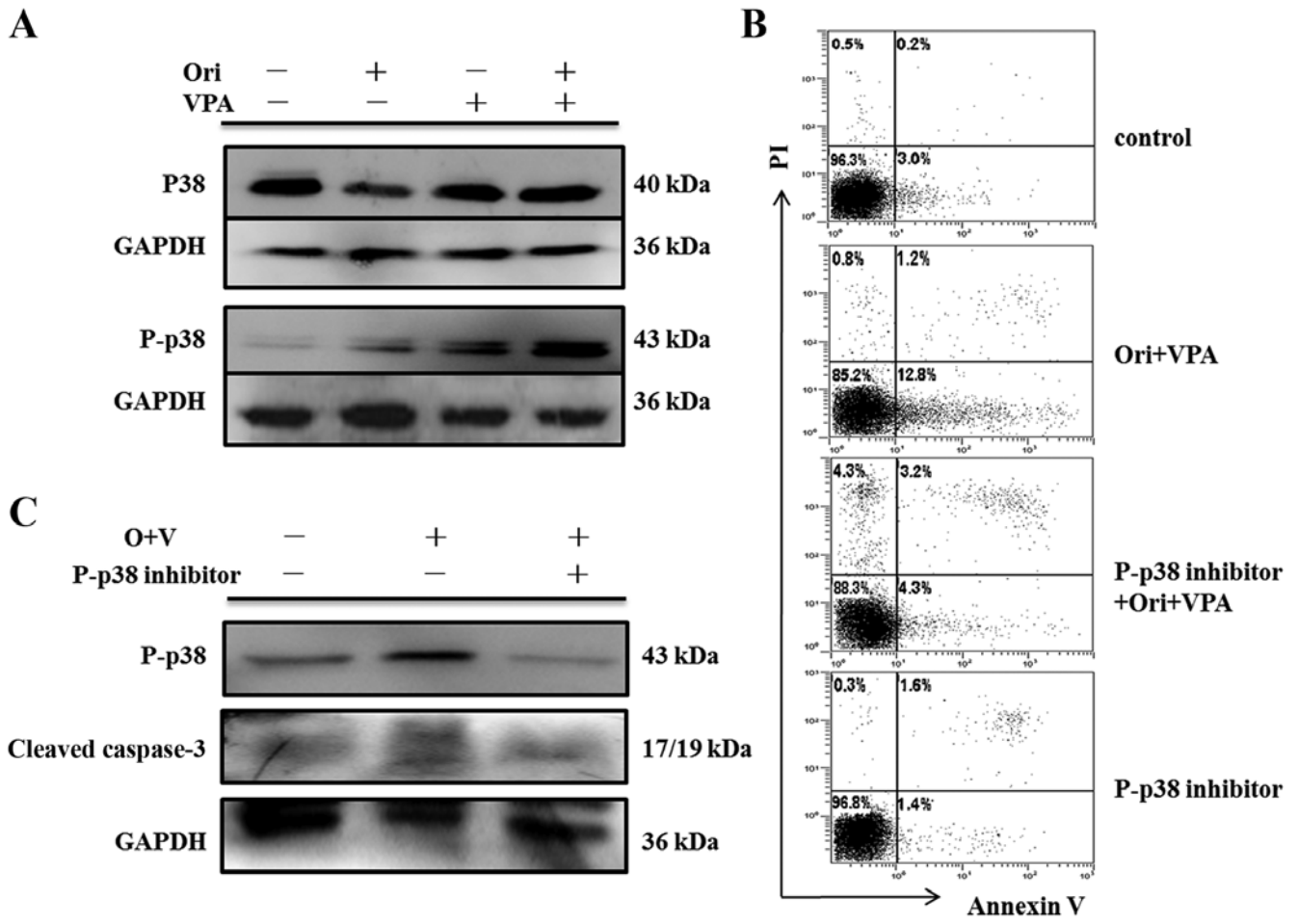


Figure 8. P38 is activated in oridonin/VPA-induced cell apoptosis in HL-60 cells. HL-60 cells were treated with oridonin and VPA alone or in combination for 24 h. (A) The cell lysates were subjected to western blotting to determine protein level of p38 and P-p38. (B) HL-60 cells were incubated for 24 h with oridonin/VPA in the absence or presence of the P38 inhibitor SB203580 (50 mM), after which the percentage of apoptotic cells was determined by Annexin V/PI staining as above. Representative FCM plots of apoptotic cells of three independent experiments are shown. (C) HL-60 cells were incubated for 24 h with oridonin/VPA in the absence or presence of the P38 inhibitor SB203580 (50 mM). Then the expression of P-p38 and cleaved caspase-3 protein was assessed by western blot analysis. GAPDH was used as the loading control. All the blots were repeated at least three times and one representative blot is presented.

of cytochrome *c* and activation of caspase-9 and downstream effectors caspase-3 and (or) -7. The extrinsic pathway involves the binding of death ligands such as Fas ligand or TNF-α to their corresponding death receptor, the cleavage of caspase-8 and then the activation of downstream effectors caspase-3 and (or) -7.

Previous studies demonstrated that oridonin induces caspase-3 activation and apoptosis via mitochondrial pathway in the gastric cancer cell line HGC-27 (26) and in gallbladder cancer cells lines (27). Consistent with these reports, we also observed increased expression of cleaved caspase-3 and -9 in HL-60 cells after exposure to oridonin for 24 h. Just as a preceding report (28), caspase-3 and caspase-9 were both activated after HL-60 cells were treated with VPA alone. Moreover, co-administration of oridonin with VPA led to increased activation of caspase-3 and -9. The findings suggested that mitochondrial pathway may contribute to enhanced apoptosis induced by combination therapy. Moreover, we observed elevated expression of Bax and reduced expression of Bcl-2, consequently downregulation of the Bcl-2/Bax ratio, together with upregulation of cytochrome *c* in the cytosol. These results further confirmed that oridonin/VPA-induced apoptosis is associated with the activation of mitochondrial pathway.

In parallel, we found that oridonin/VPA markedly induced the expression of cleaved caspase-8 fragments, concomitant with upregulation of Fas and FasL proteins. Hence, we consider that combination treatment induces apoptosis of HL-60 cells by the specific activation of Fas signaling pathway.

Despite pivotal role of caspases in apoptosis, new data also implicate that apoptosis can occur in the absence of caspase activation (25). To verify the role of caspase activation on combined treatment-induced apoptosis, HL-60 cells were treated with oridonin and VPA in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK. The results indicate that the pan-caspase inhibitor completely prevented apoptosis induced by oridonin/VPA, suggesting that combination treatment induced apoptosis in a caspase-dependent manner.

The MAPK pathway is implicated to play an important role in proliferation, differentiation, development, transformation and apoptosis (29). In mammals, the MAPK family is divided into three major subfamilies, namely ERK, JNK and p38 (30). Indeed, constitutive ERK1/2 activation has been claimed to play

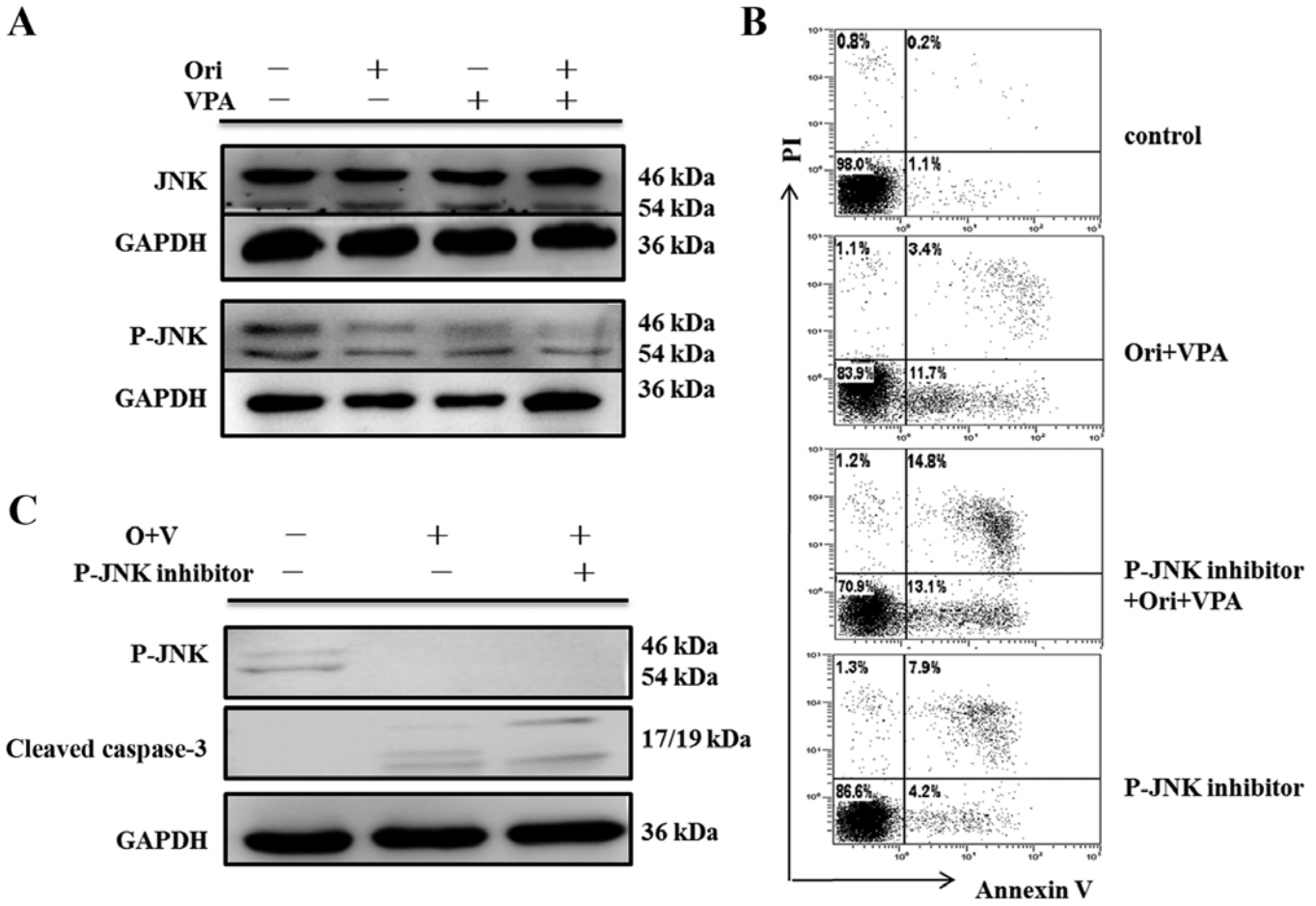


Figure 9. JNK-MAPK signaling pathway is not involved in oridonin/VPA-induced apoptosis in HL-60 cells. HL-60 cells were treated with oridonin and VPA alone or in combination for 24 h. (A) The cell lysates were subjected to western blotting to determine protein level of JNK and P-JNK. (B) HL-60 cells were incubated for 24 h with oridonin/VPA in the absence or presence of the JNK inhibitor SP600125 (100 mM), after which the percentage of apoptotic cells was determined by Annexin V/PI staining as above. Representative FCM plots of apoptotic cells of three independent experiments are shown. (C) HL-60 cells were incubated for 24 h with oridonin/VPA in the absence or presence of the JNK inhibitor SP600125 (100 mM). Then the expression of P-p38 and cleaved caspase-3 protein was assessed by western blot analysis. GAPDH was used as the loading control. All the blots were repeated at least three times and one representative blot is presented.

an important role in the progression of tumorigenesis in AML (31). Inhibition of phosphate-ERK will induce acute myeloid leukemia apoptosis (32). Previous studies have revealed that oridonin is able to inhibit ERK signaling pathway in osteosarcoma cells (33), while activating ERK signaling pathway in HepG2 cells (35). In the present study, we found that oridonin inhibited the expression of p-ERK in HL-60 cells. The difference of the effect of oridonin on ERK signaling pathway may be attributed to the specificity of cell type. Moreover, combined treatment with oridonin and VPA could more significantly suppress the phosphorylation of ERK1/2 in apoptosis induction. Finally, pretreatment with ERK inhibitor enhanced apoptosis and activity of caspase-3 induced by oridonin/VPA, which suggested that ERK acts upstream of caspase-3 in the apoptotic process induced by oridonin/VPA. Since activation of ERK indirectly allowed Bcl-2 to form homo-dimers to produce an anti-apoptotic effect (35) and herein Bcl-2 was found to be significantly downregulated, we speculated that ERK signaling may participate in apoptosis of HL-60 cells through downregulating the expression of Bcl-2 protein. It has been demonstrated that p38 and JNK are more sensitive to stress stimuli ranging from osmotic shock to inflammatory cytokines and are mostly activated during drug-induced apoptosis of leukemia cell lines (36). Evidence has been shown that oridonin is able to activate p38 MAPK and JNK signaling pathways in osteosarcoma cells (34) or HepG2 cells (34). One recent report has demonstrated that oridonin induces apoptosis in SW1990 pancreatic cancer cells via caspase-dependent induction of p38 MAPK (37). The activation of p38 was demonstrated to affect apoptotic pathway through inhibiting the expression and phosphorylation of Bcl-2 protein in human hepatoma cells (38). In the present study we found p38 MAPK also participated in oridonin/VPA-induced apoptosis, which was supported by upregulation of phosphorylation of p38 and complete block of apoptosis by p38 inhibitor. In addition, downregulation Bcl-2 may account for the mechanism by which p38 MAPK signaling affects apoptotic procedure. These findings may suggest that combination of VPA and oridonin can enhance the apoptosis induced by oridonin through p38 pathway. While our data showed that no remarkable change in P-JNK was observed. Similarly, no appreciable effect on the apoptosis and cleaved caspase-3 fragment was detected after pretreatment with JNK inhibitor

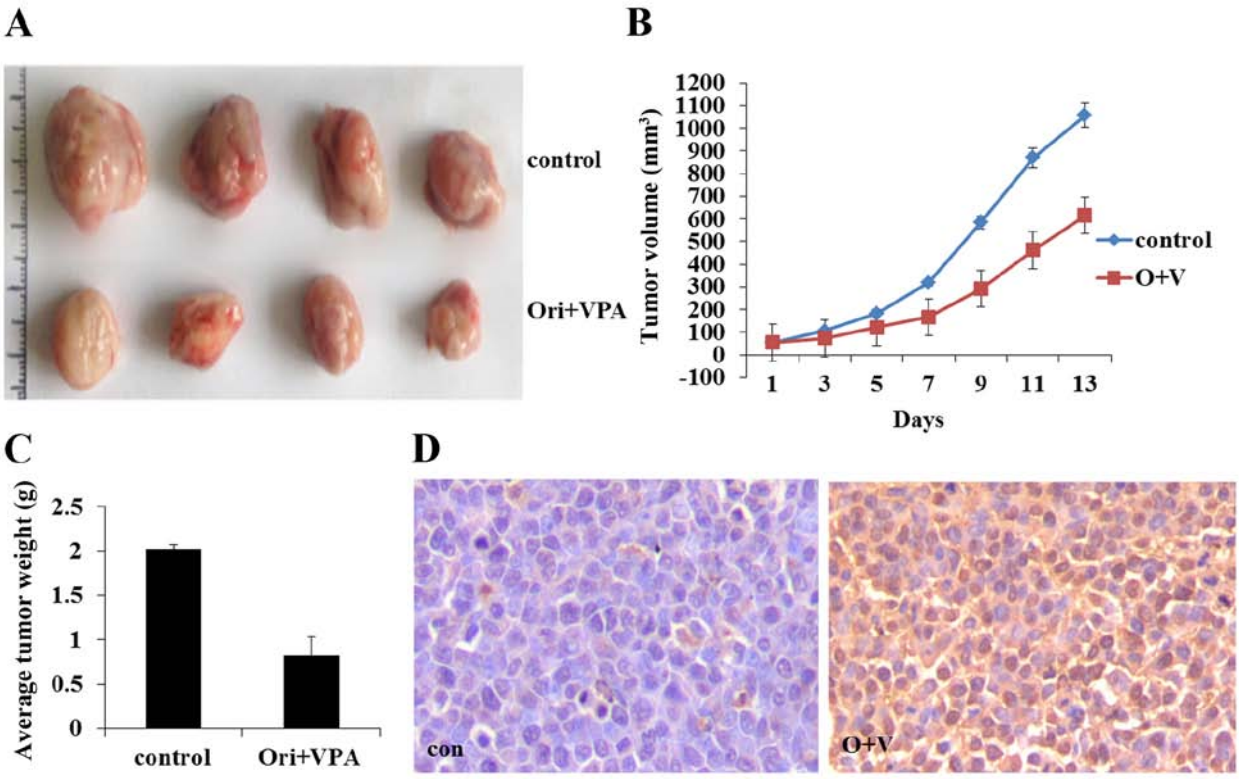


Figure 10. The *in vivo* effect of oridonin combined with VPA on murine models. HL-60 xenografts were established in mice and treated with oridonin and VPA. Tumor volume and the weight of the nude mice were measured and are presented as the mean ± SE. (A) The xenografted tumors of control and oridonin+VPA. (B) Oridonin combined with VPA could decrease the tumor size in a nude mouse model xenograft tumor. (C) The average weights of the tumor are shown. (D) TUNEL assay was performed on the specimen from mice. The results show that cotreatment with oridonin and VPA induces apoptosis of leukemic cells *in vivo*.

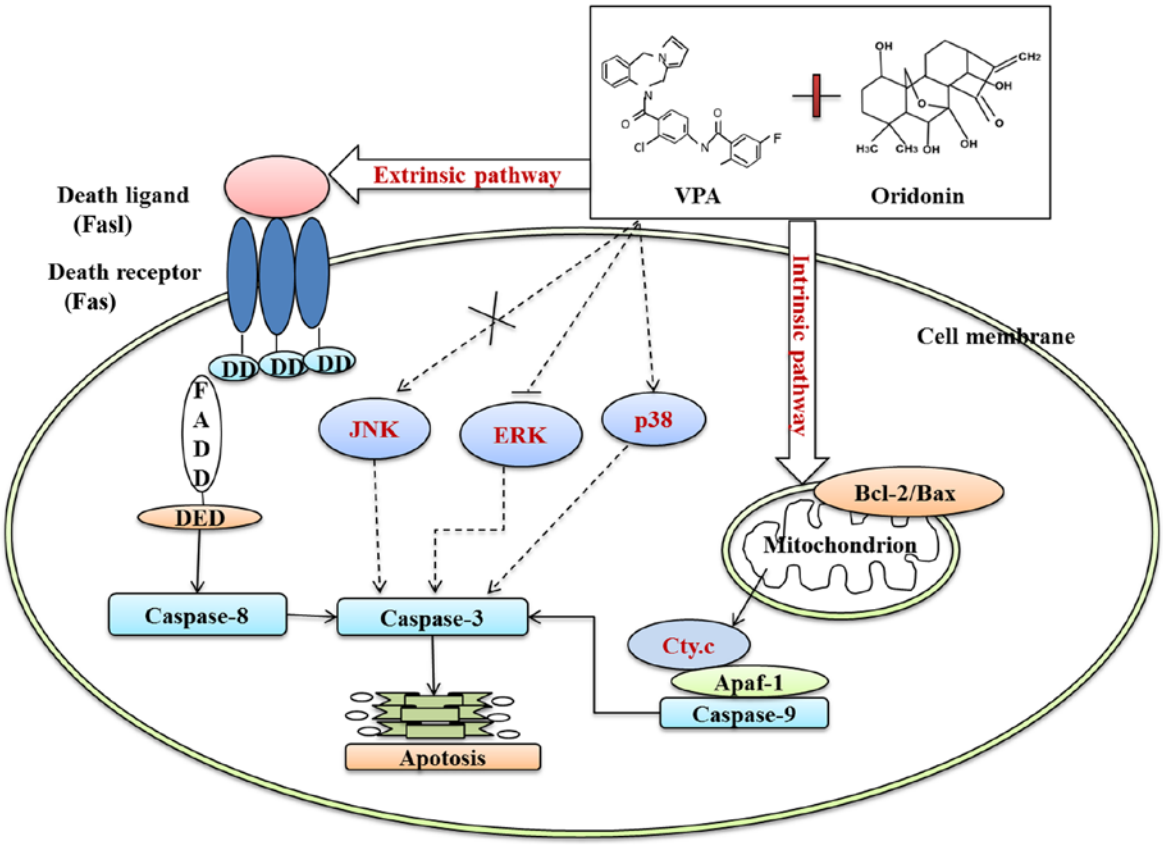


Figure 11. Model of the extrinsic and intrinsic apoptotic pathway and the related MAPK signaling pathways involved in apoptosis induced by oridonin plus VPA.

SP600125, which confirmed that apoptosis herein was not associated with JNK pathways.

To further confirm the effect of oridonin plus VPA on proliferation and apoptosis, our present study highlighted the synergistic anti-leukemia effect of oridonin in combination with VPA *in vivo* models in AML. The results indicated that treatment with oridonin and VPA resulted in significant reduction of tumor size and tumor weight of HL-60 xenograft mice, accompanied by cell apoptosis in tumor tissue.

In conclusion, oridonin plus VPA exerted more synergistic effect on inhibition of proliferation and apoptosis than that of each of them alone. Mechanically, oridonin plus VPA induced obvious caspase-dependent apoptosis by activation of the intrinsic apoptosis pathway, as evidenced by the downregulation of Bcl-2/Bax ratio, cytochrome *c* release and caspase-9 activation, as well as through the extrinsic apoptosis pathway by triggering Fas/FasL and caspase-8 activation. In addition, downregulation of p-ERK and upregulation of p-p38 also participated in enhanced apoptosis of HL-60 cells induced by oridonin plus VPA (Fig. 11). The results presented herein indicate that combination treatment with oridonin and VPA may be a potent strategy for targeted treatment of AML.

Acknowledgements

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