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ORIGINAL ARTICLE

Oridonin effectively reverses the drug resistance of cisplatin involving induction of cell apoptosis and inhibition of MMP expression in human acute myeloid leukemia cells

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KEYWORDS

Oridonin;
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Cell apoptosis

Abstract Cisplatin is the first generation platinum-based chemotherapy agent. However, the extensive application of cisplatin inevitably causes drug resistance, which is a major obstacle to cancer chemotherapy. Oridonin is a diterpenoid isolated from *Rabdosia rubescens* with potent anticancer activity. The aim of our study is to investigate the role of oridonin to reverse the cisplatin-resistance in human acute myeloid leukemia (AML) cells. The effect of oridonin on human AML cell proliferation was evaluated by MTT assay, cell migration and invasion were evaluated by transwell migration and invasion assays in cisplatin-resistant human AML cells. Furthermore, cell apoptosis was examined by flow cytometry. The inhibitive effect of oridonin *in vivo* was determined using xenografted nude mice. In addition, the expressions of MMP2 and MMP9 were detected by Western blot. There was a synergistic antitumor effect between cisplatin and oridonin on cisplatin-resistant human AML cells *in vitro* and *in vivo*. In addition, the combination of cisplatin and oridonin synergistically induced cell apoptosis. Furthermore, the combination treatment not only inhibited AML cell migration and invasion, but more significantly, decreased the expressions of MMP2 and MMP9 proteins. Our results suggest that the synergistic effect between both agents is likely to be driven by the inhibition of MMP expression and the resulting increased apoptosis.

1. Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia (Showel and Levis, 2014). Following induction of chemotherapy, high remission rates have been achieved in most of patients. However, there have been few changes in standard induction therapy and response rate over the past three decades, particularly for elderly patients (Medeiros

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et al., 2015; National Cancer Institute. SEER stat fact sheets: acute myeloid leukemia, 2015; Zeidner et al., 2014; Duan, 2016). Nowadays, the chemotherapy remains an important way to treat AML. Cisplatin is the first line platinum-based chemotherapy agent, and has significant anti-tumor activity in a variety of solid tumors (Lee et al., 2014; Previati et al., 2006). It is also one of the most commonly used drugs for therapy of AML. However, the extensive application of cisplatin has kinds of adverse gastrointestinal toxicity, including severe nausea and vomiting, renal toxicity neurotoxicity *etc*. (Joo et al., 2009). Importantly, long term use of cisplatin will inevitably cause drug resistance, which is a major obstacle to cancer chemotherapy (Kim et al., 2009; Karam et al., 2009; Zhang et al., 2012). Then, to explore more effective ways to decrease the side effects or resolve drug resistance on AML has become the most important problem.

Recently, natural product extraction from medicinal plants has drawn more and more attention in cancer therapy. Oridonin is extracted from Chinese herb *Rabdosia rubescens*, which is a natural compound with the structure of tetracycline diterpenoid (Wang et al., 2013; Gao et al., 2010). It has been reported to have antitumor effects and widely used in clinical treatment of various tumors. Qi et al. (2012) reported that oridonin could effectively induce cell apoptosis of pancreatic cancer cells, and oridonin nanosuspension was more effective than free oridonin on G_2/M cell cycle arrest and apoptosis in the human pancreatic cancer PANC-1 cell line. Gao et al. (2012) found oridonin induces apoptosis and senescence by increasing hydrogen peroxide and glutathione depletion in colorectal cancer cells. Meanwhile, some researchers reported that autophagy preceded apoptosis in oridonin-treated human breast cancer MCF-7 cells (Cui et al., 2007). In lung cancer patients, oridonin also suppressed mTOR signaling and the growth of lung cancer tumors, and inhibition of mTORC1 might be an effective target for increasing the therapeutic outcome treated with oridonin (Zhou et al., 2007; Lin et al., 2015).

In the present study, we used two cisplatin-resistant ovarian cancer cell lines MV4-11/DDP and MOLM-13/DDP, and explored the mechanism of combined therapy on oridonin and cisplatin. It is beneficial to clarify the mechanism of disease progression and also helpful for developing treatment method on reversing drug resistance.

2. Materials and methods

2.1. Agents and cell lines

Oridonin and cisplatin were obtained from the Shifeng biocorporation (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide was purchased from Sigma Chemical (St. Louis, MO, U.S.A). The cisplatin-resistant human AML subline MV4-11/DDP (Huiying corporation, Shanghai, China) and MOLM-13/DDP (Yunnan tumor hospital, Kunming, China) were used. The cells were cultured in DMEM medium (Hyclone, Beijing, China) enriched with 10% fetal bovine serum (Hyclone) at 37 °C and 5% CO_2. Cisplatin was obtained from Qilu Pharm. (Jinan, China) and 0.5 μg/mL of cisplatin was added into the medium to maintain chemoresistance.

2.2. MTT assay

The inhibitory effect of oridonin alone, cisplatin alone, oridonin plus cisplatin for human AML cells MV4-11/DDP and MOLM-13/DDP cells was measured by MTT assay. Generally speaking, cells were transferred to cisplatin-free medium 3 days before experiments. The cells (1.0×10^4 cells/well) were plated into 96-well plates. Cells were allowed to attach to the bottom overnight, and then treated with different concentrations of oridonin or cisplatin for 24 h, 48 h and 72 h, respectively. Control cells received an equal amount of DMSO only. Before test, 20 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C in the dark. After removing the supernatant, formazan crystals formed were dissolved in 100 μL DMSO and mixed thoroughly before reading on a microplate reader. The absorbance was measured at 490 nm. All *in vitro* experiments were carried out in triplicate.

2.3. Tumor inhibition assay in vivo

The protocol of the animal study was approved by the Animal Ethics Committee of Wuhan University. All experiments were conducted in accordance with institutional guidelines. Briefly, 56 male BALB/C athymic nude mice (Wuhan University Laboratory Animal Center, Wuhan, China) weighing between 18 and 23 g (4–6 weeks old) were subcutaneously engrafted with 1×10^7 MV4-11/DDP cells. Tumors were allowed to establish and grow to a volume of 100–150 mm^3. The rodents were then randomly divided into seven groups of eight mice; they received the following treatments by an intraperitoneal injection: 0.9% saline, two doses of cisplatin (1 and 2 mg/kg), two doses of oridonin (10 and 20 mg/kg), and two combinations (1 mg/kg cisplatin + 10 mg/kg oridonin; 2 mg/kg cisplatin + 20 mg/kg oridonin). The treatment for each group was administered once daily for 21 days, with a 1-day interval every 6 days. Tumor size was measured every 3 days starting on the day of first treatment (day 0) and ending 28 days after the first treatment (day 28) according to the published literature (Estey, 2014). At day 28, the animals were killed and tumor weight was determined.

2.4. Apoptosis

Flow cytometry was used to detect the apoptosis rate. MV4-11/DDP cells (2×10^4 cells/well) were plated into 6-well plates. They were cultured for 6–8 h and treated with cisplatin alone, oridonin alone, or cisplatin in combination of oridonin for 48 h. The cells were analyzed after being treated with RNAse (Sigma) and stained with annexin v and propidium iodide (Sigma) before test.

2.5. Cell migration and invasion assays

For the transwell migration assay, 5×10^4 MV4-11/DDP cells were placed in the upper chamber of each insert (Corning). For the invasion assay, 1×10^5 cells were placed on the upper chamber of each insert coated with 40 μL matrigel (Clontech), which was diluted to 2 μg/μL with RPMI-1640 medium. Medium supplemented with 20% FBS (600 μL) was added to the lower chambers. After several hours of incubation, the upper

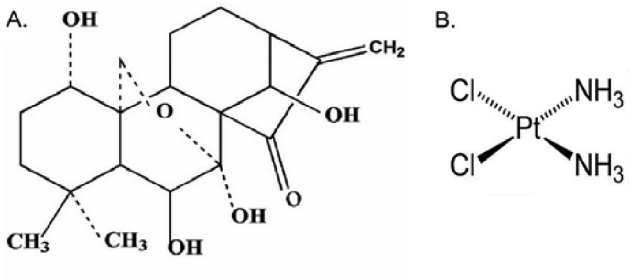


Figure 1 Chemical structures of oridonin and cisplatin. (A) Chemical structure of oridonin. (B) Chemical structure of cisplatin.

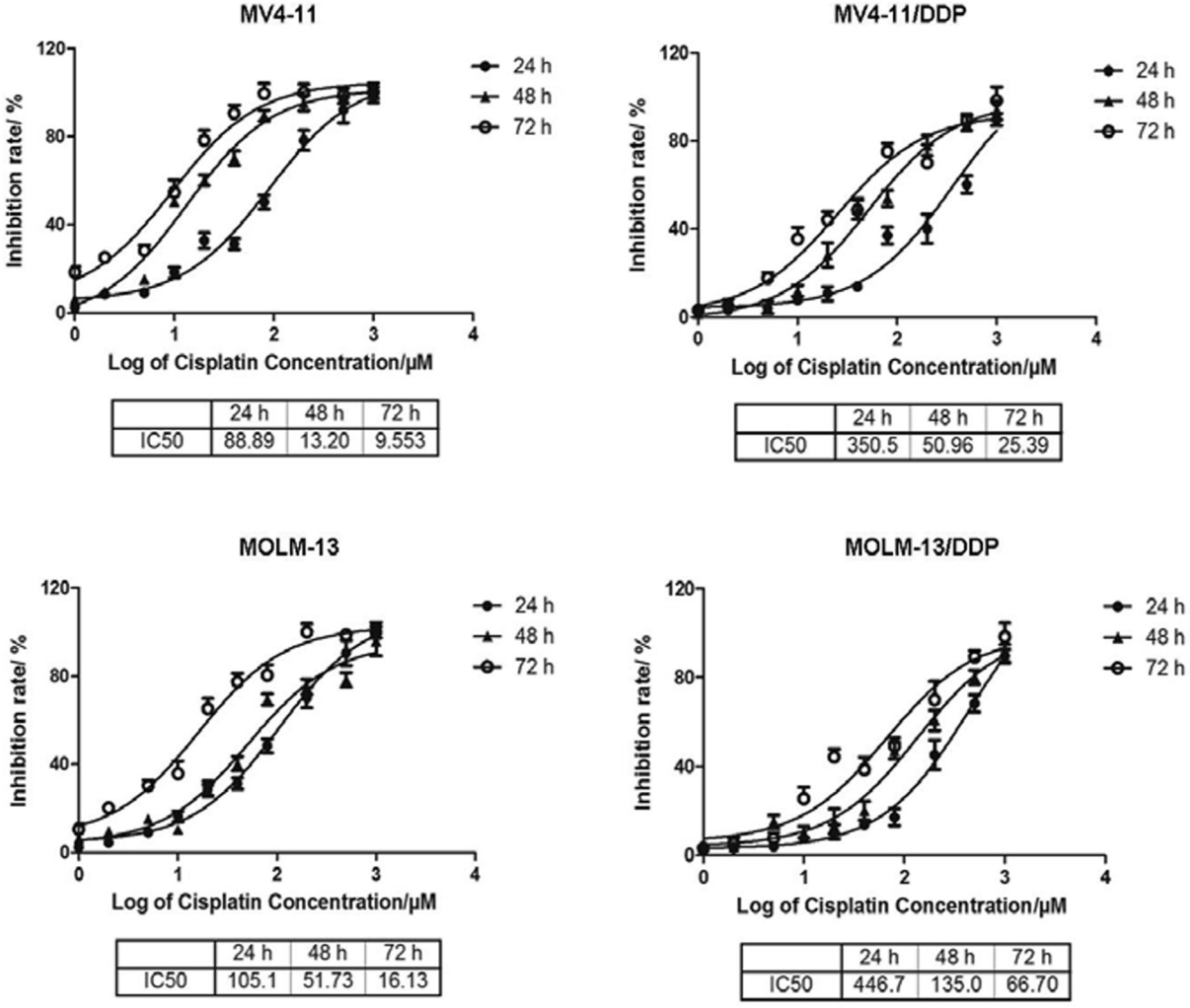
surface of the membrane was wiped with a cotton tip and cells attached to the lower surface were stained for 20 min with crystal violet. Cells in 5 random fields of view at 100× magnification were counted and expressed as the average number of cells per field of view.

2.6. Western blotting

Western blots were performed as described previously (20–22). Briefly, total protein extract for each tissue sample or cell line was dissolved in lysis buffer and equal amounts of protein (60 μg) were analyzed by immunoblotting. Rabbit polyclonal antibodies against Bcl-2and Bax, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A). The horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) was obtained from Abgent corporation.

2.7. Statistical analysis

All data were processed using the SPSS 17.0 (IBM, Armonk, NY, USA). Statistical analysis was performed by ANOVA and Student's *t*-test for continuous data. The data were shown as the mean ± standard error of the mean. $P < 0.05$ was considered to be significant.



	24 h	48 h	72 h
IC50	88.89	13.20	9.553

	24 h	48 h	72 h
IC50	350.5	50.96	25.39

	24 h	48 h	72 h
IC50	105.1	51.73	16.13

	24 h	48 h	72 h
IC50	446.7	135.0	66.70

Figure 2 IC50 values of cisplatin on AML cells are determined by MTT assay. The human AML cells MV4-11, MV4-11/DDP, MOLM-13 and MOLM-13/DDP cells (3×10^4 cells/well) were plated into 96 wells and the cells were exposed for increasing concentrations of cisplatin for 24 h, 48 h and 72 h, respectively.

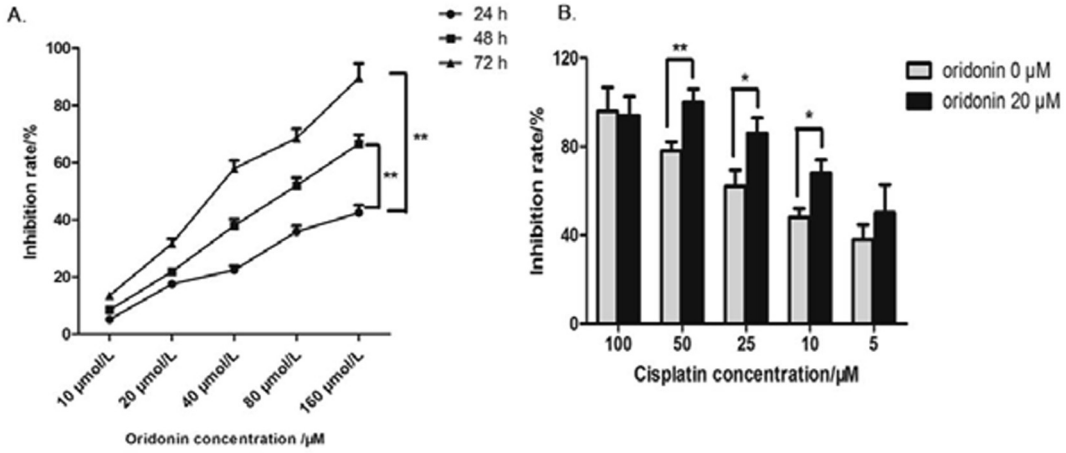


Figure 3 The proliferation of MV4-11/DDP cells is determined by MTT assay. (A) The DDP resistant AML cells MV4-11/DDP (3×10^3 cells/well) were plated into 96-well plates and treated with increasing concentrations of oridonin for 24 h, 48 h and 72 h, respectively. Data are shown as the mean values ± SD. in triplicate. $^{**}p < 0.01$, compared with the values of 24 h. (B) MV4-11/DDP cells were treated with 20 μM of oridonin in combination with increasing concentrations of cisplatin for 48 h. The inhibition rate of oridonin in combination of cisplatin was determined by MTT assay. The cells treated without oridonin were used as controls. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with untreated cells.

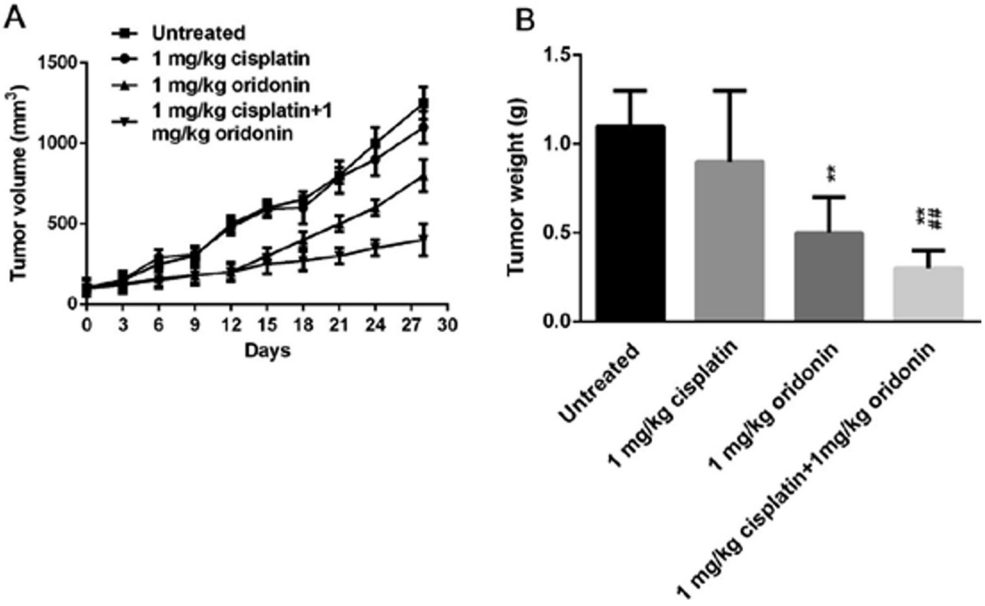


Figure 4 Cisplatin, oridonin, and the combination of both agents inhibit the growth of xenografted A2780/DDR cells in nude mice. (A) Tumor growth after treatment with cisplatin alone and/or oridonin. The treatment for each group was administered once daily for 21 days, with a 1-day interval every 6 days. (B) Tumor weight at 28 days after treatment. Data are expressed as mean ± SD ($n = 8$). $^{**}p < 0.01$, compared with the untreated group; $^{\#\#}p < 0.01$, compared with the cisplatin-alone group.

3. Result

3.1. The IC50 value of cisplatin is determined in human AML cells

In order to detect the antitumor effects of cisplatin in ovarian cancer cells, the human AML cell lines and their cisplatin-resistant sublines, MV4-11 and MV4-11/DDP, MOLM-13 and MOLM-13/DDP were used as cell models. The AML cells were treated with increasing concentration of cisplatin and the inhibitory rate was determined by MTT assay. The structure of oridonin and cisplatin is shown in Fig. 1. Cisplatin had an increasing antitumor effect in human AML cell lines MV4-11 and MV4-11/DDP in a dose and time dependent manner. As shown in Fig. 2, the IC50 values of cisplatin were 88.89 μM, 13.20 μM and 9.55 μM for 24 h, 48 h and 72 h in sensitive cell line MV4-11, respectively. However, the IC50 values were 350.5 μM, 50.96 μM and 25.39 μM in cisplatin-resistant cell line MV4-11/DDP, being treated for 24 h, 48 h and 72 h, respectively. The IC50 values of MOLM-13 and

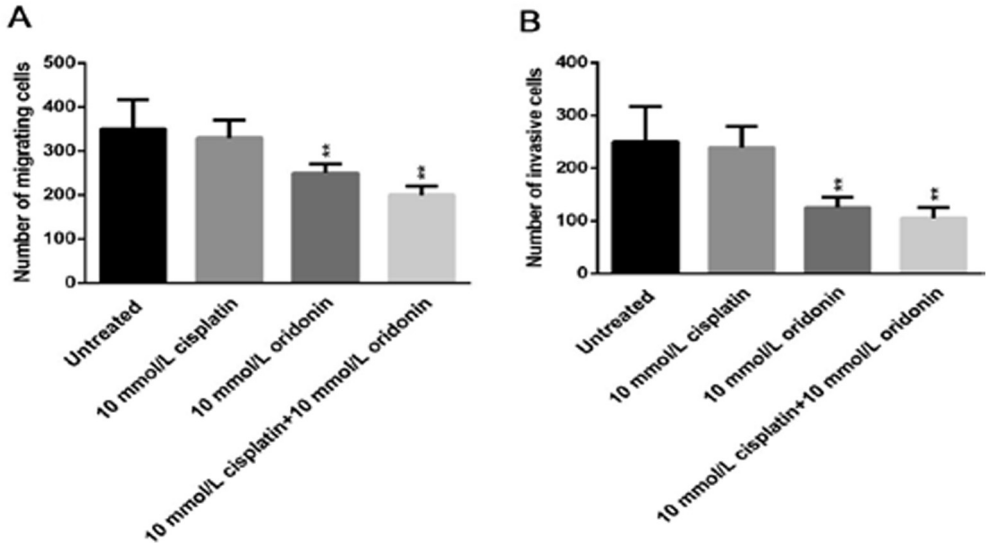


Figure 5 Cisplatin and oridonin synergistically inhibit ovarian cancer cell migration and invasion *in vitro*. (A) Transwell migration assay of A2780/DDR cells treated with cisplatin alone, oridonin alone, or cisplatin and oridonin combination. (B) Transwell invasion assay of A2780/DDR cells treated with cisplatin alone, oridonin alone, or cisplatin and oridonin combination. $^{**}p < 0.05$.

MOLM-13/DDP were consistent with those of MV4-11 and MV4-11/DDP. IC50 values in were MOLM-13 and MOLM-13/DDP 105.10 μM, 51.73 μM, 16.13 μM and 446.7 μM, 135.0 μM, 66.70 μM, respectively, for 24 h, 48 h and 72 h.

3.2. Oridonin synergistically increases the antitumor effects of cisplatin in MV4-11/DDP cells

The inhibitory effects of oridonin in human AML cells were also detected by MTT assay. The cisplatin resistant human AML cells MV4-11/DDP were treated with increasing concentrations of oridonin for 24 h, 48 h and 72 h, respectively. The concentrations of oridonin were 10 μM, 20 μM, 40 μM, 80 μM and 160 μM. As shown in Fig. 3A, the inhibitory effects were enhanced as the increasing concentration of oridonin.

In order to detect whether oridonin could play the synergistically antitumor effects with cisplatin in human AML cells, we chose 20 μM as the appropriate concentration for oridonin. The concentrations of cisplatin were 5 μM, 10 μM, 25 μM, 50 μM and 100 μM, respectively. Compared with the group treated with oridonin alone, the inhibitory effects were enhanced as the increasing concentrations of cisplatin in combination with 20 μM of oridonin for 48 h (Fig. 3B), which demonstrated that oridonin and cisplatin showed synergistical antitumor effects in human AML cells.

3.3. Cisplatin and oridonin synergistically inhibit the growth of xenografted MV4-11/DDP cells in nude mice

Next, we confirmed the synergistic antitumor activity of cisplatin and oridonin in nude mice with xenografted MV4-11/DDR cells. As shown in Fig. 4, 1 mg/kg cisplatin alone did not significantly inhibit the growth of xenografted/DDR cells. In contrast, 1 mg/kg oridonin demonstrated significant inhibitive effects (33 decrease in tumor volume and 31 decrease in tumor weight, respectively) after 28 days of treatment. However, compared with oridonin alone, cisplatin and oridonin combination induced a considerably higher reduction in tumor volume (72%) and tumor weight (84%). Taken together, these findings indicate a synergistic antitumor effect between cisplatin and oridonin *in vivo*.

3.4. Cisplatin and oridonin synergistically inhibit ovarian cancer cell migration and invasion in vitro

Furthermore, we investigated the effect of oridonin on migration and invasion of cisplatin-resistant ovarian cancer cells. Transwell migration assay revealed that cisplatin alone did not inhibit cancer cell migration compared with the untreated group. However, 10 mmol/L oridonin demonstrated significant inhibitive effects. In addition, compared with oridonin alone, cisplatin and oridonin combination induced more significant inhibitive effects on ovarian cancer cell migration (Fig. 5A). Similarly, transwell invasion assay demonstrated that oridonin alone and cisplatin and oridonin combination markedly decreased the invasive capacity of MV4-11/DDR cells (Fig. 5B).

3.5. Oridonin and cisplatin synergistically induces cell apoptosis in MV4-11/DDP cells

We observed that the cell death rates significantly increased in cisplatin-oridonin group. In order to identify whether cell apoptosis in MV4-11/DDP cells was induced being treated with oridonin alone, cisplatin alone, oridonin in combination with cisplatin for 48 h, FACS analysis was performed to detect the cell apoptosis rate in MV4-11/DDP cells. As shown in Fig. 6A, the cells were treated with 50 μM of cisplatin alone, 50 μM of oridonin alone, or 50 μM of cisplatin plus 50 μM of oridonin for 48 h, and the late apoptosis rate was 32.48% in oridonin-cisplatin group, which was much higher than that of the oridonin group or cisplatin group. As shown in Fig. 6B, the early and late cell apoptosis rates were 42.67%, 40.73% and 71.24% in oridonin group, cisplatin group and oridonin plus cisplatin group, respectively. All the results demonstrated

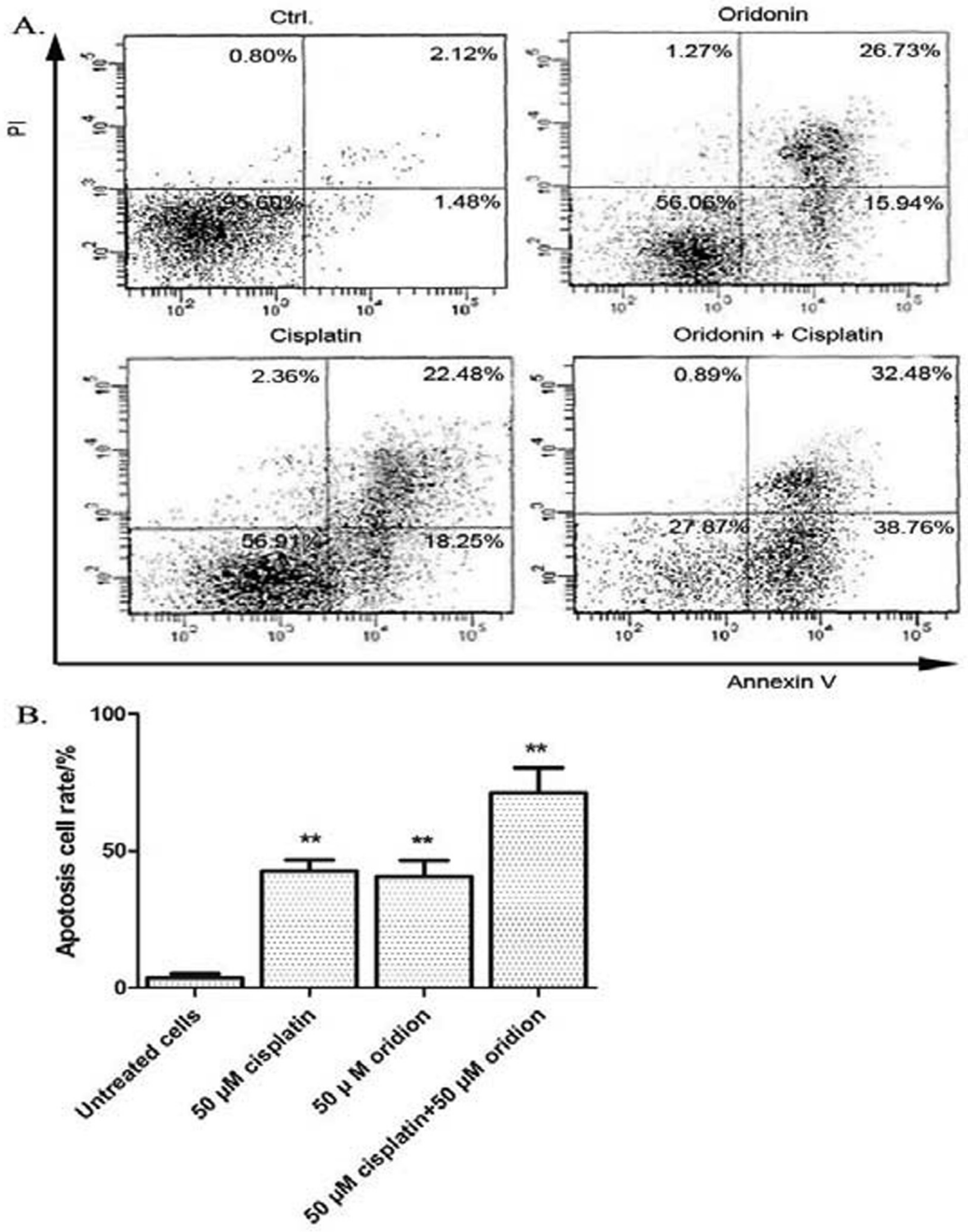


Figure 6 Cell apoptosis rates are detected by flow cytometry of MV4-11/DDP cells. (A) MV4-11/DDP cells (2×10^4cells/well) were plated into 6-well plates. They were cultured for 6–8 h and treated with cisplatin alone, oridonin alone, or cisplatin in combination of oridonin for 48 h. The untreated cells were used as negative controls. Cell apoptosis rate were detected by FACS analysis with Annexin V and PI dual staining method. (B) The histogram of cell apoptosis rates is shown. $^{**}p < 0.01$, compared with untreated cells.

that oridonin had a synergistical role with chemotherapy drug cisplatin to induce cell apoptosis in MV4-11/DDP cells.

3.6. Oridonin induces cell apoptosis of human AML cells and induces cell-cycle arrest in G0/G1 phase

In order to further clarify the mechanism of cell apoptosis in cisplatin resistant human AML cells, the MV4-11/DDP cells were treated with oridonin at the concentration of 10 μM, 20 μM, 40 μM, 80 μM and 160 μM for 48 h. As shown in Fig. 7A, the result showed that oridonin indeed induced cell apoptosis of MV4-11/DDP cells and the apoptosis rate increased in a dose-dependent manner with oridonin.

The distribution of cell cycle of MV4-11/DDP cells was also analyzed by propidium iodide-staining method. As shown in Fig. 7B, G0/G1 phase arresting of the cells was induced and the number of cells in G0/G1 phase increased as increasing concentrations of oridonin.

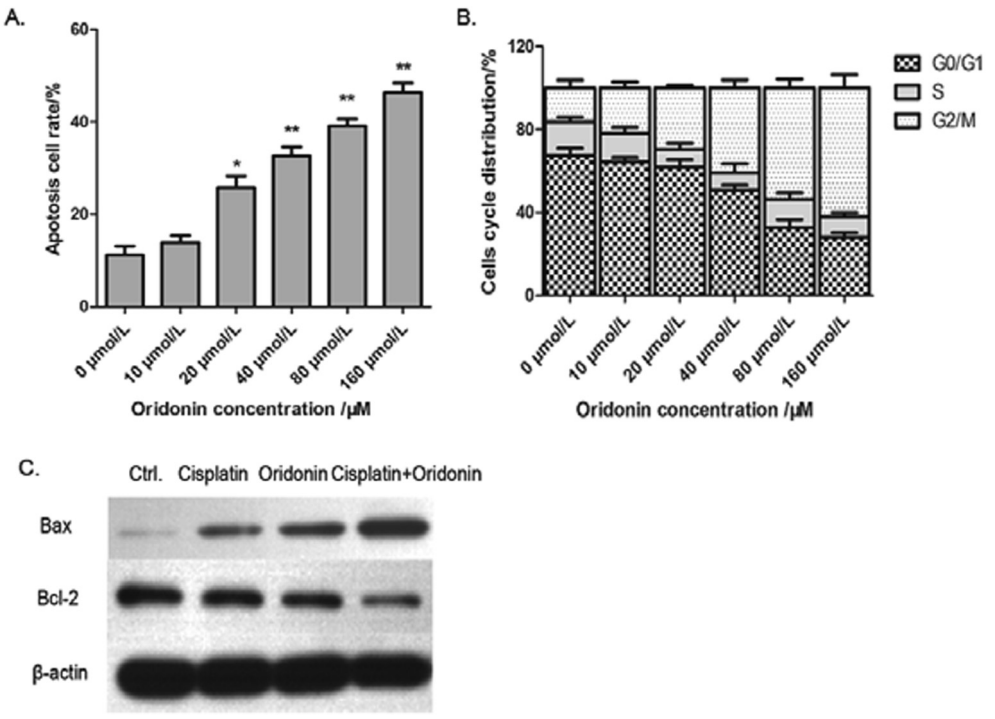


Figure 7 The cell apoptosis rate and cell cycle of MV4-11/DDP cell is determined. (A) MV4-11/DDP cells were treated with increasing concentrations of oridonin for 48 h, and the untreated cells were used as negative controls. Cell apoptosis rate was determined by FACS analysis with Annexin V-FITC and PI dual staining. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with untreated cells. (B) Cell cycle of MV4-11/DDP cells was determined by propidium iodide-staining method. MV4-11/DDP cells were treated with increasing concentrations of oridonin for 48 h. Proportion of the cells in each phase was determined. The cell distribution was shown in the histogram. (C) Expression of apoptosis related proteins in MV4-11/DDP cells was detected by western blotting analysis. MV4-11/DDP cells were treated with 20 μM of oridonin and 10 μM of cisplatin for 48 h. Expressions of Bax and Bcl-2 in MV4-11/DDP cells were detected by western blotting. The experiment is repeated for 3 times.

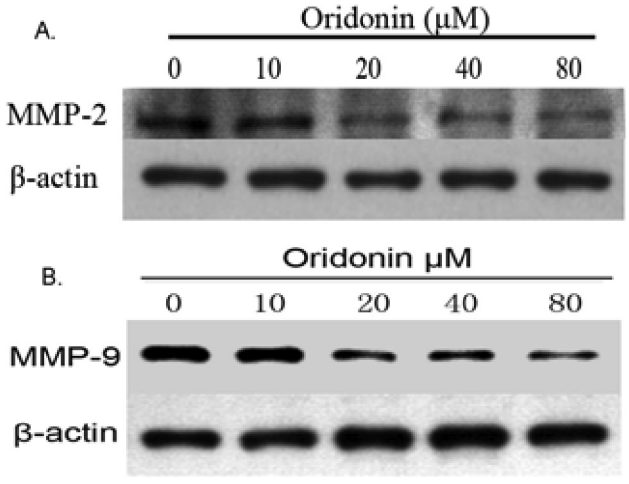


Figure 8 The expression levels of MMP-2 and MMP-9 in MV4-11/DDP cells are detected by western blotting analysis. MV4-11/DDP cells were plated into 48-well plates and cultured for 8 h. The cells were treated with increasing concentrations of oridonin for 48 h. The untreated cells were used as negative controls.

3.7. Oridonin and cisplatin synergistically down-regulated expression of Bcl-2 and upregulated the expression of Bax

We also detected the expression of Bcl-2 family proteins by Western blot analysis. As shown in Fig. 7C, treatment with oridonin resulted in down regulation of Bcl-2 protein and the up-regulation of Bax protein. This was consistent with those treated by cisplatin. More importantly, MV4-11/DDP cells were treated with 20 μM of oridonin and 10 μM of cisplatin for 48 h, the ratio of Bax/Bcl-2 was obviously higher than that treated with cisplatin or oridonin alone. All the data revealed that oridonin and cisplatin synergistically down-regulated expression of Bcl-2 and upregulated the expression of Bax.

3.8. The expression levels of MMP-2 and MMP-9 decrease in dose-dependent manner treated with oridonin

MMPs such as MMP-2 and MMP-9 are involved in the invasion and metastasis of several human malignancies, as degradation of collagen IV in basement membrane and extracellular matrix facilitates tumor progression. Finally, we detected the expression levels of MMP-2 and MMP-9 in MV4-11/DDP cells treated with increasing concentration of oridonin. MV4-11/DDP cells were treated with increasing concentrations of

oridonin for 48 h. The concentrations of oridonin were 10 μM, 20 μM, 40 μM and 80 μM, respectively. As shown in Fig. 8, the expression levels of MMP-2 and MMP-9 were decreased as the increasing concentration of oridonin suggesting that oridonin could have the ability to suppress the invasion and metastasis of human AML cells.

4. Discussion

Adverse side effects of chemotherapy and resistance to chemotherapy drugs have been the pressing problems in AML treatment (Jamieson et al., 2016). As we have known, cisplatin resistance is a major obstacle in the treatment of AML. New chemotherapy strategies are urgently needed for AML treatment (Arriazu et al., 2016; Mamdani et al., 2016; Ding et al., 2014). In the present study, we tried to investigate the new method to reverse the cisplatin-resistance by combination therapy with oridonin and cisplatin in human AML cells. Additionally, our goal is also focused on reducing the side effects of single medication. Here, we used two cisplatin-resistant AML cell lines MV4-11/DDP and MOLM-13/DDP as *in vitro* cell models. Our results demonstrated that oridonin had a synergistical role with chemotherapy drug cisplatin to inhibit proliferation and induce cell apoptosis of cisplatin - resistant human AML cells.

Firstly, the combination therapy of oridonin and cisplatin has a synergistical anti-tumor effect, which could decrease the dose of single medication. It is effective to kill tumor cells at a relatively low dose, which is useful to decrease the side effects of chemotherapy drugs. The results demonstrated that cell death significantly increased in cisplatin-oridonin group partly because cell apoptosis was induced in oridonin and cisplatin treated groups. The apoptosis rates were 71.24% in oridonin plus cisplatin group, significantly higher than those of 42.67%, 40.73% in oridonin alone group or cisplatin alone group. This was consistent with that detected by Western blotting analysis. The down-regulated expression of Bcl-2 and upregulated the expression of Bax showed combination therapy of oridonin and cisplatin promoted cell apoptosis in drug-resistant MV4-11/DDP cells (Hoshyar et al., 2013; Lu et al., 2010). We also detected cell phase by FACS, and the results demonstrated oridonin induces cell-cycle arrest in G0/G1 phase and the apoptosis rate increased in a dose-dependent manner with oridonin. All of the results demonstrated that the combination therapy is an effective way to inhibit the proliferation of human AML cells.

Additionally, the preferential cell growth inhibitory effect might be at least partly attributed to the inhibition of MMPs. MMPs are involved in the invasion and metastasis of human malignancies, and we found the expression levels of MMP-2 and MMP-9 were decreased in MV4-11/DDP cells treated with increasing concentration of oridonin. The data revealed that oridonin could have the ability to suppress the invasion and metastasis of human AML cells, which was probably effective to inhibit the growth of drug-resistant AML cells.

In conclusion, combination therapy with oridonin and cisplatin was a useful method to treat cisplatin-resistant human AML cells. Both of the compounds exerted synergistic anti-tumor effects and effectively reverse the cisplatin resistance in human AML cells. Meanwhile, the drug toxicity was to be decreased under these experimental conditions. Therefore, it would be a beneficial and useful treatment for the drug-resistant human AML.

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