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Effects of oridonin on proliferation of HT29 human colon carcinoma cell lines both *in vitro* and *in vivo* in mice

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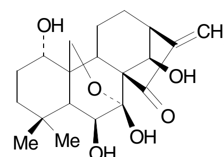
Oridonin, an active ditepenoid component isolated from *Rabdosia rubescens* which is currently one of the most important Chinese traditional herbs, has been reported to exhibit anti-tumor effects *in vitro*. In this study, the anti-proliferation effect of oridonin against the human colorectal carcinoma cells HT29 was investigated both *in vitro* and *in vivo*. MTT assay showed that oridonin inhibited HT29 cells in a time- and dose-dependent manner. Flow cytometric analysis demonstrated that oridonin induced a G2/M phase arrest. Apoptotic bodies were observed by Hoechst 32258 fluorescence staining. Notable apoptosis and decrease of mitochondrial membrane potentials was also detected by flow cytometry. In the *in vivo* experiments, oridonin (10, 15, 20 mg kg^{-1} of body weight, on days 1–12) was injected intraperitoneally into mice 24 h after the mice were incubated with HT29 cells. Inhibition of the solid tumor was observed. As a result, oridonin could inhibit the proliferation of HT29 cells both *in vitro* and *in vivo*, and induce apoptosis partly via the mitochondrial pathway.

1. Introduction

Rabdosia rubescens, which is a traditional Chinese herb originating in He-Nan province (Northeastern region of China), has been clinically used to treat colorectal carcinoma, esophageal carcinoma and some other kinds of tumors by herbalist doctors for many years (Huang 1992). Oridonin, an *ent*-kaurane diterpenoind isolated from *Rabdosia rubescens*, has been identified as the most important bioactive component of this herb. It has not only exhibited various pharmacological and physiological functions such as immunoregulatory, anti-inflammation and anti-bacteria bioactive effects (Kubo et al. 1974; Han et al. 2003; Fujita et al. 1976a), but also been reported to be a very effective reagent with profound anti-tumor effects (Fujita et al. 1976a, 1976b, 1988). Previous studies have shown that oridonin can inhibit the proliferation of a number of malignant cells such as breast, ovarian, prostate, non-small cell lung, leukemia, and glioblastoma cancers (Ikezoe et al. 2003; Chen et al. 2005; Liu et al. 2003). It was also indicated that oridonin had reversal effects on multidrug resistant cell lines such as K562/A02 (Guo et al. 2002). *In vitro* studies showed that oridonin could inhibit sodium pump activity of tumor cells (Wu et al. 1994), induce apoptosis effects on some tumor cell lines (Wang et al. 2001), facilitate the phagocytic activity against apoptotic cells (Liu et al. 2005), and prevent DNA damage in the mammalian cells (Yan et al. 2005).

Although *Rabdosia rubescens* has been mainly used for folk therapy and clinical trials on colorectal carcinoma for a long time in China, up to now, there has been no available information which addresses the anti-tumor effect and its functional mechanisms of oridonin in colorectal carcinoma.



In a continuation work of investigating the anti-melanoma mechanisms of oridonin (Ren et al. 2006), present studies were initiated to explore the inhibitory function of oridonin on proliferation of human colorectal carcinoma HT29 cell lines *in vitro*. In order to clarify some of its anti-proliferation mechanisms, we investigated the effects of oridonin on HT29 cell cycle progression, cell apoptosis, and changes of mitochondrial membrane potentials. We also did the *in vivo* experiment to observe the inhibitory effects of oridonin on transplanted tumors in mice. This may shed light on elucidating the mechanism of oridonin's anti-colorectal carcinoma effects.

2. Investigations and results

2.1. *Inhibitory of oridonin on cell growth in vitro*

As a result of MTT assay, oridonin could inhibit the growth of HT29 cells both in time-dependent and dose-dependent manners. It was noticed that the inhibition was not very distinct until the present concentration of oridonin reached 30 μM. Strong inhibition was observed with an increase in concentration and time (Fig 1). Cells could hardly grow in the presence of 40 μM oridonin. The inhi-

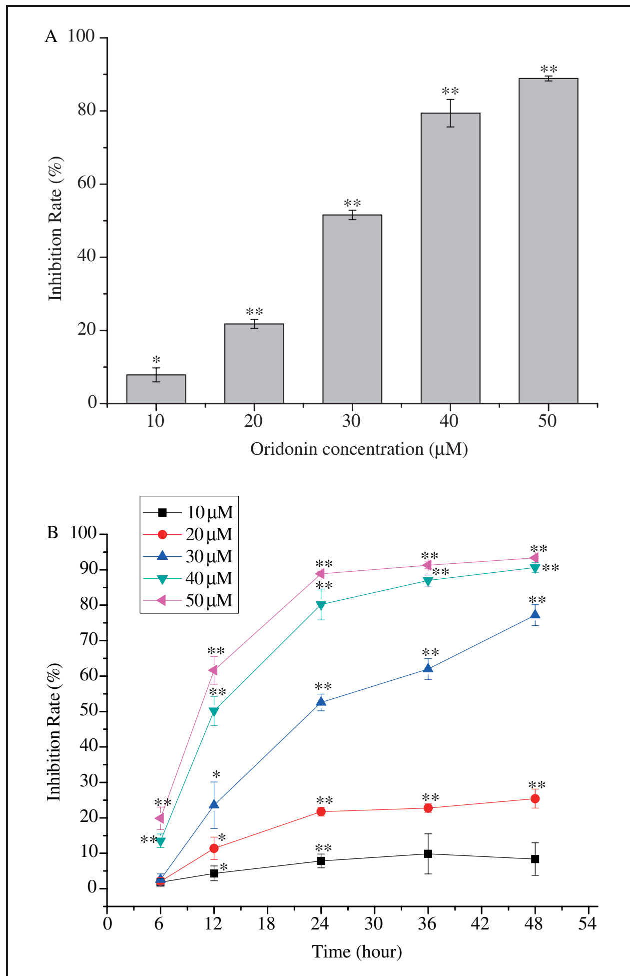


Fig. 1: Effects of oridonin on cell growth of HT29 cell lines *in vitro*.
Cells in logarithm growing period were treated with oridonin. After incubation for the desired time, the inhibitory rate was determined by MTT assay as described in Experimental. (A) with 10–50 μM oridonin for 24 h in the dose-dependent study. (B) with 10–50 μM oridonin in the time-dependent study. (mean ± S.D., n = 5, * $p < 0.05$, ** $p < 0.01$ vs. control)

bition rate could be (50.2 ± 4.1)% after 40 μM oridonin treatment for 12 h, and (80.1 ± 4.4)% for 24 h (mean ± S.D., n = 5). These results demonstrated the potency of oridonin to inhibit the proliferation of HT29 cells *in vitro*.

2.2. Effects of oridonin on cell cycle distribution

Flow cytometry analysis revealed that HT29 cells were arrested in G2/M phase after oridonin treatment for 24 h. However, this arrestment was unconspicuous until the concentration of oridonin reached 30 μM (Fig. 2). (35.62 ± 0.69)% HT29 cells were stationed in the G2/M phase at 30 μM, while only (14.54 ± 1.52)% cells were stationed at 20 μM. The cells stationed in the S phase at 30 μM [(13.29 ± 0.85)%] were less than the half of control [(39.34 ± 0.53)%] (mean ± S.D., n = 3).

2.3. Effects of oridonin on apoptosis

To determine if exposing HT29 cells to oridonin results in apoptosis, HT29 cells were incubated with 0,10 μM, 20 μM, 30 μM, 40 μM and 50 μM oridonin for 24 h, respectively. Marked morphological changes of cell apoptosis including condensation of chromosome and nuclear fragmentation were found using Hoechst 32258 staining

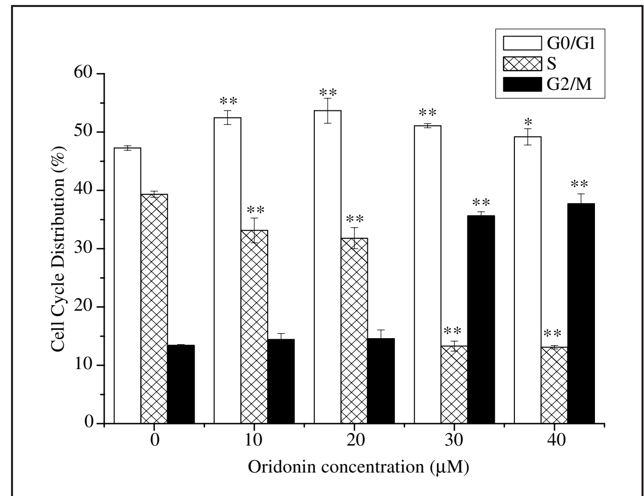
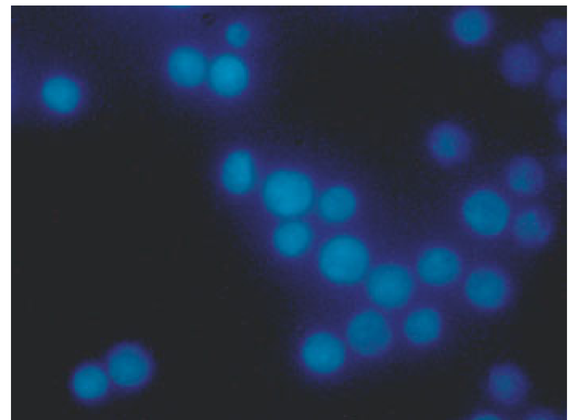


Fig. 2: Effects of oridonin on cell cycle distribution in HT29 cells.
Cells have been grown in 0–40 μM of oridonin for 24 h, and then analyzed by flow cytometry as described in Experimental. (mean ± S.D., n = 3, * $p < 0.05$, ** $p < 0.01$ vs. control)

when HT29 cells were exposed to 30 μM for 24 h. But the above mentioned morphological changes of cell apoptosis were absent in the control group (Fig. 3). The results of dual-color flow cytometry analysis showed that oridonin can induce apoptosis in HT29 cells significantly when the



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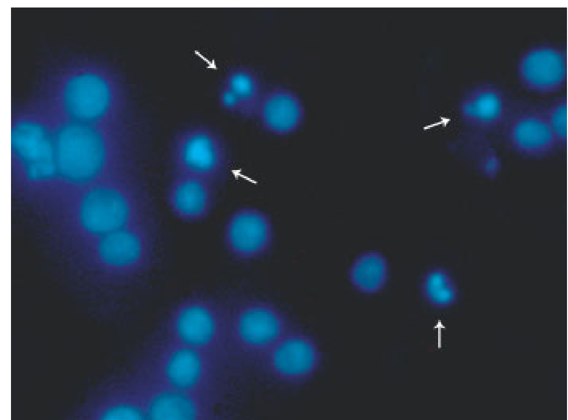

B

Fig. 3: Morphology changes of cells treated by oridonin (Hoechst 32258 staining, × 630).
(A) Control group (B) Cells were incubated with 30 μM oridonin for 24 h. Apoptotic bodies were observed, including condensation of chromosome and fragmentation of nuclei

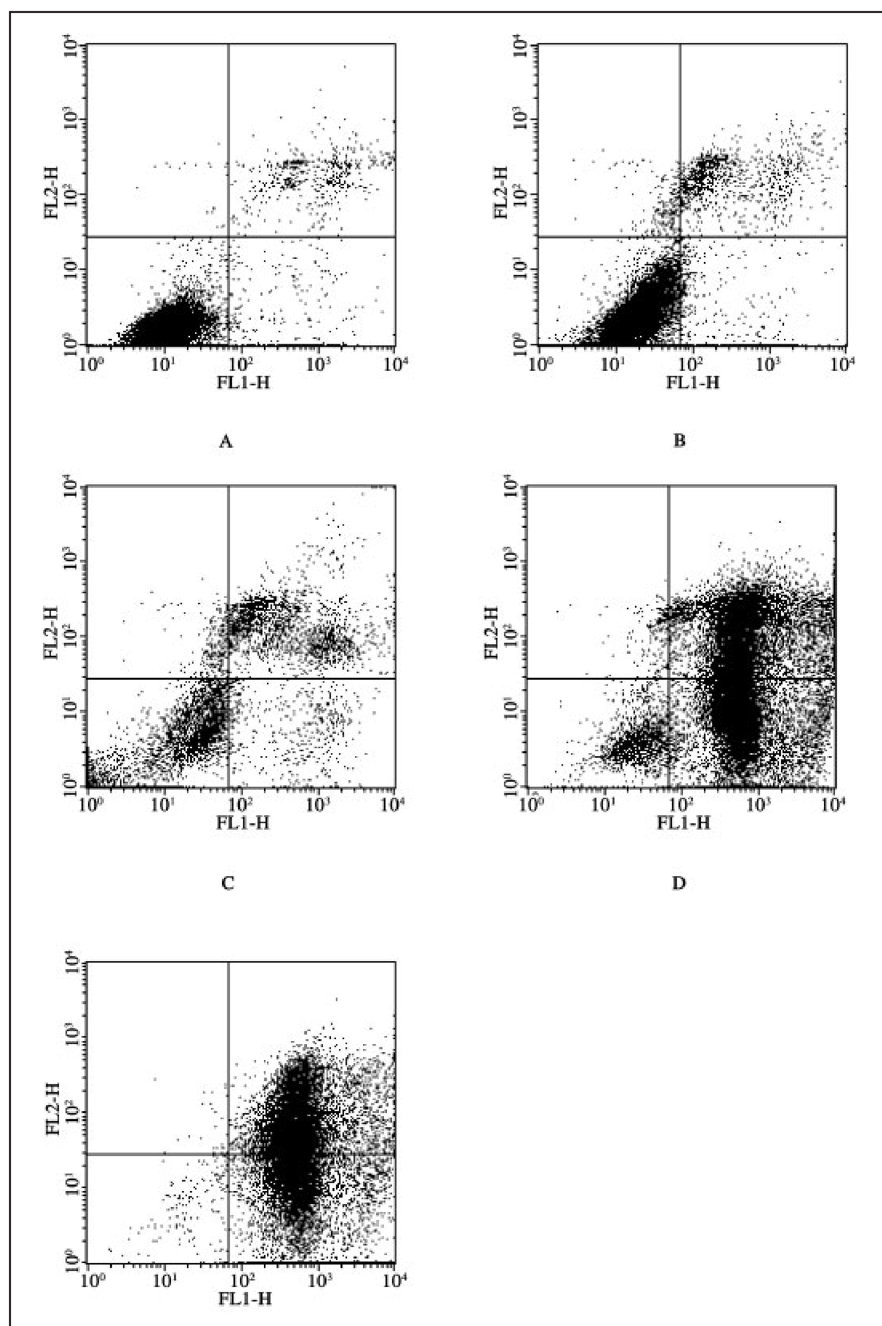

Fig. 4:
Effects of oridonin on apoptosis of HT29 cells.
(A) Control (B) 10 μM (C) 20 μM (D) 30 μM (E) 40 μM. Cells have been grown in the presence or absence of oridonin for 24 h addition: The FLI-H axis denotes FITC-Annexin-V, while the FL2-H axis denotes PI. The fraction of cells that were FITC-Annexin-V positive and PI negative were considered apoptotic

concentration of oridonin reached 30 μM (Fig. 4). Hence, it was shown that oridonin added over 30 μM could cause apoptosis on HT29 cells prominently from both morphology observation and dual-color flow cytometry.

2.4. Changes of mitochondria membrane potentials ($\Delta\psi m$)

In our work, we investigated the change of $\Delta\Psi m$ of HT29 cells treated by oridonin. Quantitation of $\Delta\Psi m$ was determined by rhodamine-123 retention. Rhodamine-123 is a cationic fluorescent dye, which localizes in the mitochondria of viable cells because of the relatively high negative electric potential across the mitochondria inner membrane (Johnson et al. 1980). Thus, depolarization of $\Delta\Psi m$ drives the down-regulation of the accumulation in mitochondria of cationic dyes such as rhodamine-123. From our research, we could see that oridonin did cause the decline in rhodamine-123 uptake of mitochondria, as compared with control (Fig. 5). The mean value of searched fluorescent intensity was 712.19 ± 12.81 without oridonin, 605.04 ± 6.15, 527.42 ± 13.48, 339.01 ± 8.11 and 308.67 ± 17.23 after incubating with 10 μM, 20 μM, 30 μM and 40 μM oridonin for 24 h, respectively (mean ± S.D., n = 3). From these data we noticed that the decrease in fluorescent intensity was striking after the present concentration of oridonin reached 30 μM.

2.5. Effect of treatment with oridonin on growth of HT29 cells in mice

The impact of oridonin on the growth of implanted HT29 cells was evaluated. The treatment with oridonin inhibited primary tumor growth of HT29 cells on the oxters of mice after intraperitoneal injection. We supervised that the relative tumor inhibition obtained with 10, 15 and 20 mg kg^{-1} of oridonin was 19.97%, 27.35%, and 53.19%, respectively (Table). The tumor weight of mice treated with

Table: Effects of oridonin on the growth of HT29 human colorectal carcinoma cells in BALB/C nu/nu mice (5 – 7 weeks)

Oridonin (mg kg^{-1})	Number of mice		Body weight (g)		Tumor weight (g)	Inhibition (%)
	Beginning	End	Beginning	End		
–	10	10	20.2 ± 0.37	28.3 ± 1.91	2.168 ± 0.34	–
10	10	10	20.0 ± 0.57	28.0 ± 2.5	1.735 ± 0.26	19.97*
15	10	10	20.0 ± 0.47	27.5 ± 2.63	1.575 ± 0.98	27.35*
20	10	10	20.1 ± 0.42	27.2 ± 1.65	1.015 ± 0.46	53.19**

Values are reported as mean ± S.D., * $p < 0.05$, ** $p < 0.01$

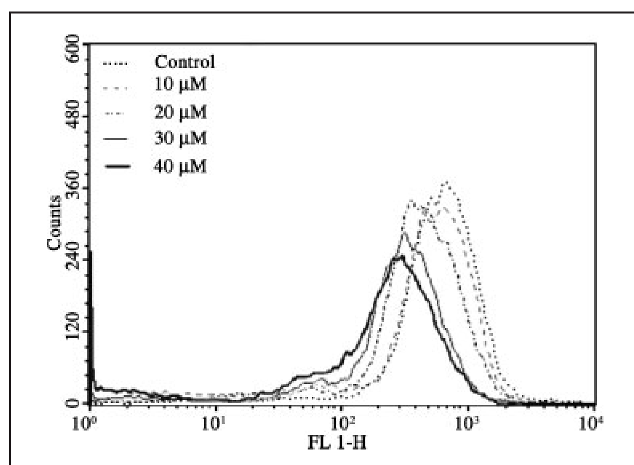

Fig. 5: Mitochondrial membrane potentials changes of cells treated by oridonin.
Cells have been grown in the presence or absence of oridonin for 24 h. Data in this figure are representative of three independent experiments

15 and 20 mg kg^{-1} of oridonin was significantly reduced ($p < 0.05$) compared with that of the control group. During this experiment, physiological behavior, appetite and body weight were not disturbed. The *in vivo* experiment indicated that oridonin played a role in the successful completion of primary tumor proliferation or growth and also had a therapeutic potential for the treatment of a solid tumor.

3. Discussion

Cell proliferation plays an important role in multistage carcinogenesis with multiple genetic changes (Moore and Tsuda 1998). Therefore, it is very important to control cell growth for cancer prevention (Mori et al. 1999). Oridonin has been reported to be one of the most effective diterpenoind derivatives extracted from *Rabdosia rubescens* and was proved to have anti-proliferation activity against lots of tumor cells. Our present research showed that oridonin can significantly inhibit growth of colorectal carcinoma HT29 cells both *in vitro* and *in vivo*.

From MTT assay, we found that oridonin could inhibit the proliferation of HT29 cell lines in a dose- and time-dependent manner *in vitro*. However, the inhibition was not very distinct until the present concentration of oridonin reached 30 µM. In the cell cycle distribution experiment, cells were found retarded at G2/M phase eminently when the added oridonin reached above 30 µM. This was coincident with our study fact that the growth inhibition was not very remarkable until this concentration. Thus, it can be deduced that inhibitory effects of oridonin came from G2/M phase arrestment. This finding conforms to the earlier observations from prostate (Chen et al. 2005) and melanoma (Ren et al. 2006) cancers. Cell cycle progression requires phosphorylation events carried out by cyclin-dependent kinases (CDKs), the universal cell cycle regulators, which function as key targets of the check-points response (Murray 2004; Nurse 2002). It has been demonstrated that G2 arrest is triggered by the maintenance of the inhibitory phosphorylations of CDK1, the CDK responsible for the G2/M transition (Nurse 2002; Norbury and Zhivotovsky 2004). CDKs activity is closely dependent on association with cyclin subunits (Pines 1995). It is also reported that the formation of CDK1-cyclin B complex is required for G2/M transition and for the cells to enter mitosis (Panaro et al. 1999). So a possible mechanism for G2/M arrest induction by oridonin is to affect the phosphorylation reactions of CDK1, or to prevent the formation or activation of CDK1-cyclin B complex.

Apoptosis is a programmed form of cell death, morphologically characterized by cell shrinkage and condensation of both nuclear chromatin and cytoplasm. Both morphology observation and dual-color flow cytometry announced that oridonin added in a concentration above 30 µM could cause apoptosis on HT29 cells remarkably. Mitochondria play an important role in the particular apoptosis pathway (Green and Reed 1998). Depolarization of the inner mitochondrial membrane potential ($\Delta\Psi m$) facilitated by the opening of large mitochondrial permeability transition pores is regarded as one of the signs of cell death (Hirsch et al. 1997). Cytochrome C, apoptosis inducing factor (AIF) and some other proapoptotic factors are released after disruption of the mitochondrial membrane, and this will lead to caspase-3 activation and eventually apoptotic cell death (Crompton 1999). It has been reported that oridonin can induce human melanoma A375-S2 cell apoptosis via mitochondria-regulated caspase-3 activation (Zhang et al. 2004). During our research, we also detected that the downtrend of mitochondrial membrane potentials of HT29 cells treated by oridonin was obvious to some extent, especially when the oridonin concentration reached 30 µM, which meant that the apoptosis induction of HT29 cells by oridonin was mediated by mitochondrial pathway. Taken together, it seems a reasonable speculation that the anti-tumor effects of oridonin are associated with apoptosis induction, which may be mediated by the mitochondrial pathway. But the details on how oridonin causes apoptosis of HT29 cells in this pathway need further investigation.

On the other hand, like what has been expatiated, another distinct apoptotic signaling pathway is widely recognized besides the mitochondrial pathway on a molecular basis, the death receptor (Fas-Fas ligand) pathway (Hengartner 2000). Fas (CD95) is one member of the tumor necrosis factor (TNF) receptor family. The death signal is known to be transduced through Fas-FasL binding and downstream caspase-8 activation in this pathway. It has also been detected that oridonin can induce dose-dependent growth inhibition in Fas-positive surface expressing GBC-SD (gallbladder carcinoma cells-Shandong) cells (Xue

et al. 2005). Throughout the course of our present study, we have mainly focused on mitochondria-centered apoptotic induction. However, this does not mean that the possibility of the involvement of the death receptor pathways in oridonin-induced HT29 cell death could be neglected.

Furthermore, the *in vivo* results show that systemic administration of 15 and 20 mg kg^{-1} of oridonin resulted in significant retardation of HT29 cells growth with the relative tumor inhibition being 27.35%, and 53.19%, respectively. The *in vivo* experiment pointed out that oridonin was a potent inhibitor of primary tumor proliferation or growth, and it may have therapeutic activity for the treatment of a solid tumor.

Colorectal carcinoma is one of the most frequent causes of cancer deaths in industrialized countries. About 30% patients are diagnosed when they are already at a latter stage, and about half of early stage patients present disease relapse after surgery therapy (Russo et al. 2005). Our current study has indicated that oridonin can inhibit the proliferation of human colorectal carcinoma cell lines HT29 effectively through cell cycle arrestment and apoptosis induction. These results represent the potential of oridonin to treat and prevent colorectal carcinoma.

4. Experimental

4.1. Reagents

Oridonin was isolated from *Rabdosia rubescens* and its purity was higher than 98.0%. Oridonin was dissolved in dimethylsulfoxide (DMSO) to make stock solutions, and then diluted in cell culture medium at different concentrations and used immediately. 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), ribonuclease A (RNase A), Hoechst 32258 and rhodamine-123 were purchased from Sigma Chemical Co. (St Louis, MO). PRIM-1640 medium, trypsin, fetal bovine serum (FBS) and some other culture reagents were purchased from Hyclone (Logan, UT, USA). Forty female C57BL/6 mice (6 weeks old; weight: 18 – 22 g), maintained in the laboratory animal unit of our department, were used.

4.2. Cell cultures

The human colorectal carcinoma cells HT29 (obtained from the medical school of Peking Univ.) were cultured in PRIM-1640 medium *in vitro*, pH 7.2, and the cell cultures were supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Cells were grown at 37 °C under 5% CO_2 atmosphere. Culture media were changed every 2 d. When they reached confluence, cells were dissociated by 0.25% trypsin-0.02% ethylenediamine tetraacetic acid (EDTA) and replated at 1 : 5 dilution.

4.3. MTT assay

The inhibition of cell proliferation by oridonin was determined with the MTT assay, which monitored the numbers of cells based on the reduction of MTT by the mitochondrial dehydrogenases presenting in viable cells. Briefly, cells were plated into 96-well flat-bottom microplates at a density of 1×10^4 cells/well in 180 μl medium. After plating, cells were allowed to attach and grow for 24 h. Oridonin of desired concentration was added using DMSO as the vehicle, with the maximum concentration of DMSO kept to below 0.01% so as not to exert any detectable changes to the cell growth. Incubation with oridonin continued for 48 h, and then 20 μl of MTT (5 mg/ml) was added to each well. After incubation at 37 °C for 4 h, the supernatants were removed and the formazan crystals were dissolved by the addition of 200 μl DMSO. The plate was then read on a microplate reader (Bio-RAD, model 550) at 490 nm. Cell inhibitory rate was calculated according to the formula below:

Inhibition rate (%) = [A490 (control)-A490 (oridonin)]/A490 (control) × 100.

4.4. Cell cycle progression analysis

HT29 cells were cultured in the presence of oridonin of desired concentration and harvested with trypsin/EDTA, washed with phosphate buffered saline (PBS) and fixed with 70% ethanol. The fixed cells were spun down and resuspended in PBS at a concentration of 1×10^6 cells/ml and incubated with RNase A at a final concentration of 100 μg/ml at 37 °C for 30 min, then filtered with nylon meshwork of 300 screen meshes. The cell suspension was stained by propidium iodide (PI), before being measured by a flow cytometer (Partec, CCA-II).

4.5. Hoechst 32258 staining

The morphology of HT29 cells were exposed to oridonin (30 μM) for 24 h and were stained by Hoechst 32258 to observe the apoptotic morphology. Cells were harvested and then fixed in methanol for 10 min at room temperature. After washing by PBS, they were incubated with Hoechst 32258 (0.1 mg/ml) for 30 min at 37 °C, and subjected to fluorescence microscopy (Leica, DM-IRB) through UV-filter to detect condensation of chromosome and fragmentation of nuclei.

4.6. Apoptosis analysis

Apoptosis was determined by the dual-color flow cytometric procedure with staining of FITC-Annexin-V and propidium iodide (PI). FITC-Annexin-V was used to probe the appearance of phosphotidylserine (PS) residues on the outer leaflet of the plasma membrane. As necrotic cells also exposed PS, according to the loss of membrane integrity, cells were simultaneously stained with PI. This allowed differentiation of necrotic and apoptosis population. Cells were prepared according to the manufacturer's instruction. Briefly, approximately 5×10^5 cells per experimental condition were harvested, washed with cold PBS twice, and resuspended with 200 μl binding buffer. Cells were then incubated for 15 min at 37 °C or for 30 min at 4 °C in the dark, at the presence of FITC labeled Annexin-V and PI. Another 300 μl binding buffer was added to the cell suspension. The cells were then analyzed by a flow cytometer (Partec, CCA-II).

4.7. Measurement of mitochondrial membrane potentials ($\Delta\Psi m$)

HT29 Cells were cultured in the presence of oridonin of desired concentration and harvested with trypsin/EDTA. After washing by cold PBS, they were incubated with rhodamine-123 at a final concentration of 10 μg/ml at 37 °C for 30 min in the dark, rinsed by cold PBS twice, and analyzed by a flow cytometer (Partec, CCA-II). The probes were excited with a laser at 488 nm and the fluorescence emission was measured through a 520 nm bandpass filter.

4.8. In vivo experiments

Forty female BALB/C nu/nu mice were used when they were 5 – 7 weeks old. Each group consisted of 10 mice. HT29 human colorectal carcinoma cells (20 μl, 1×10^6 cells) were transplanted into the oxter of the right fore limb of mice. After 24 h, the tumor-bearing mice were treated with an intraperitoneal injection of oridonin (10, 15 and 20 mg kg^{-1} daily) for 12 d. Control mice received injection with saline. The body weights of mice in each group were measured every 24 h during the experiment. The mice were killed on day 12 and the local tumors were removed carefully from the transplanted oxter and the net mass weights of the tumors were quantified. Relative tumor inhibition was calculated as follows:

Relative tumor inhibition (%) = [(Control mice tumor weight) − (Treated mice tumor weight)]/(Control mice tumor weight) × 100.

4.9. Statistical analysis

The results represent at least three independent experiments and are expressed as the mean ± S.D., accompanied by the number of tests. The Student's t-test was performed to determine the level of significance between groups and a value of $p < 0.05$ was considered to indicate a statistically significant difference.

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