# Oridonin-induced apoptosis in leukemia K562 cells and its mechanism*

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Oridonin, an extract from the Chinese herb *Rabdosia rubescens*, is currently one of the most important traditional Chinese herbal medicines. Recently oridonin has been reported to have anti-tumor effects in a large variety of malignant diseases. In this study, we investigated the apoptotic inducing effect of oridonin in leukemia K562 cells and its mechanism. Cell growth inhibition was measured using a microculture tetrazolium assay, apoptosis was measured by flow cytometry and electron microscopy as well as by DNA fragmentation analysis. Telomerase activity was measured by TRAP-enzyme-linked immunosorbent assay, and the expression of Bcl-2 and Bax proteins was detected by western blot analysis. The results showed that oridonin could inhibit the proliferation and induce apoptosis on leukemia K562 cells remarkably. Telomerase activity as well as Bcl-2 expression was down-regulated, while Bax expression was up-regulated concurrently, when apoptosis ocurred. We therefore conclude that oridonin demonstrated anti-proliferative and apoptosis-inducing effects on K562 cells *in vitro*, and that changes in bcl-2 and bax protein levels as well as telomerase activity may play an important role in its mechanism of action.

*Key words: oridonin, apoptosis, Bcl-2, Bax, telomerase*

Oridonin, a diterpenoid compound ($C_{20}H_{28}O_6$) (Fig. 1), is extracted and purified from traditional Chinese herb, *Rabdosia rubescens* or *Isodon japonicus* [7, 25]. It is one of the most important traditional Chinese herbs currently used in clinical practice. More than half a century ago, oridonin showed a variety of biological effects, such as immunoregulatory and anti-inflammatory functions as well as anti-viral activities, especially in the upper respiratory tract infection. Recent laboratory and clinical data suggest that oridonin is a very effective anti-tumor reageant with profound effects on a number of malignant diseases such as prostate, breast, non-small cell lung cancers [10].

Though oridonin has been proved to be very effective in treating a variety of malignancies, many of its anti-tumor mechanisms have not been demonstrated. Currently there is no detailed laboratory evidence showing the mechanisms of oridonin on leukemic K562 cells. In order to clarify some of its anti-leukemia mechanisms, we investigated the apoptotic effects of various concentrations of oridonin (8–32 μmol/l) on K562 cells *in vitro*, and detected the variation of telomerase activity, Bax and Bcl-2 expressions to provide laboratory evidence of oridonin for its mechanisms in the treatment of leukemia.

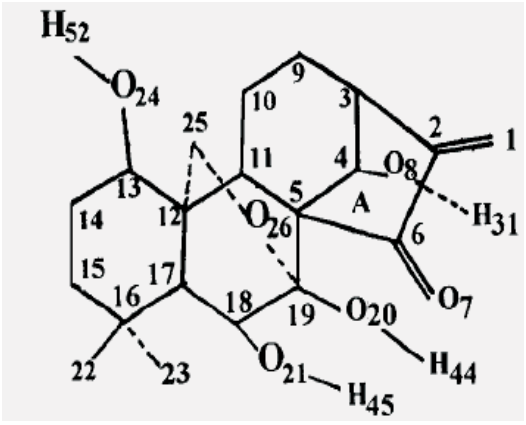


**Figure 1. Chemical structure of oridonin ($C_{20}H_{28}O_6$).**

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## Material and methods

*Reagents.* Oridonin was provided by Prof. Pan Xiang-lin, and leukemic K562 cells were applied by central laboratory of the Sun Yat-sen University cancer center.

*Cell culture.* K562 cells were cultured in RPMI-1640 medium (Sigma, St. Louis, Mo.) supplemented with 10% heat-inactivated calf serum (Sijiqing Biochemical Factory, Hangzhou, China) and 100 U/ml penicillin (Qilu Pharmaceutical Factory, Jina, China). Cells were incubated in a humidified 5% $CO_2$ incubator (Hearous, Germany) at 37 °C. Cells were passaged twice weekly and routinely examined for mycoplasma contamination.

*Inhibition of cell proliferation.* The inhibition of cell growth was measured using the microculture tetrazolium (MTT) method. K562 cells in logarithmic growth phase were collected, and $2x10^5$ cells were dispensed into each well of a 96-well culture plate in 100-µl aliquots. Oridonin was added to the wells at a range of concentrations (8 µmol/l, 16 µmol/l, 24 µmol/l, and 32 µmol/l) and the control wells received tissue culture medium. Each concentration was regarded as one observed group. Each observed and control group consisted of six parallel wells. Culture plates were incubated for 0, 24, 48 and 72 h prior to the addition of tetrazolium reagent. The MTT working solution was prepared as follows: MTT (5 mg/ml) in phosphate buffered saline (PBS) was sterilized by filtering it through a 0.45 µm filter unit. A 20-µl aliquot of MTT working solution was added to each well and the wells were incubated continuously for 4 h. The culture medium supernatant was removed from the wells and replaced with 100 µl of DMSO. Following thorough solubilization, the absorbance (*A* value) of each well was measured using a microculture plate reader at 570 nm. The rate of cell growth inhibition was calculated according to the following formula:

Rate of growth inhibition = 100 x (*A* value of control group – *A* value of treated group)/*A* value of control group.

*Flow cytometric assessment of DNA degradation.* To determine the DNA content, cells treated with different concentrations of oridonin were harvested, centrifuged to a pellet, washed with PBS, and resuspended in PBS containing 20 mg/l PI and 1 g/l ribonuclease A. A total of $1x10^6$ fixed cells were examined per experimental condition by flow cytometry. The percentage of degraded DNA was determined by the number of cells displaying subdiploid (sub-$G_1$) DNA divided by the total number of cells examined.

*Electron microscopy.* K562 cells treated with oridonin 16 µmol/l for 24 h, 48 h and 72 h were collected. Then the cells were fixed in half-strengh Karnofsky's fixative, postfixed in 1% collidine-buffered osmium tetroxide, dehydrated in a graded series of ethanol and propylene oxide, and embedded in LR White Resin. Sections were cut and stained with both uranyl acetate and Reynold's lead stain. Electron micrographs were obtained with a JEM-1200 electron microscope.

*DNA fragmentation assay.* Apoptosis was confirmed by detection of fragmentation of chromosomal DNA with the classic DNA ladder method. Briefly, $2x10^6$ cells were immersed in cytolysis buffer (Tris-HCL 1 mmol/l pH 8.0, edetic acid 10 mmol/l pH 8.0, proteinase K 200 mg/l, 0.5% SDS) and incubated for 3 h at 50C. DNA was extracted with phenol-chloroform, precipitated in 1/10 volume of NaAc 2 mol/l and 2 volumes of ethanol at –20 °C overnight, recovered by centrifugation at 1000 x g for 30 min at 4, and then resuspended in TE buffer. RNase A was then added at a concentration of 200 mg/l, then the treated extract was incubated at 37 °C for 30 min and electrophoresed on a 1.2% agarose gel.

*Western blot analysis.* Cells were washed with ice-cold PBS twice and lysed for 30 min at 4 °C. The debris was removed by centrifugation (15,000 g at 4 °C for 15 min). Equivalent amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membrane was first stained to confirm uniform transfer of all samples and then incubated in blocking solution for 2 h at room temperature. The filter was incubated with monoclonal antibodies, anti-bcl-2 and anti-bax, at a dilution of 1:1000 for 2 h, and then washed with PBS twice and TBST twice. The filter was then incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1000 for 1 h, washed with TBST and developed using the Super Signal West Pico Kit.

*Telomerase activity assay.* Telomerase activity was measured quantitatively by the TRAP-enzyme-linked immunosorbent assay (ELISA) based on the modified telomere repeat amplification protocol (TRAP). Telomerase polymerase chain reaction (PCR)-ELISA kit was obtained from Boehringer-Mannheim, Germany. The sequences of the primers were: CX5'-CCCTTACCCTTACCCTTACCCTTA-3' and TS 5'-AATCCGTCGAGCAGAGTT-3'. Cell extracts were prepared according to TRAP. Telomerase added telomeric repeats TTGGG to the 3'-end of the biotin-labeled synthetic P1-TS primer. Then these elongation products were amplified by PCR using the primers P1-TS and P2-CX to generate PCR products with the telomerase-specific 6-nucleotide increments. Then an aliquot of the PCR product was denatured and hybridized to a dioxigenin-DIG-labeled, telomeric repeat-specific detection probe. The final product was immobilized to a streptavidin-coated microtiter plate via the biotin-labeled primer and detected with an antibody against dioxigenin (anti-DIG POD) that was conjugated to peroxidase. The probe was visualized by virtue of peroxidase metabolizing TMB to form a colored reaction product. Finally, the absorbance of the samples was measured at 450 nm (with a reference wave length of approx. 690 nm) using a microtiter plate (ELISA) reader within 30 min after addition of the stop reagent. Absorbance values were reported as the $A_{450\ nm}$–$A_{690\ nm}$.

*Statistical analysis*. All experiments were performed in triplicate and the results were expressed as mean ±SD. Statistical analysis was performed with a Student's t-test using SAS 6.12 software. Statistical significance was accepted at the level of $p<0.05$.

## Results

*Cell inhibitory rate caused by oridonin.* Oridonin below 8 μmol/l had little inhibitory rate on K562 cells, but it could inhibit the cell proliferation significantly at a higher concentration (between 24–32 μmol/l oridonin), especially the concentration was 32 μmol/l. The inhibitory rate of oridonin between 16–32 μmol/l is much higher than that of lower concentrations of oridonin ($p<0.01$) (Fig. 2).

*Apoptotic rate caused by oridonin.* Oridonin (over 16 μmol/l) could induce apoptosis when cultured with K562 cells after 24–72 h. The apoptotic rate was high and over 50% when cultured with cells for 72 h (Fig. 3).

*Morphological analysis by electron microscopy.* After K562 cells were incubated with oridonin (over 16 μmol/l) for 48–72 h, typical morphological changes of apoptosis were observed using electron microscope. The cytoplasm shrinked and the chromatin of cells became condensed and marginated especially the cells were exposed to 32 μmol/l oridonin for 48 h (Fig. 4A), and "apoptotic bodies" were also observed (Fig. 4B). When the cells incubated with oridonin for 72 h, dead cells increased remarkably (Fig. 4C).

*DNA fragmentation.* The intergrity of DNA was assessed by agarose gel electrophoresis. Incubation of K562 cells with oridonin 16–32 μmol/l for 48 h elicited a characteristic "ladder" of DNA fragments representing integer multiples of the internucleosomal DNA length (about 180–200 bp). DNA ladder was observed in a dose-manner (Fig. 5).

*Detection of apoptotic related genes.* After treatment with different concentrations of oridonin especially when the cells were incubated for 48 h, the protein levels of bcl-2 and bax were examined by Western blotting when apoptosis ocurred.

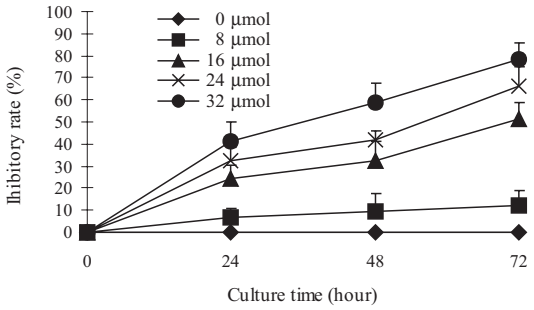


**Figure 2. Cell inhibitory rate caused by oridonin. Cells were treated with different concentrations of oridonin for 24, 48 and 72 h. Experiments were done in triplicate. The cell growth inhibitory rate was then determined by MTT assay, as described in the Methods.**

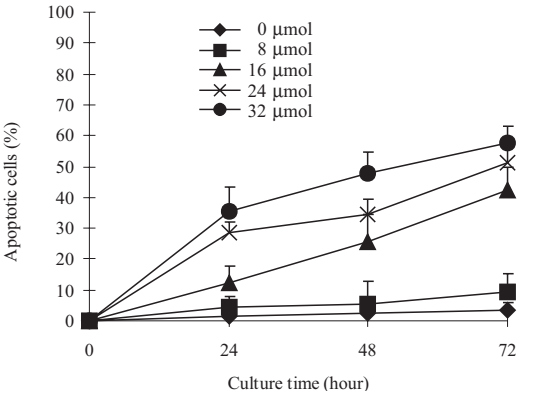


**Figure 3. Cell apoptotic rate caused by oridonin. After cells treated with oridonin, flow cytometry (FCM) analysis was used to detect apoptotic rate. The cells were stained with PI and then analyzed by FCM, the percentage of sub-G1 cells was high and over 50%, when the cells were treated with 32 μmol/l oridonin for 72 h. The experiments were repeated three times and the results were presented as mean ±SD.**

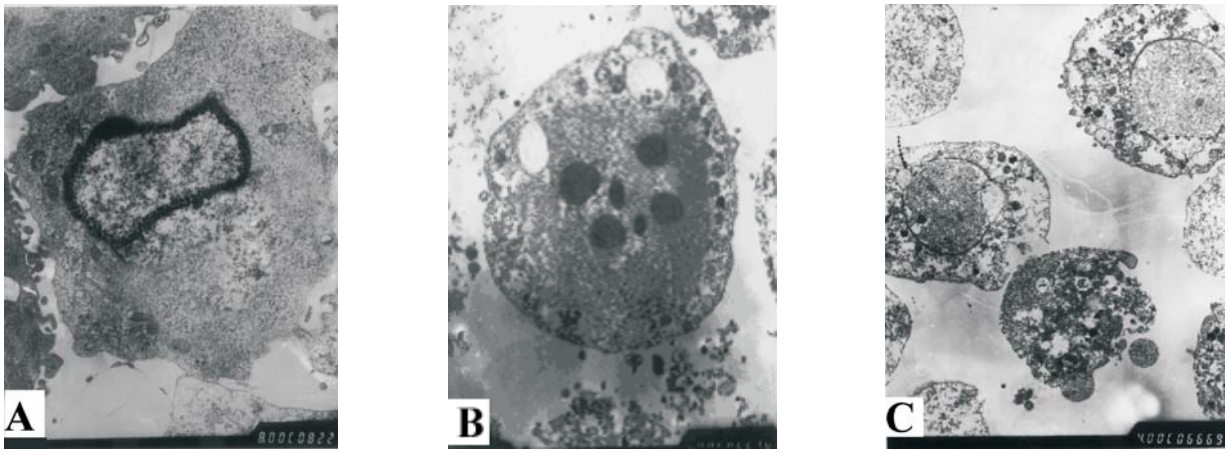


**Figure 4. The morphological changes of cell apoptosis (observed by electron microscope x 6000) after the cells treated with 32 μmol/l oridonin for different time. A: cells were treated for 24 h, the chromatin became condensed and marginated. B: cells were treated for 48 h, "apoptotic bodies" were observed clearly. C: cells were treated for 72 h, viable cells were decreased remarkably.**

Western blot analysis revealed that downregulation of bcl-2 and upregulation of bax protein were induced by oridonin in K562 cells (Fig. 6).

*Telomerase activity caused by oridonin.* Telomerase activity of K562 cells decreased remarkably when exposed to oridonin, the more higher the oridonin concentration, the more lower telomerase activity of K562 cells, especially at 72 h the telomerase activity is approximately zero(oridonin concentration is 32 μmol/l) (Fig. 7).

## Discussion

There are many compounds extracted from *Rabdosia rubescens*. Oridonin is one of its most effective derivatives which is recently proved to have activity against a number of cancer cells. In this study we found that oridonin could inhibit the proliferation and induce apoptosis in leukemia K562 cells remarkably when the concentration of oridonin was over 16 μmol/l, and Western blot analysis revealed that downregulation of bcl-2 and upregulation of bax protein were induced by oridonin in K562 cells; Telomerase activity was gradually down-regulated along with the increased concentration of oridonin, especially when the cells were treated with 32 μmol/l oridonin for 72 hours telomerase activity is approximately zero. The suppression was both in time-and in dose-dependent manner. Marked morphological changes of cell apoptosis including condensation of chromatin and nuclear fragmentation were observed clearly by electron microscope especially after the cells were treated with oridonin for 48–60 hours, and typical DNA "ladder" was observed when apoptosis ocurred. These results suggest that oridonin may be effective agent for the treatment of leukemia.

Apoptosis plays an important role in the development and maintenace of homeostasis, and impaired apoptosis is now recognized to be a key step in tumorigenesis [2, 3]. Recent data [4] indicated that activation of apoptosis pathways is a key mechanism by which cytotoxic drugs kill tumor cells. The Bcl-2 family consists of about 20 homologues of important pro- and anti-apoptotic regulators of programmed cell death [14]. Bax is a 21 kDa protein that shares homolog with bcl-2 clustered conserved regions including BH1 and BH2 [3]. When bax was overexpressed in cells, apoptotic death in response to death signals was accelerated, earning its designation as a death agonist. When bcl-2 was overexpressed, it heterodimerized with bax and death was repressed, thus the ratio of bcl-2 to bax is important in determining susceptibility to apoptosis [13, 18]. Many laboratory data showed that a major problem in the treatment of leukemia is caused by the development of drug resistance to chemotherapeutic agents [8]. Apart from classical drug resistance mechanisms, the failure of tumor cells to undergo apoptosis also plays an important role in drug resistance [11]. Mutation of the tumor suppressor gene p53 and overexpression of bcl-2 are important factors in this drug resistant pathway [22, 24]. Down-

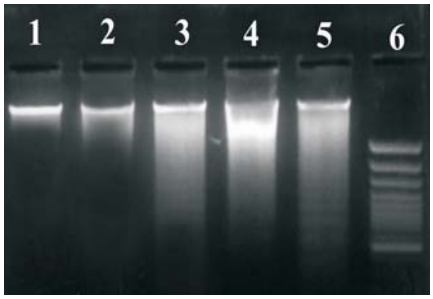


**Figure 5. DNA ladder of K562 cells treated with oridonin for 48 h. Lane 1 – control. Lane 2, 3, 4 and 5 were 8, 16, 24 and 32 μmol/l oridonin. Lane 6 – marker, 100 bp DNA ladder Plus.**

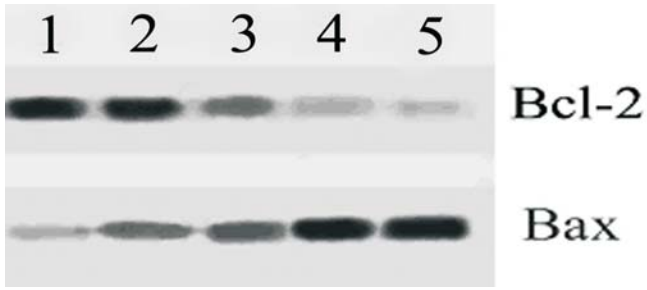


**Figure 6. Western blot analysis of Bcl-2 and bax of K562 cells treated with different concentrations of oridonin for 48 h. Analysis showed downregulation of Bcl-2 and upregulation of Bax. Lane 1 – control. Lane 2–5 were 8, 16, 24 and 32 μmol/l oridonin.**

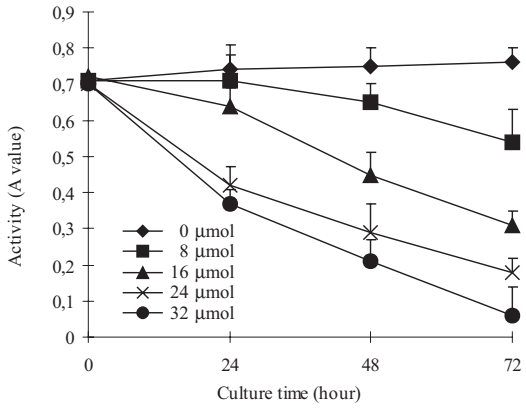


**Figure 7. Telomerase activity caused by oridonin. Telomerase activity detected by TRAP-ELISA, as described in the Methods. Telomerase activity of K562 cells decreases remarkably, especially when the cells were treated with 32 μmol/l oridonin for 72 h, the telomerase activity is approximately zero.**

regulation of bcl-2 and upregulation of bax expression may play an important role in chemotherapeutic drug induced apoptosis.

In malignant hematologic diseases such as leukemia and lymphoma, high telomerase activity almost always correlates with disease severity, and the activity of telomerase is a very

useful index for the diagnosis and clinical staging in hematologic malignancies [19, 21]. New data [20] suggest that telomerase inhibitor can promote the apoptosis in many hematologic malignancies such as acute leukemia. This indicates that anti-telomerase therapy can not only enhance apoptosis in tumor cells but also be one of the most important and effective markers for the selection of new anti-tumor drugs. It is reasonable that inhibition of telomerase increases the susceptibility of tumors to DNA-damaging drugs that induce apoptosis [19, 23]. The regulatory factors for telomerase activity are not clarified. Some laboratory data [1] revealed that the regulatory factors for apoptosis might be key for telomerase regulation, and telomerase may be activated as an early event of carcinoma progression. Expression of some apoptosis related genes such as bcl-2 may be related to the telomerase activity of carcinoma [5].

Oridonin is an ent-kaurane diterpenoid as shown in Figure 1. It is used mainly in the treatment of some solid tumors such as esophageal cancer [9, 12] in the clinical practice with the dosage between 20–40 μmol/kg/d [26]. Furthermore, oridonin is often used with other chemotherapeutic drugs such as nitrocaphane and cisplatin. The anti-tumor effects usually increase remarkably when oridonin is used synergistically with these chemotherapeutic drugs [6, 26]. Previously, some of our studies [15, 16, 17] demonstrated that oridonin had considerable anti-proliferation effects on some leukemia cells *in vitro.* Increasing caspase-3 activity of leukemia cells may be its important anti-leukemia mechanisms. In this study, we found that 16–32 μmol/l oridonin could inhibit leukemia K562 cell growth by induction of apoptosis. The ratio of Bcl-2 to Bax expression as well as telomerase activity was decreased dramatically after the cells were treated with oridonin. The results suggest that oridonin may serve as a potential therapeutic agent for the treatment of leukemia. *In vivo* anti-leukemia effects of oridonin as well as its potential clinical effectiveness needs further and profound investigation.

In summary, the results indicate that oridonin has apparent anti-proliferation and apoptosis-inducing effects on K562 cells *in vitro,* downregulation of bcl-2 and upregulation of bax as well as decreasing telomerase activity may be its important mechamisms. These results will provide strong laboratory evidence of oridonin for clinical treatment of leukemia.

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