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Oridonin induces human epidermoid carcinoma A431 cell apoptosis through tyrosine kinase and mitochondrial pathway

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Oridonin, a diterpenoid isolated from the plant *Rabdosia rubescens*, induces human epidermoid carcinoma A431 cell death through apoptosis and tyrosine kinase pathway. To examine the pathway of oridonin-induced A431 cell death, morphologic observation, lactate dehydrogenase activity-based assay, DNA agarose gel electrophoresis and Western blot analysis were carried out. When A431 cells, which overexpress epidermal growth factor receptor (EGFR), were treated with oridonin, caspase-3 was activated followed by the degradation of caspase-3 substrates, inhibitor of caspase-activated DNase (ICAD) and poly(ADP-ribose) polymerase (PARP) in a time-dependent manner. Oridonin promoted the release of cytochrome *c* and the down-regulation of mitochondrial transmembrane potential ($\Delta\Psi$m). Oridonin up-regulated the expression ratio of mitochondrial proteins, Bax/Bcl-2. In addition, the total tyrosine kinase activity of A431 cellular proteins and the expression of EGFR were markedly reduced after oridonin treatment. Taken together, oridonin induced apoptosis in A431 cells via mitochondrial pathway, activation of caspase-3 and inhibition of tyrosine kinase activities.

Keywords: Oridonin; Tyrosine kinase; Apoptosis; Mitochondria

1. Introduction

Herbal medicine, Donglingcao (*Rabdosia rubescens*), has been traditionally used in China. Diterpenoids are the major active constituent of *R. rubescens*. Oridonin [1] (figure 1) is one of the diterpenoids isolated from *R. rubescens*, which has various pharmacological and physiological effects such as anti-inflammation, anti-bacteria and anti-tumour [2–4], and has been used for the treatment of human cancers, especially oesophageal carcinoma [5]. However, the exact mechanisms of the diterpenoid-induced tumour cell death are still not clear. In this study, we investigated the mechanism of oridonin-induced death of human epidermoid carcinoma A431 cells.

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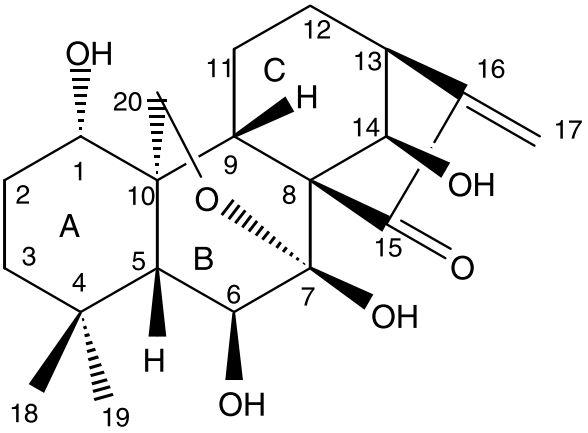

Figure 1. The structure of oridonin.

2. Results and discussion

2.1 Oridonin-induced apoptotic cell death in A431 cells

Apoptosis, or programmed cell death, is a genetically regulated, self-destructive cellular death process that is important in organic development, tissue remodelling, immune regulation and many other diseases [6–9]. Some anticancer drugs and a variety of cell differentiation inducers have been shown to induce apoptosis of cancer cells [10,11]. To determine the characteristics of oridonin-induced A431 cell death, the morphologic changes of stained cell nuclei by Hoechst 33258, lactate dehydrogenase (LDH) activity-based assay and DNA fragmentation were examined (figure 2). In A431 cells, exposure to oridonin 20 μmol·L^{-1} for 24 h resulted in morphologic alterations [12], characteristic of apoptosis, including membrane blebbing, nuclear condensation and granular apoptotic bodies (figure 2A–b). The ratio of lactate dehydrogenase (LDH) released from viable cells, floating dead cells and the culture medium might be used to distinguish the number of apoptotic and necrotic cells [13]. In this study, in the presence of oridonin (20 μmol·L^{-1}), the numbers of apoptotic cells were 30.1% at 24 h and reached the peak at 48 h (52.6%), then down-regulated at 60 h, with a nearly invariable necrotic ratio (below 14.0%). However, oridonin at 40 μmol·L^{-1} induced more significant necrosis rather than apoptosis (figure 2B). Consistent with the above result, in gel electrophoresis, DNA fragmentation was also observed in oridonin treated cells (figure 2C). It was found that 20 μmol·L^{-1} oridonin-induced apoptosis was in a time-dependent manner, and the most obvious fragmentation occurred at 48 h. These results demonstrated that the major cause of 20 μmol·L^{-1} oridonin-induced A431 cell death was apoptosis from 12 h to 48 h.

2.2 Activation of caspase-3 is required for oridonin-induced apoptosis

It is well known that in caspase family, caspase-3 plays a central role. Once activated, caspase-3 performed a number of executioner functions, including the activation of a latent cytosolic endonuclease, poly(ADP-ribose) polymerase (PARP) and inhibitor of caspase-activated DNase (ICAD). Moreover, the ICAD cleavage and the degradation of PARP are

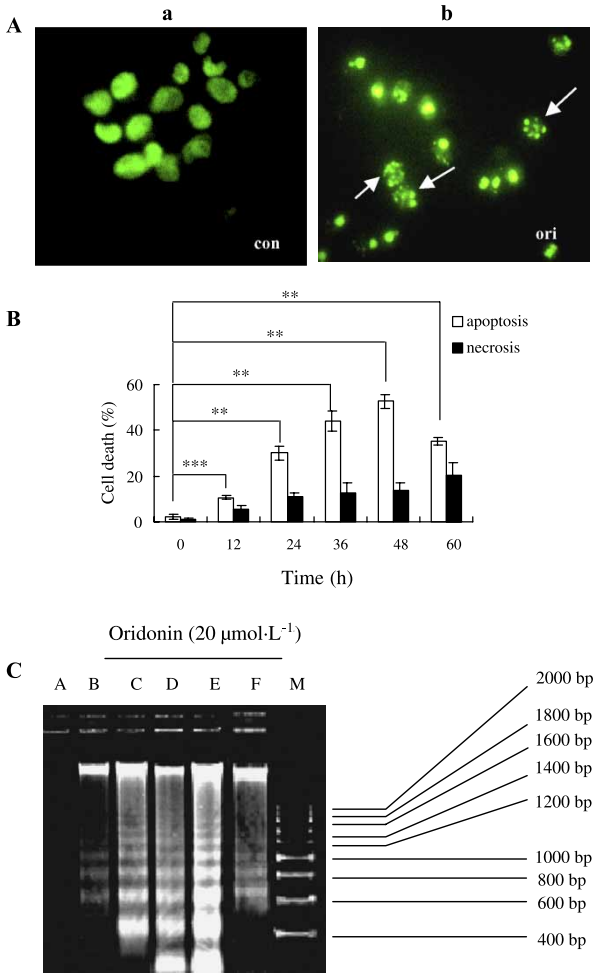

Figure 2. Oridonin-induced A431 cell death was characterised mainly by apoptosis. (A) Cells were cultured with medium alone or with 20 μmol·L^{-1} oridonin for 24 h. Morphologic changes were observed by fluorescent microscopy (× 200 magnification). Ori: 20 μmol·L^{-1} oridonin, con: medium. Arrows indicate condensed nuclei. (B) Cells treated with 20 μmol·L^{-1} oridonin for 0, 12, 24, 36, 48, and 60 h, and then were measured by LDH activity-based assay. (open square: apoptosis, closed square: necrosis). All data were presented as mean ± S.D. (bars) and considered statistically significant between the cells treated with vehicle and oridonin. ***$p < 0.001$, **$p < 0.01$ (C) The cells were cultured in the presence of oridonin at 20 μmol·L^{-1} for 12, 24, 36, 48, and 60 h. Genomic DNA was extracted and analysed via electrophoresis on 2% agarose gels. Lanes: M: DNA molecular marker; A: medium; B: 12 h; C: 24 h; D: 36 h; E: 48 h; F: 60 h.

consistent with DNA fragmentation, which results in the morphologic and biochemical features of apoptosis [14,15]. To assess whether caspase-3 participated in the apoptotic pathway, western blot analysis was carried out. The 32-kDa band of procaspase-3 began to degrade and activated caspase-3 expression began to increase after treatment with oridonin for 12 h (figure 3), indicating the activation of caspase-3. Then the expression of ICAD protein, one of caspase-3 substrates, decreased with culturing time. This suggests that ICAD was cleaved by caspase-3 and induced DNA fragmentation, consistent with the result of figure 2C. Another characteristic associated with the execution phase of the apoptotic pathway is the specific PARP cleavage by the caspase. This cleavage leads to inactivation of the enzyme, thus preventing futile DNA repair cycle [16]. It has been reported that caspase-3

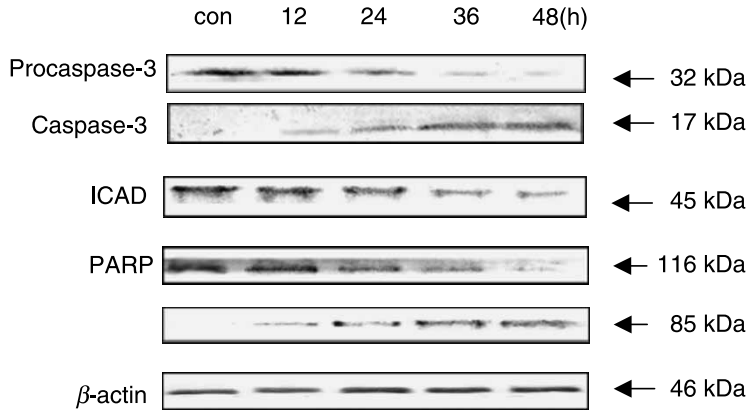

Figure 3. Effect of oridonin on caspase-3, ICAD, and PARP expression. A431 cells were incubated with 20 μmol·L^{-1} oridonin for different time periods. Cell lysates were separated by 10% SDS–PAGE, and caspase-3, ICAD, and PARP proteins were detected by Western blot analysis. Triplicate experiments gave similar results.

is the most efficient processing enzyme for PARP [17]. As expected, the amount of 116-kDa-protein decreased and the 85 kDa degraded product increased after 12 h oridonin-treatment, suggesting that PARP was involved in oridonin-induced cell death. All these results suggested that the caspase cascade played a pivotal role in oridonin mediated A431 cell apoptosis.

2.3 The release of cytochrome c following altering Bax/Bcl-2 expression ratio in oridonin-treated A431 cells

It is widely accepted that alterations of mitochondrial function, such as breakdown of the mitochondrial transmembrane potential ($\Delta\Psi$m) and release of proapoptotic cytochrome *c*, constitute key events of the mitochondrial apoptosis signalling cascade [18]. The Bcl-2 protein family constitutes an important control mechanism in the regulation of apoptosis. This large family is comprised of apoptosis-regulating proteins that modulate the mitochondrial pathway, including antiapoptotic proteins, such as Bcl-2, Bcl-X_L; and other proapoptotic proteins, such as Bax and Bid. Therefore, Bcl-2 protein family has been demonstrated to play a critical role in inhibiting or promoting apoptosis [19–21]. Additionally, these proteins regulate the mitochondrial pathway that induces intrinsic activation of caspase [22]; and the balance between these two groups determines the fate of cells in many apoptotic systems [23]. To confirm whether such a mechanism is involved in oridonin-induced apoptosis, A431 cells were incubated with 20 μmol·L^{-1} oridonin for 12, 24, 36 and 48 h. As shown in figure 4, 24 h after treatment with oridonin, the release of cytochrome *c* began to increase, while pro-apoptotic Bax protein showed no change in its expression. However, the Bcl-2 expression markedly decreased at 24 h. These observations indicated that oridonin-induced apoptosis involved the initial phase mediated by the balance between Bax and Bcl-2, resulting in cytochrome *c* release from the mitochondria. Thus, treatment of A431 cells with oridonin up-regulated the ratio of Bax/Bcl-2 and induced the release of cytochrome *c*, indicating mitochondrial signal pathways participation in oridonin-induced apoptosis.

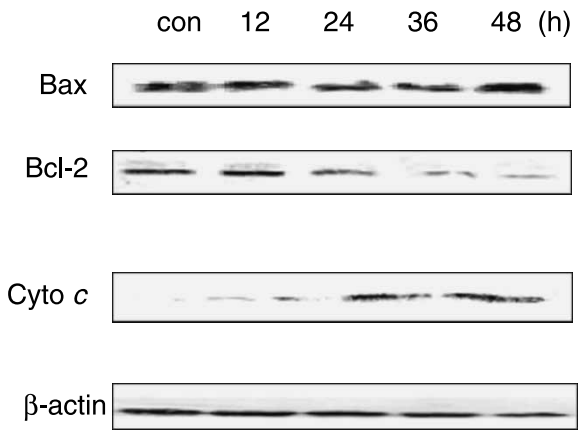

Figure 4. The balance of Bax/Bcl-2 and the release of cytochrome *c* regulated by oridonin. A431 cells were treated with 20 μmol·L^{-1} oridonin for 12, 24, 36, and 48 h. Cell lysates were separated by 12% SDS–PAGE, and Bax, Bcl-2, and cytochrome *c* proteins were detected by Western blot analysis. Triplicate experiments gave similar results.

2.4 Mitochondrial transmembrane potential (ΔΨm) was markedly reduced by oridonin treatment

Kinetic data indicate that mitochondria undergo major changes in membrane integrity before classical signs of apoptosis become manifest [24]. These changes concern both the inner and the outer mitochondrial membranes, leading to a disruption of the inner transmembrane potential (ΔΨm) and the release of intermembrane proteins through the outer membrane. In this study, changes of ΔΨm were detected by using rhodamine 123 (a cell permeable, cationic, fluorescent dye which can incorporate into mitochondria depending on the inner transmembrane potential) by fluorescence microscopy. The declining uptake of rhodamine 123 reflects a reduction of ΔΨm, which is a signal for the changes of mitochondrial transmembrane potential (MMP). As shown in figure 5, remarkable loss of fluorescent intensity was observed in the oridonin treatment group and the fluorescent intensity became weaker as time progressed (figures 5-c, 5-d, 5-e). These observations further indicated that down-regulation of mitochondrial transmembrane potential was caused by oridonin-induced A431 cell apoptosis.

2.5 Effect of oridonin on the total tyrosine kinase activities

Protein phosphorylation/dephosphorylation is implicated in a large number of cellular processes, particularly signal transduction pathways and enzymatic regulation. Signal transduction involving protein phosphorylation is generally triggered by the activation of tyrosine kinase of cell surface receptors and, as a consequence, the plasma membrane has been considered the main subcellular site of such activities [25]. One of the major classes of protein tyrosine kinases (PTKs) is the ligand-stimulated tyrosine kinase, exemplified by the epidermal growth factor receptor (EGFR), which is one of the transmembrane receptor protein tyrosine kinases. Once EGFR overexpresses or is stimulated by EGF, it will lead to activation of mitogenic signalling and other proliferation-related cellular events [26,27].

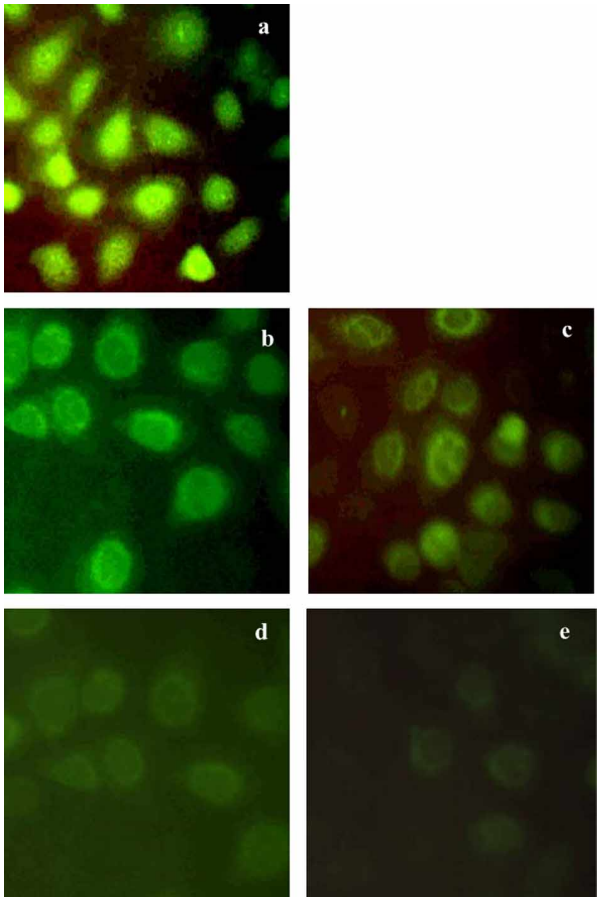

Figure 5. Oridonin-induced the loss of mitochondrial transmembrane potential in A431 cells. The cells were incubated in 6-well culture plates. Fluorescent images were observed by fluorescence microscope (× 200 magnification) at 12 (b), 24 (c), 36 (d) and 48 h (e) in the absence (a) or presence of oridonin 20 μmol·L^{-1}.

It has been reported that enhancing of tyrosine kinase activities is an important regulator in mitochondrial metabolism [28]. In the absence of PTK protection, mitochondrial membrane potential was lowered and cytochrome *c* was released from mitochondria to the cytosol. In addition, it has been elucidated that some tyrosine kinase inhibitor, such as genistein, could induce apoptosis of RPE-J cells (Rat retinal pigment epithelial cell line), perhaps due to opening of the mitochondrial permeability transition pore, resulting in the down-regulation of MMP and promoting cytochrome *c* release [25]. These suggested that the function of mitochondria to some extent, could be mediated by some tyrosine kinases. Therefore, in this study, in order to determine whether this mechanism was involved in oridonin treated A431 cells, the total tyrosine kinase activities of the cellular proteins were measured by tyrosine kinase activity assay with 20 μmol·L^{-1} oridonin for different time periods. Results showed that although pre-treatment with EGF in the reaction mixture led to a significant increase in the total tyrosine kinase activities as compared with the control, oridonin time-dependently inhibited the total tyrosine kinase activities in A431 cells (figure 6A). This phenomenon demonstrated that oridonin-induced the down-regulation of MMP and the release of cytochrome *c* might affect the total tyrosine kinase activity of A431 cells.

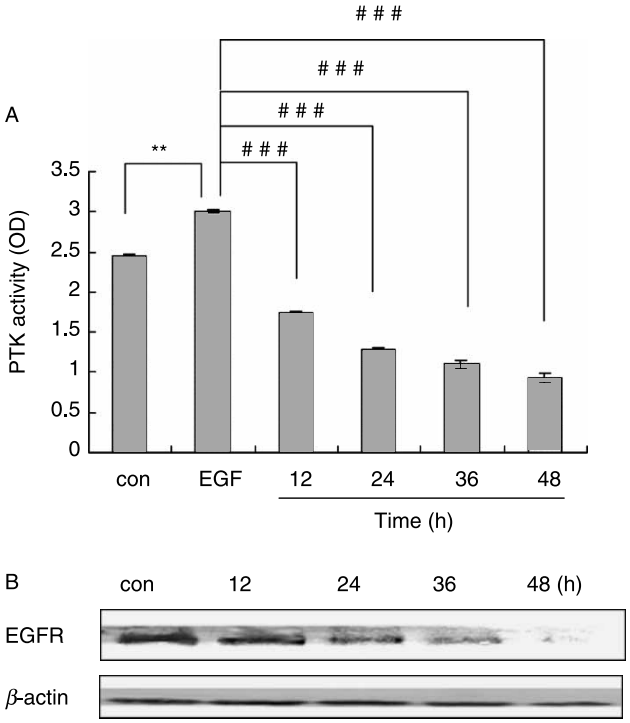

Figure 6. Effect of oridonin on the tyrosine kinase activities of A431 cellular proteins. (A) The cells were stimulated by 15 nmol·L^{-1} EGF for 20 min, and then 20 μmol·L^{-1} oridonin was added. After incubation for different time periods, the optical density values were measured by ELISA at 450 nm. (B) The expression of EGFR was detected by Western blot analysis. $^{**}p < 0.01$, $^{\#\#\#}p < 0.001$.

On the other hand, activation of PKC δ-holoenzyme resulted in cytochrome *c* release and mitochondrial membrane depolarisation, which is the result of down-regulation of MMP [29,30]. However, activation of PKC, to some extent, exerted inhibitory effects on EGFR binding or EGF-stimulated tyrosine kinase activity [31]. To assay whether this mechanism is involved in oridonin-induced apoptosis, Western blot analysis was performed (figure 6B). As expected, the expression of EGFR was down-regulated with culturing time. These results indicated that in this study, the down-regulation of MMP might, to some extent, be caused by the activation of PKC δ-holoenzyme, which led to the down-regulation of EGFR in oridonin-induced apoptosis. Moreover, oridonin-induced inhibition of the total tyrosine kinase activity might also inhibit the expression of EGFR.

In conclusion, our data showed that tyrosine kinase, caspase and mitochondrial pathways were involved in oridonin-induced apoptosis. The activation of caspase-3, release of cytochrome *c* and increasing in Bax/Bcl-2 ratio contributed to apoptosis in response to oridonin. Changes of mitochondrial transmembrane potential might connect apoptosis-inducing effect with an inhibitory effect of the total tyrosine kinase activities in oridonin treated A431 cells. On the one hand, the down-regulation of MMP might be caused by oridonin-induced inhibition of the total tyrosine kinase activities. On the other hand, it was controlled by oridonin-induced apoptosis and the release of cytochrome *c*. However, elucidation of the functional relationship between tyrosine kinase and mitochondria will be required in further study.

3. Experimental

3.1 Chemicals

Oridonin was obtained from the Beijing Institute of Biological Products (Beijing, China). The structure of oridonin was assigned by comparing the chemical and spectral data (^{1}H NMR, ^{13}C NMR) with those reported in the literature [32]. The purity of the oridonin was measured by HPLC and determined to be 99.4%, and oridonin was dissolved in the medium.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), ribonuclease (RNase), proteinase K, and Hoechst 33258 were purchased from Sigma Chemical (St. Louis, MO, USA). Polyclonal antibodies against EGFR, caspase-3, PARP, ICAD, Bax, Bcl-2, cytochrome *c* and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tyrosine kinase activity assay kit was obtained from Chemicon International (Temecula, CA, USA). Human epidermal growth factor (EGF) was from Pepro Tech (Rocky Hill, NJ, USA).

3.2 Cell culture

Human epidermoid carcinoma A431 cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Ham's F-12 medium (Hyclone, Logan, UT, USA) supplemented with 10% heated inactivated foetal bovine serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.03% L-glutamine at 37°C with 5% CO_2 in a humidified atmosphere.

3.3 Drug solutions

Oridonin was dissolved in DMSO to make stock solutions, then diluted in cell culture medium at different concentration and immediately used. In all the assays, the final concentrations of DMSO in the culture medium were below 0.05%.

3.4 DNA fragmentation assay

A431 cells (1×10^6) were collected by centrifugation at 1500 × *g* for 5 min, and washed with phosphate-buffered saline (PBS). The cells were pelleted and suspended in 10 mmol·L^{-1} Tris–HCl (pH 7.4), 10 mmol·L^{-1} EDTA (pH 8.0) and 0.5% Triton X-100, then kept at 4°C for 10 min. The supernatants were incubated with 40 μg·ml^{-1} RNase A (2 μl) and 40 μg·ml^{-1} proteinase K (2 μl) at 37°C for 1 h. Then they were kept in 5 mol·L^{-1} NaCl (20 μl) and isopropanol (120 μl) at − 20°C overnight, and centrifuged at 15,000 × *g* for 15 min. DNA was dissolved in TE buffer [10 mmol·L^{-1} Tris–HCl (pH 7.4), 10 mmol·L^{-1} EDTA (pH 8.0)], subjected to 2% agarose gel electrophoresis at 50 V for 40 min, and stained with ethidium bromide.

3.5 LDH activity-based cytotoxicity assays

LDH (lactate dehydrogenase) activity was assessed using a standardised kinetic determination kit (Zhongsheng LDH kit, Beijing, China). LDH activity was measured in

both floating dead cells and viable adherent cells. The floating cells were collected from culture medium by centrifugation (240 × *g*) at 4°C for 5 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp) [33]. The LDH released in the culture surpernatant (extracellular LDH, or LDHe) was used as an index of necrotic death, and the LDH present in the adherent viable cells as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\text{Apoptosis}\% = \text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$
$$\text{Necrosis}\% = \text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

3.6 Nuclear damages observed by Hoechst 33258 staining

Apoptotic nuclear morphology was assessed using Hoechst 33258 staining. The cells were fixed with 3.7% paraformaldehyde at room temperature for 30 min, and then washed and stained with 167 $\mu mol \cdot L^{-1}$ Hoechst 33258 at 37°C for 30 min. The cells were again washed and resuspended in PBS for morphologic observation using a fluorescent microscope (Olympus, Tokyo, Japan).

3.7 Fluorescence microscopic analysis of rhodamine 123-stained A431 cells

Cells were washed twice with PBS, fixed in 3.7% formaldehyde solution at room temperature for 10 min, permeabilized with 0.1% Triton X-100, and stained with 10 $mg \cdot L^{-1}$ rhodamine 123 [34]. Fluorescent images were monitored using fluorescence microscope (Olympus, Tokyo, Japan).

3.8 Tyrosine kinase activity assay

A431 cells were treated with lysis buffer (RIPA buffer: 50 $mmol \cdot L^{-1}$ Tris–HCl (pH 8.0), 150 $mmol \cdot L^{-1}$ NaCl, 0.5 $mmol \cdot L^{-1}$ EDTA, 1 $mmol \cdot L^{-1}$ DTT, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 $\mu g \cdot ml^{-1}$ phenylmethylsulfonyl fluoride (PMSF), 1 $\mu g \cdot ml^{-1}$ aprotinin, 2 $\mu g \cdot ml^{-1}$ leupeptin, 100 $\mu mol \cdot L^{-1}$ sodium vanadate) on ice for 10 min. Then mixed with 5 × assay buffer (HEPES 20 $mmol \cdot L^{-1}$, $MgCl_2$ 10 $mmol \cdot L^{-1}$, $MnCl_2$ 3 $mmol \cdot L^{-1}$, Na_3VO_4 0.1 $mmol \cdot L^{-1}$, DTT 1 $mmol \cdot L^{-1}$) before 10 µl of 5 × ATP/$MgCl_2$ solution was added. After reactions the absorbance of each microwell were read through ELISA at 450 nm.

3.9 Western blot analysis

A431 cells were treated with 20 $\mu mol \cdot L^{-1}$ oridonin for 12, 24, 36 and 48 h. Both adherent and floating cells were collected, and then Western blot analysis was carried out as previously described with some modification. Briefly, the cell pellets were resuspended in lysis buffer consisting of 20 $mmol \cdot L^{-1}$ Tris–HCl (pH 7.5), 1% Triton-X 100, 1 $mmol \cdot L^{-1}$ EDTA, 1 $mmol \cdot L^{-1}$ EGTA, 10 $mmol \cdot L^{-1}$ β-mercaptoethanol, 1 $mmol \cdot L^{-1}$ sodium orthovanadate, 10 $\mu g \cdot ml^{-1}$ leupeptin, 1 $mmol \cdot L^{-1}$ PMSF and lysed on ice for 1 h. After centrifugation of the cell suspension at 12,000 × *g* for 20 min, the protein content of the supernatant was

determined by a protein assay reagent (Bio-Rad, Laboratories, Hercules, CA, USA). The protein lysates were separated by electrophoresis in 7.5, 10 or 12% SDS–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane [35]. Proteins were detected using polyclonal antibody and visualised using anti-rabbit or anti-mouse IgG conjugated with peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

3.10 Statistical analysis

Statistical analysis was conducted using Student's *t*-test for analysis of significance between the different values. Values were expressed as the mean ± S.D., and they were considered significant at a p of less than 0.05. All ps are two-tailed.

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References

[1] J. Huang, L.J. Wu, S.I. Tashiro, S. Onodera, T. Ikejima. *Biol. Pharm. Bull.*, **28**, 2068 (2005).
[2] C.L. Zhang, L.J. Wu, S.I. Tashiro, S. Onodera, T. Ikejima. *Chin. Pharmacol. Bull.*, **19**, 525 (2003).
[3] K. Osawa, H. Yasuda, T. Maruyama, H. Morita, K. Takeya, H. Itokawa. *Phytochemistry*, **36**, 1287 (1994).
[4] K. Fuji, M. Node, M. Sai, E. Fujita, S. Takeda, N. Unemi. *Chem. Pharm. Bull. (Tokyo)*, **37**, 1472 (1989).
[5] C.L. Zhang, L.J. Wu, S.I. Tashiro, S. Onodera, T. Ikejima. *Biol. Pharm. Bull.*, **27**, 1527 (2004).
[6] S.O. Kim, J. Han. *J. Endotoxin Res.*, **7**, 292 (2001).
[7] N. Kawazoe, M. Watabe, Y. Masuda, S. Nakajo, K. Nakaya. *Oncogene*, **18**, 2413 (1999).
[8] P.A. Hill, A. Tumber, M.C. Meikle. *Endocrinology*, **138**, 3849 (1997).
[9] S. Mizukami, K. Kikuchi, T. Higuchi, Y. Urano, T. Mashima, T. Tsuruo, T. Nagano. *FEBS Lett.*, **453**, 356 (1999).
[10] J.A. Hicjmann. *Cancer Metast. Rev.*, **11**, 121 (1992).
[11] Z. Chen, M. Naito, T. Mashima, T. Tsuruo. *Cancer Res.*, **56**, 5224 (1996).
[12] D. Li, L.J. Wu, S.I. Tashiro, S. Onodera, T. Ikejima. *Biol. Pharm. Bull.*, **30**, 254 (2007).
[13] C.L. Zhang, L.J. Wu, S.I. Tashiro, S. Onodera, T. Ikejima. *Acta Pharmacol. Sin.*, **25**, 691 (2004).
[14] G. Kroemer, P. Petit, N. Zamzami, J.L. Vayssiere, B. Mignotte. *FASEB J.*, **9**, 1277 (1995).
[15] W.W. An, M.W. Wang, S.I. Tashiro, S. Onodera, T. Ikejima. *J. Korean Med. Sci.*, **19**, 560 (2004).
[16] Y.A. Lazebnik, S.H. Kaufmann, S. Desnoyers, G. Poirier, W.C. Earnshaw. *Nature*, **371**, 346 (1994).
[17] T. Muneesh, T.Q. Long, O.R. Karen, D. Serge, Z. Zhi. *Cell*, **81**, 801 (1995).
[18] C. Gruber, M. Henkel, W. Budach, C. Belka, V. Jendrossek. *Biochem. Pharmacol.*, **67**, 1859 (2004).
[19] J. Schlegel, I. Peters, S. Orrenius, D.K. Miller, N.A. Thornberry, T.T. Yamin, D.W. Nicholson. *J. Biol. Chem.*, **271**, 1841 (1996).
[20] E.S. Alnemri, D.J. Livingston, D.W. Nicholson, G. Salvesen, N.A. Thornberry, W.W. Wong, J. Yuan. *Cell*, **87**, 171 (1996).
[21] Z.N. Oltvai, C.L. Milliman, S.J. Korsmeyer. *Cell*, **74**, 609 (1993).
[22] D.W. Chan, S.C. Son, W. Block, R. Ye, K.K. Khanna, M.S. Wold, P. Douglas, A.A. Goodarizi, J. Pelley, Y. Taya, M.F. Lavin, S.P. Lees-Miller. *J. Biol. Chem.*, **275**, 7803 (2000).
[23] G. Kroemer. *Nat. Med.*, **3**, 614 (1997).
[24] S.A. Susin, N. Zamzami, G. Kroemer. *Biochim. Biophys. Acta*, **1366**, 151 (1998).
[25] M. Salvi, A.M. Brunati, L. Bordin, N.L. Rocca, G. Clari, A. Toninello. *Biochim. Biophys. Acta*, **1589**, 181 (2002).
[26] N. Moghal, P.W. Sternberg. *Curr. Opin. Cell Biol.*, **11**, 190 (1999).
[27] A. Wells. *Int. J. Biochem. Cell Biol.*, **31**, 637 (1999).

[28] A. Livigni, A. Scorziello, S. Agnese, A. Adornetto, A. Carlucci, C. Garbi, I. Castaldo, L. Annunziato, E.V. Avvedimento, A. Feliciello. *Mol. Biol. Cell.*, **17**, 263 (2006).
[29] L. Leonard, S. Luydmila, B. Christina, M. I, S. Mervyn, W. Trevor. *Anti-Cancer Drug*, **17**, 495 (2006).
[30] L.W. Li, P.S. Lorenzo, K. Bogi, P.M. Blumberg, S.H. Yuspa. *Mol. Cell. Biol.*, **19**, 8547 (1999).
[31] P. Morrison, A.R. Saltiel, M.R. Rosner. *J. Biol. Chem.*, **271**, 12891 (1996).
[32] E. Fujita, Y. Nagao, T. Kohno, M. Matsuda, M. Ozaki. *Chem. Pharm. Bull. (Tokyo)*, **29**, 3298 (1981).
[33] Y.M. Kim, R.V. Talanian, T.R. Billiar. *J. Biol. Chem.*, **172**, 31138 (1997).
[34] A. Pastore, G. Tozzi, L.M. Gaeta, E. Bertini, V. Serafini, S.D. Cesare, V. Bonetto, F. Casoni, R. Carrozzo, G. Federici, F. Piemonte. *J. Biol. Chem.*, **278**, 42588 (2003).
[35] E.J. Meuillet, B. Mania-Farnell, D. George, J.I. Inokuchi, G.E. Brenmer. *Exp. Cell Res.*, **256**, 74 (2000).