J Pharmacol Sci **105**, 22 – 33 (2007)

Journal of Pharmacological Sciences

Full Paper

Inactivation of Ras and Changes of Mitochondrial Membrane Potential Contribute to Oridonin-Induced Autophagy in A431 Cells

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Received December 18, 2006; Accepted June 19, 2007

Abstract. We have previously shown that oridonin isolated from *Rabdosia rubescens* augmented apoptosis while inhibiting autophagy within 24 h in HeLa cells. However, the mechanisms between apoptosis and autophagy induced by oridonin in A431 cells are largely unknown. Here, it was found that autophagic level is significantly upregulated when A431 cells are pretreated with manumycin A (Ras specific inhibitor) compared with oridonin alone treatment, whereas cells precultured with GW5074 (Raf inhibitor) or PD98059 (ERK inhibitor) did not exhibit such an effect. Ras, but not Raf or ERK, was engaged in the control of oridonin-induced autophagy. At the same time, manumycin A contributes to oridonin-induced down-regulation of Ras protein expression. Treatment with the combination of oridonin and manumycin A downregulated phosphorylation of Akt, downstream of phosphatidylinositol 3-OH kinase (PI3-K). Preincubation with the PI3-K inhibitor wortmannin and Akt inhibitor KP372-1 enhanced oridonin-induced apoptosis, whereas it inhibited oridonin-induced autophagy. However, under oridonin treatment, the expression of Beclin-1, which has autophagy-inducing activity, was reduced, suggesting that Beclin-1 did not participate in the oridonin-induced autophagy. Morphologic observations, DNA fragmentation analysis, and LDH activity-based assay showed that 3-methyladenine (3-MA), an inhibitor of autophagy, increased the apoptotic sensitivity of A431 cells to oridonin. In addition, manumycin A contributed to oridonin-induced decrease of mitochondrial membrane potential ($\Delta\psi$m), consistent with the upregulation of Bax/Bcl-2 ratio. In conclusion, Ras negatively regulated autophagy in oridonin-treated A431 cells, which might be associated with activation of class I PI3-K. Downregulation of $\Delta\psi$m and increasing of the ratio of Bax/Bcl-2 might also be partially responsible for the initiation of the autophagic process.

Keywords: oridonin, apoptosis, autophagy, Ras, mitochondrial permeability transition, class I phosphatidylinositol 3-OH kinase (PI3-K)

Introduction

Oridonin, a diterpenoid isolated from *Rabdosia rubescens*, has many kinds of pharmacological and physiological effects and has been traditionally used for treatment of many diseases, such as leukemia, in China (1 – 3). Previous reports have demonstrated that oridonin exerted a variety of biological effects, including anti-tumor activity, scavenging active oxygen free radicals, and anti-bacterial action (4). In our previous study, oridonin induced apoptosis of A431 cells, which overexpress epidermal growth factor receptor, through inhibiting total tyrosine kinase activity and suppressing the phosphorylation of EGFR (5), thereby inhibiting activation of the downstream phosphatidylinositol 3-OH kinase (PI3-K)/Akt signaling pathway, which mediates

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Published online in J-STGAGE
doi: 10.1254/jphs.FPJ06022X

autophagy. Therefore, in this study, we analyzed the mechanism responsible for regulating the balance between oridonin-induced apoptosis and autophagy in A431 cells.

Autophagy, a process for bulk degradation of proteins and organelles, is essential for cell homeostasis. In addition to its normal physiological functions in catabolism, autophagy has been shown to be associated with pathologic conditions such as certain neurodegenerative diseases, cardiomyopathies, and infectious diseases (6 – 9). Martinet proposed a cell death classification scheme that contains three types of cell death, also referred to as type I, type II, and type III cell death (10). Type I cell death is regarded as apoptosis. Type II cell death is named autophagy, which has been thought occur with apoptosis sometimes in the process of programmed cell death. Conversion of microtubule-associated protein 1 light chain 3 (LC3-I) to LC3-II was considered a general marker for initiation of autophagy. Type III cell death is a specific biochemical pathway that has not yet been identified. Necrosis, initially described as a distinct subgroup of cell death, can be recognized as a terminal stage of apoptosis and autophagy.

Apoptosis serves as a targeted elimination of individual cells during physiological circumstances, followed by endocytosis of cellular remnants by macrophages and subsequent degradation in lysosomes (11). Autophagy is a constitutive event that is responsible for the degradation of cytoplasmic cargo or organelles through their sequestration within autophagosomes and subsequent fusion with lysosomes (12). In the past decade, there has been much growth in the field of studying the close relationships between autophagy, tumorogenesis, and disease progression. However, the detailed knowledge of molecular phenomena that initiate autophagy is lacking.

The mitochondria is considered to be a pivotal organelle in determining cell destiny and may act as an 'on–off' switch modulating autophagy and apoptosis in the process of cell death. Change of the $\Delta\psi$m is responsible for these depolarizations and the opening of mitochondrial permeability transition (MPT) pore signals the initiation of the autophagic process (13).

PI3-Ks are a family of enzymes that catalyze phosphorylation of phosphotidylinositols, changing them into phosphoinositols by phosphorylating the inositol ring's free OH groups. In this way, PI3-K plays a key enzymatic role in production of phosphoinositol 3,4,5-trisphosphate. Activated PI3-K converts plasma membrane lipid phosphoinositol-4,5-bisphosphate [PI(4,5)P2] into phosphinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. There are three structurally distinct classes of PI3-K. The class I PI3-K (p110α, p110β, p110δ, and p110γ) is activated by a cell surface receptor tyrosine kinase (such as EGFR) or G protein-coupled receptors to generate (PIP3). Once activated by these receptors, class I PI3-K phosphorylates plasma membrane phosphoinositides and then activates serine-threonine kinase Akt (14 – 17). The class II PI3-K is a p170 molecule. These proteins are predominately found in the membrane of cells and are activated by insulin, EGF, or platelet-derived growth factor. Class III PI3-Ks play a key role in intracellular trafficking through the synthesis of PI(3)P and PIP2 (18). Although all of them are the members of the PI3-K family, they have adopted different mechanisms in autophagy. When class I PI3-K is activated, autophagy is inhibited, while class III PI3-K is required for both autophagic vesicle formation and vesicular transport to the lysosome.

Ras proteins exert a pivotal regulatory function in signal transduction involved in cell proliferation, and Ras-Raf-MAPK pathways have been the most extensively studied. However, Ras also activates PI3-K, which has been implicated in mitogenic signaling and inhibition of apoptosis and regulates autophagy (19). Therefore, Ras must be involved in autophagy, an intracellular protein degradation process in cell growth control.

Materials and Methods

Reagents

Oridonin was obtained from the Kunming Insititute of Botany, the Chinese Academy of Sciences (Kunming, China). The purity of oridonin isolated from *Rabdosia rubescens* was confirmed by HPLC to be higher than 99%. The structure of oridonin was assigned by comparing the chemical and spectral data (^{1}H-, ^{13}C-NMR) with those reported in the literature (20). Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The DMSO concentration was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or cell death.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), ribonuclease (RNase), proteinase K, monodansylcadaverine (MDC), wortmannin (an inhibitor of PI3-K), KP372-1 (an inhibitor of Akt), 3-methyladenine (3-MA), rhodamine 123, and acridine orange were purchased from Sigma Chemical (St. Louis, MO, USA). Polyclonal antibodies against Akt, phospho-Akt, Bcl-2, Bax, Beclin-1, LC3, Ras, and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit, goat-anti-mouse) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The human epidermoid carcinoma A431 cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in Ham's F-12 medium (Hyclone, logan, UT, USA) supplemented with 10% heated inactivated fetal bovine serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.03% L-glutamine and maintained at 37°C with 5% CO_2 in a humidified atmosphere. The cells were preincubated for 1 h with specific inhibitors before treatment with oridonin. Manumycin A (Ras inhibitor) was obtained from Calbiochem (CA, La Jolla, USA). The effective doses of manumycin A was determined by preliminary studies and found to be comparable to those used in other studies (21).

Cell growth assay

The cytotoxic effect of oridonin on A431 cells was measured by MTT assay as described previously (11). The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well. After 24-h incubation, they were treated with various concentrations of oridonin for different time periods. Four hours before the end of incubation, 20 μL MTT solution (5.0 mg/L) was added to each well. The resulting crystals were dissolved in DMSO. Optical density was measured by MTT assay using a plate microreader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

Cell death (%) = {1 − [*A492* (control) − *A492* (oridonin)]} / *A492* (control) × 100

DNA fragmentation assay (22)

A431 cells (1×10^6) were collected by centrifugation at 1,000 × *g* for 5 min and washed with phosphate-buffered saline (PBS). The cells were pelleted and suspended in 100 μl cell lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L EDTA (pH 8.0) and 0.5% Triton X-100] and then kept at 4°C for 10 min. The lysate was centrifuged at 15,000 × *g* for 20 min. The supernatant was incubated with RNase A (20 μg/μL) at 37°C for 1 h and then incubated with proteinase K (20 μg/μL) at 37°C for 1 h. Then the supernatant was again mixed with 0.5 M NaCl (20 μL) and 50% isopropanol (120 μl) at −20°C overnight, followed by centrifugation at 15,000 × *g* for 15 min. After drying, the DNA was dissolved in TE buffer, pH 7.8 [10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0)], separated by 2% agarose gel electrophoresis at 100 V for 40 min, and stained with 0.1 mg/L ethidium bromide.

LDH activity-based cytotoxicity assays (4)

LDH (lactate dehydrogenase) activity was assessed using a standardized 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). The LDH released in the culture surpernant (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:

Apoptosis% = LDHp / (LDHp + LDHi + LDHe) × 100

Necrosis% = LDHe / (LDHp + LDHi + LDHe) × 100

Fluorescence morphologic examination of apoptosis and mitochondrial permeability transition (MPT)

Apoptotic morphology was studied by staining the cells with the fluorescent, DNA-binding dye acridine orange (AO). Cells were harvested and washed three times with PBS after incubation with 20 μmol/L oridonin in the presence or absence of 2 mmol/L 3-MA for 24 h, and they were then stained with 20 μg/mL AO (Sigma) for 15 min. Then, the color and structure of the different cell types were observed under an OLYMPUS IX70 reverse fluorescence microscope (Olympus, Tokyo) (23). On the other hand, we examined mitochondrial membrane potential ($\Delta\psi$m), which depends on the opening of MPT pores. Cells were washed twice with PBS after being incubated with 20 μmol/L oridonin in the presence or absence of 10 μmol/L manumycin A for 24 h, fixed in 3.7% para-formaldehyde solution for 10 min at room temperature, permeabilized with 0.1% Triton X-100, and stained with 10 mg/L rhodamine 123 (a cell permeable, cationic, fluorescent dye, which can incorporate into mitochondria depending on the inner transmembrane potential). Fluorescent images were monitored by using a fluorescence microscope (Olympus) (24).

Flowcytometric analysis of autophagy and $\Delta\psi m$

A431 cells were treated with 10 μmol/L manumycin A, wortmannin, or KP372-1 for 1 h before 20 μmol/L oridonin administration. After 24 h, the cells were harvested by trypsin and rinsed with PBS by centrifugation at 1,500 × *g*. For measuring autophagy, the resulting cell pellet from 1×10^6 cells was suspended with 0.05 mmol/L MDC at 37°C for 60 min as described previously (25). For measuring $\Delta\psi$m, the cells were dyed by rhodamine 123. About one million cells were

harvested and washed twice with PBS. Then cell samples were incubated with 10 mg/L rhodamine 123 at 37°C in the dark for at least 15 min and then analyzed by FACScan flowcytometer (Becton Dickinson, Franklin Lakes, NJ, USA) (26).

Western blot analysis

A431 cells were preincubated for 1 h with specific inhibitors before stimulation with 20 μmol/L oridonin. Both adherent and floating cells were collected, and then Western blot analysis was carried out as previously described with some modifications. Cell lysates were prepared to examine the expression of Akt, phospho-Akt, Beclin-1, LC3, Ras, Bax and Bcl-2. In brief, A431 cells washed twice with PBS, and lysed in ice-cold lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 1% Triton-X 100, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-mercaptoethanol, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, 1 mmol/L phenyl-methyl-sulfonylfluoride (PMSF) (27). After 60 min of incubation on ice, the cells were swelled, and then centrifuged 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), and then separated by electrophoresis in 12% SDS polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose membrane (28). Proteins were detected using polyclonal antibody and visualized using anti-rabbit or anti-mouse IgG conjugated with peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

Statistical analysis of the data

The data are expressed as means ± S.D. Statistical comparisons were made by Student's *t*-test. $P<0.05$ was considered significant.

Results

Growth inhibitory effect of oridonin on A431 cell growth

Oridonin induced A431 cell death in a time- and dose-dependent manner. Oridonin from 5 to 80 μmol/L exerted a potent inhibitory effect on A431 cell growth (Fig. 1). By 24 h after A431 cells were added by 20 μmol/L oridonin, the inhibitory ratio reached nearly 50%. Therefore, 24-h incubation with oridonin is sufficient for half inhibition of cell growth.

Inhibition of autophagy contributed to oridonin-induced apoptosis

Autophagy is characterized by the accumulation of cytoplasmic acidified lysosomes. To determine this phenomenon in A431 cells that had undergone treatment

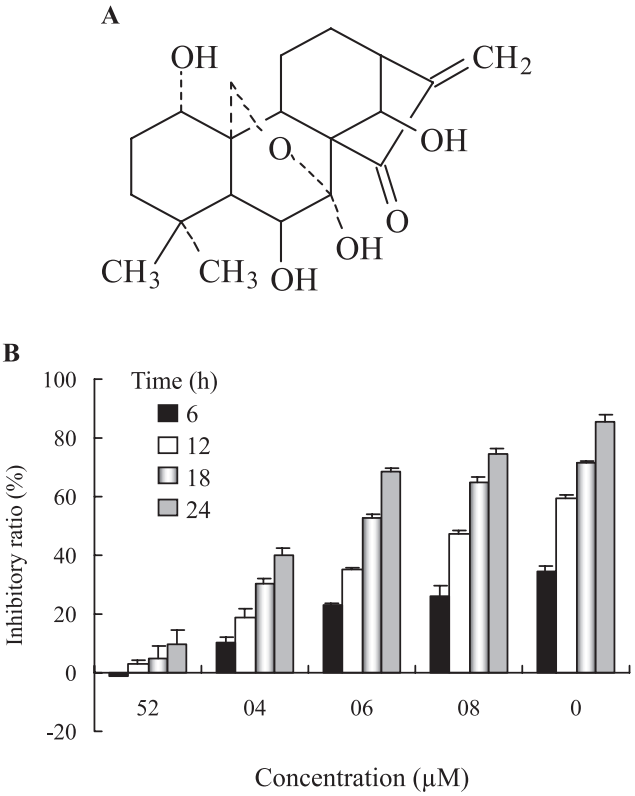

Fig. 1. Cytotoxic effect of oridonin on A431 cells. A: Chemical structure of oridonin. B: A431 cells (2×10^4 cells/well) were treated with various doses of oridonin for 6, 12, 18, or 24 h. Cell numbers were measured by MTT assay and the results show the calculated cell growth inhibitory ratio. n = 3, mean ± S.D.

with oridonin, we performed flowcytometric analysis using MDC, a specific fluorescent dye for acidified lysosomes, on control and oridonin-treated cells after 24 h. The results showed that oridonin at 20 μmol/L not only induced autophagy (Fig. 2A), but also induced apoptosis (Fig. 2B). However, when A431 cells were preincubated with 3-MA (2 mmol/L) for 60 min, the autophagy-induced ratio by oridonin was markedly suppressed (Fig. 2A) while apoptotic ratio was significantly enhanced (Fig. 2B), compared with the oridonin alone treatment group. Moreover, morphological changes by AO staining showed that control cells exhibited uniformly green fluorescence (Fig. 2Ca), whereas oridonin-induced apoptotic cells with condensed, fragmented chromatin were stained orange (Fig. 2Cb) and increased numbers of apoptotic bodies were observed in the 3-MA combination with oridonin treatment group (Fig. 2Cd). The cells in the 3-MA alone treated group did not show typical apoptotic features in A431 cells (Fig. 2Cc).

In addition, DNA ladder, another hallmark of typical apoptosis, was also analyzed. After the cells were treated

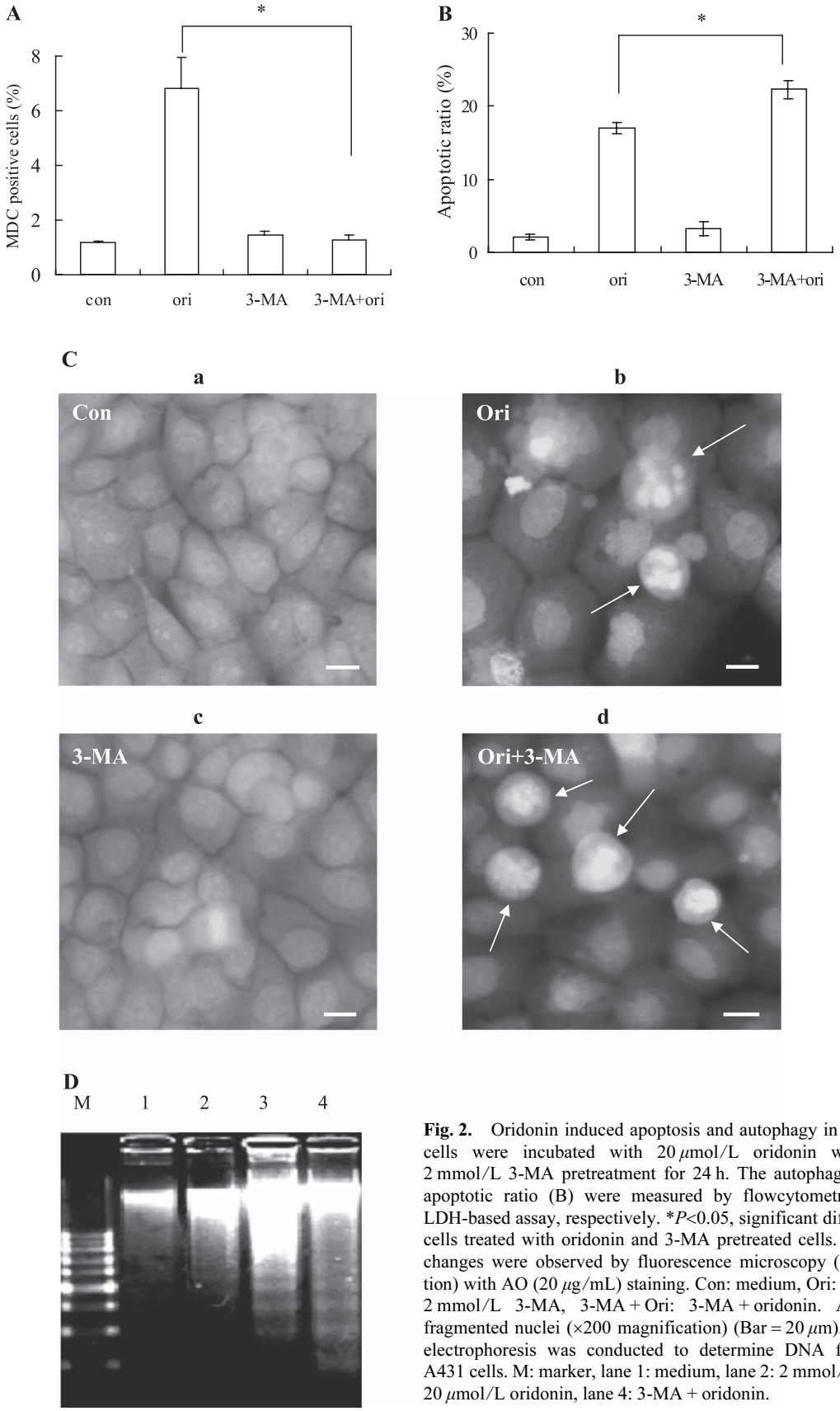

Fig. 2. Oridonin induced apoptosis and autophagy in A431 cells. The cells were incubated with 20 μmol/L oridonin with or without 2 mmol/L 3-MA pretreatment for 24 h. The autophagic ratio (A) and apoptotic ratio (B) were measured by flowcytometric analysis and LDH-based assay, respectively. **P*<0.05, significant difference between cells treated with oridonin and 3-MA pretreated cells. C: Morphologic changes were observed by fluorescence microscopy (×200 magnification) with AO (20 μg/mL) staining. Con: medium, Ori: oridonin, 3-MA: 2 mmol/L 3-MA, 3-MA + Ori: 3-MA + oridonin. Arrows indicate fragmented nuclei (×200 magnification) (Bar = 20 μm). D: Agarose gel electrophoresis was conducted to determine DNA fragmentation in A431 cells. M: marker, lane 1: medium, lane 2: 2 mmol/L 3-MA, lane 3: 20 μmol/L oridonin, lane 4: 3-MA + oridonin.

with oridonin for 24 h, internucleosomal DNA fragmentation was observed (Fig. 2D, lane 3) and this DNA fragmentation was significantly augmented when cells were pretreated with 3-MA (Fig. 2D, lane 4). The cells in the control (Fig. 2D, lane 1) or 3-MA (Fig. 2D, lane 2) treatment group exhibited slight smear-like ladder. These results indicated that apoptosis was facilitated by 3-MA preincubation in oridonin-treated A431 cells, whereas cell autophagy was suppressed.

Increased autophagy in response to oridonin is dependent on inhibition of Ras, but not Raf or ERK

A431 cells were pre-treated with 10 μmol/L manumycin A for 60 min, followed by 20 μmol/L oridonin treatment. After 24 h, the autophagic ratio was significantly upregulated, compared with the oridonin alone treatment (Fig. 3A), indicating that activation of Ras antagonized oridonin-induced autophagy.

Once autophagy happens, phosphatidylethanolamine is covalently linked to the cytosolic protein LC3-I to yield LC3-II, which associates with the autophagosome. This conversion is often regarded as a marker for autophagy (29 – 31). In order to further confirm the autophagy-augmention effect of manumycin A, Western blot analysis was performed to detect the expression of LC3 protein. Consistent with the flow cytometric analysis results in Fig. 3A, pretreatment with manumycin A contributed to the LC3 processing in oridonin-treated cells (Fig. 3B).

However, in contrast to inhibition of Ras that increased oridonin-induced autophagy, pretreatment with 10 nmol/L Raf inhibitor, GW5074, or 10 μmol/L ERK inhibitor, PD98059, had no appreciable effect on oridonin-induced autophagy in A431 cells (Fig. 4: A and B), indicating that the Ras-Raf-ERK pathway is not engaged in the control of oridonin-induced autophagy.

Effect of manumycin A on oridonin-induced expression of Ras protein

Based on the above results, Ras protein expression

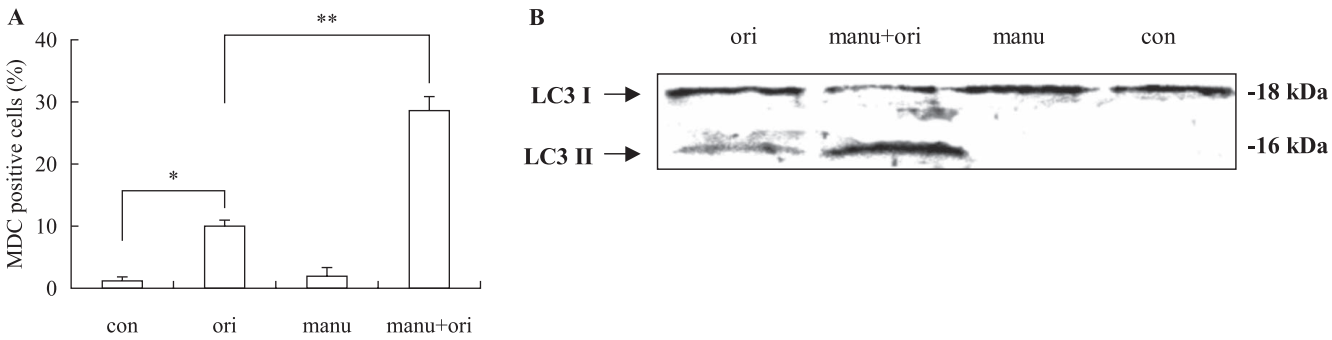

Fig. 3. Effect of Ras on oridonin-induced autophagy in A431 cells. The cells were incubated with or without 10 μmol/L Ras inhibitor (manumycin A) for 60 min before 20 μmol/L oridonin was added. After 24 h, the autophagic ratio was measured by flowcytometric analysis (A). *P<0.05, **P<0.01. The protein expression of LC3 was detected by Western blot analysis (B). con: medium, ori: oridonin, manu: manumycin A.

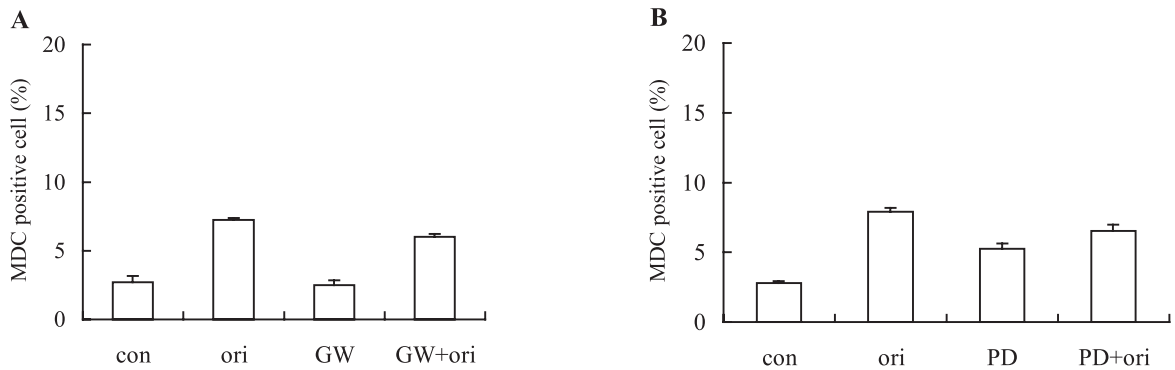

Fig. 4. Effect of Raf and ERK on oridonin-induced autophagy in A431 cells. The cells were incubated with or without 10 nmol/L Raf inhibitor (GW5074) (A) or 10 μmol/L ERK inhibitor (PD98059) (B) for 60 min before 20 μmol/L oridonin was added. After 24 h, the autophagic ratio was measured by flowcytometric analysis. con: medium, ori: oridonin, GW: GW5074, PD: PD98059.

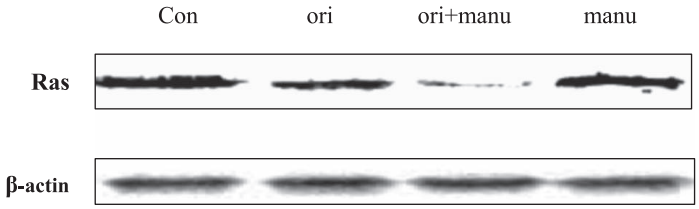

Fig. 5. Effect of manumycin A on the expression of Ras protein in oridonin-treated A431 cells. The cells were pre-treated with or without 10 μmol/L Ras inhibitor (manumycin A) for 60 min, and then 20 μmol/L oridonin was added for 24 h. The expressions of Ras protein and β-actin were detected by Western blot analysis. Con: medium, ori: oridonin, manu: manumycin A, ori + manu: oridonin + manumycin A.

was detected by introduction of manumycin A in oridonin-treated cells. The effect of manumycin A on the expression of Ras protein showed that inhibition of its activity contributed to oridonin-induced downregulation of its expression (Fig. 5).

Effects of Ras on oridonin-induced inhibition of Akt in A431 cells

Ras, except its function in the activating Raf-ERK signaling pathway, signals by directly binding to and activating PI3-K/Akt and regulates autophagy in many types of tumor cells. Here, Western blot analysis was performed to detect the expression of Akt and its phosphorylation. As shown in Fig. 6, Akt phosphorylation was reduced by oridonin treatment, and this suppressive effect was more significant when Ras was inhibited, whereas the expression of Akt was unchanged.

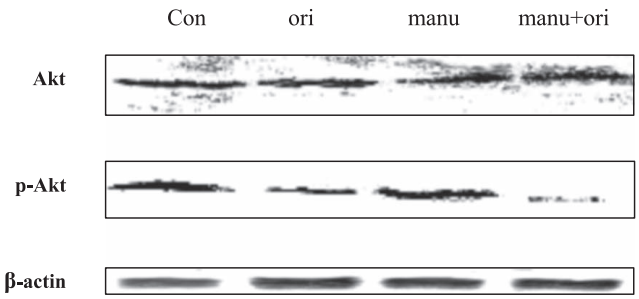

Fig. 6. The expression of Akt in oridonin-treated A431 cells. The cells were pre-treated with or without 10 μmol/L Ras inhibitor (manumycin A) for 60 min, and then 20 μmol/L oridonin was added for 24 h. The expressions of Akt, phosphorylated Akt and β-actin were detected by Western blot analysis. Con: medium, ori: oridonin, manu: manumycin A, manu + ori: manumycin A + oridonin.

Inhibition of PI3-K activity reduced autophagy and enhanced apoptosis

In order to further confirm the effect of PI3-K/Akt on oridonin-induced autophagy and apoptosis, the cells were pretreated with 200 nmol/L wortmannin and 10 μmol/L KP372-1, respectively, for 60 min before 20 μmol/L oridonin was added. After 24-h incubation, the autophagic ratios were strongly suppressed by oridonin co-treated with wortmannin or KP372-1 (Fig. 7A), whereas the inhibitory ratios were markedly increased by inclusion of wortmannin or KP372-1 (Fig. 7B).

Changes in mitochondrial membrane potential in oridonin-treated A431 cells

It was reported that changes of $\Delta\psi$m, which reflected the MPT, triggered diverse autophagic or apoptotic signals. Therefore, both autophagy and apoptosis were induced upon opening of the MPT pores (13, 32). In order to assess whether inactivation of Ras participated in changes of $\Delta\psi$m, A431 cells were treated with

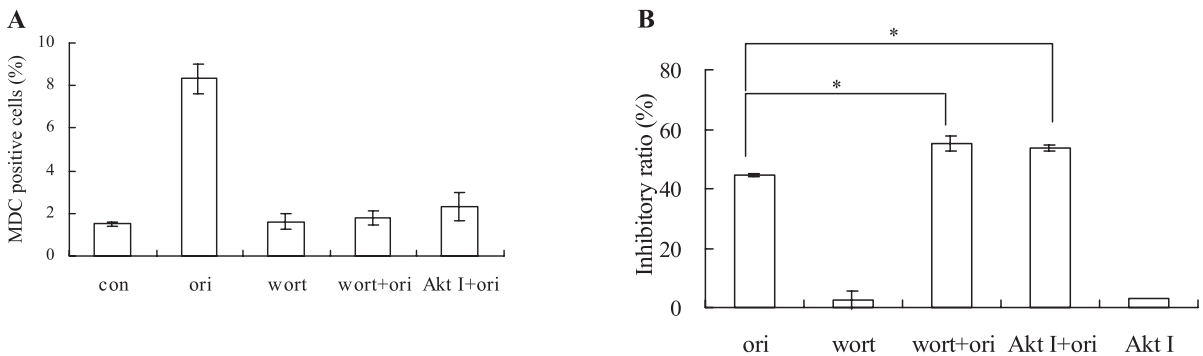

Fig. 7. Effect of wortmannin and KP372-1 on oridonin-induced autophagy and apoptosis in A431 cells. The cells were incubated with or without 200 nmol/L wortmannin or 10 μmol/L KP372-1 for 60 min before 20 μmol/L oridonin was added. After 24 h, the autophagic ratio (A) and apoptotic ratio (B) were measured by flowcytometric analysis and MTT assay, respectively. con: medium, ori: oridonin, wort: wortmannin, Akt I: KP372-1. *P<0.05.

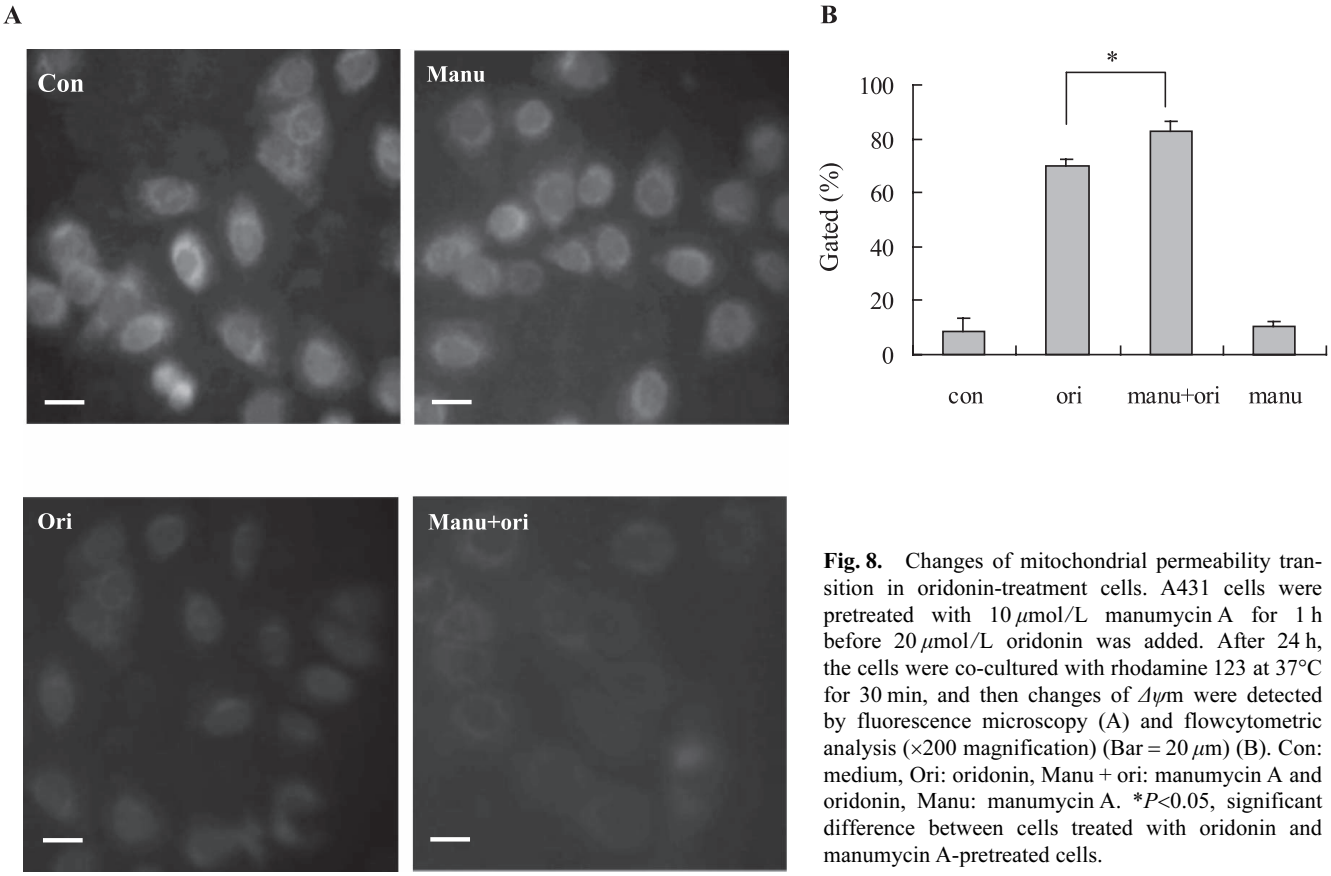

Fig. 8. Changes of mitochondrial permeability transition in oridonin-treatment cells. A431 cells were pretreated with 10 μmol/L manumycin A for 1 h before 20 μmol/L oridonin was added. After 24 h, the cells were co-cultured with rhodamine 123 at 37°C for 30 min, and then changes of $\Delta\psi$m were detected by fluorescence microscopy (A) and flowcytometric analysis (×200 magnification) (Bar = 20 μm) (B). Con: medium, Ori: oridonin, Manu + ori: manumycin A and oridonin, Manu: manumycin A. *P<0.05, significant difference between cells treated with oridonin and manumycin A-pretreated cells.

20 μmol/L oridonin for 24 h with or without manumycin A, and then morphologic observation was carried out by using rhodamine 123 staining. Remarkable loss of fluorescent intensity was observed in the oridonin alone treatment group or oridonin and manumycin A pretreatment group (Fig. 8A). In order to further confirm the above results, the changes in $\Delta\psi$m were also detected through flowcytometric analysis (Fig. 8B). Inhibition of Ras contributed to oridonin-induced downregulation of $\Delta\psi$m, indicating that changes of $\Delta\psi$m might partially be caused by the increased autophagy by inactivation of Ras.

Inactivation of Ras enhanced oridonin-induced the ratio of Bax/Bcl-2

The Bcl-2 family not only were known to play an essential role in DNA damage-induced apoptosis, but also are apoptosis-regulating proteins that modulate the mitochondrial pathway, including antiapoptotic proteins such as Bcl-2 and Bcl-X_L and other proapoptotic proteins such as Bax and Bid. These proteins regulate mitochondrial permeability transition, and the balance between these two groups determines the fate of cells (33, 34). To confirm whether such a mechanism is involved in oridonin-induced cell death, Western blot analysis was performed. As shown in Fig. 9, the expression of Bcl-2 protein was reduced in oridonin-treated group and this suppressive effect was more significant after manumycin A preincubation. However, the level of Bax expression did not change.

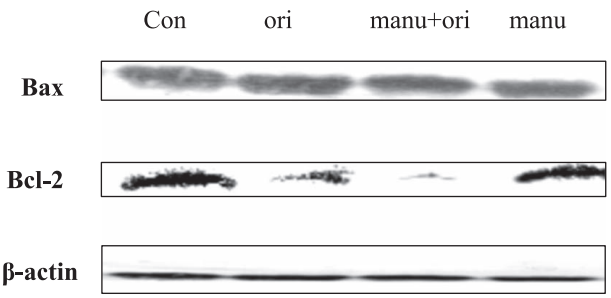

Fig. 9. The expression of Bcl-2 and Bax in oridonin treated cells. A431 cells were pretreated with 10 μmol/L manumycin A for 1 h before 20 μmol/L oridonin was added for 24 h. Then the expressions of Bax, Bcl-2 and β-actin were detected by Western blot analysis. Con: medium, ori: oridonin, manu + ori: manumycin A + oridonin, manu: manumycin A.

Beclin-1 did not participate in autophagy process in A431 cells

Beclin-1, associated with PI3-K, has been identified

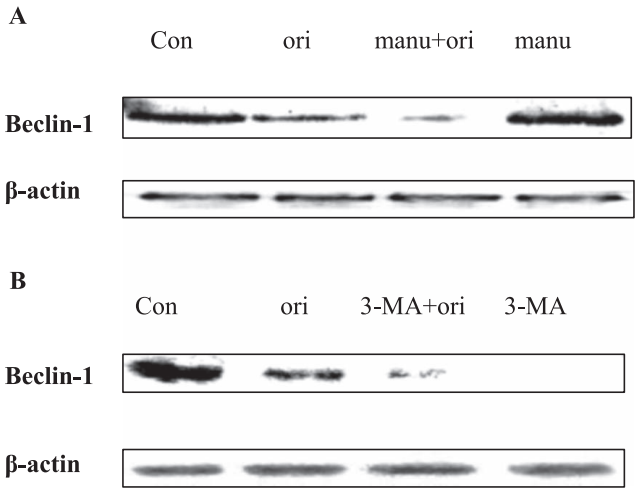

Fig. 10. The expression of Beclin-1 in oridonin treated cells. A431 cells were pretreated with 10 μmol/L manumycin A (A) or 3-MA (B) for 1 h before 20 μmol/L oridonin was added for 24 h. Then the expressions of Beclin-1 and β-actin were detected by Western blot analysis. Con: medium, ori: oridonin, manu + ori: manumycin A and oridonin, manu: manumycin A, 3-MA + ori: 3-MA and oridonin.

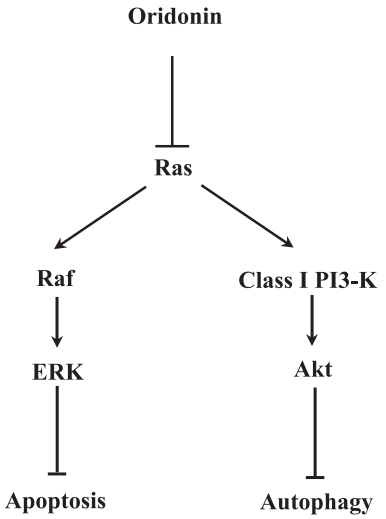

Fig. 11. The role of Ras in oridonin-induced apoptosis and autophagy in A431 cells.

to be responsible for autophagy (35). To investigate whether the augmented autophagy induced by inactivation of Ras was associated with Beclin-1, we therefore examined the protein expression of Beclin-1 by Western blot analysis. Manumycin A had no effects on the expression of Beclin-1 (Fig. 10A). However, after oridonin treatment, Beclin-1 protein expression was significantly downregulated and this suppressive effect became more significant by further treatment of manumycin A. Addition of 3-MA, as an autophagy specific inhibitor, into A431 cells significantly suppressed Beclin-1 expression (Fig. 10B). When A431 cells were pretreated with 3-MA, the expression of Beclin-1 was more significantly downregulated, compared with the oridonin alone treatment group.

Discussion

Autophagy, being an evolutionarily ancient cellular response to extra- and intracellular stimuli, may precede or co-exist with apoptosis, and this process may be induced by apoptotic stimuli. Cellular autophagy is a physiological degradative process, like apoptosis, in embryonic growth and development, cellular remodeling, and the biogenesis of some subcellular organelles (36). However, in some cases, apoptosis and autophagy coincide in vivo in certain tissues or cell groups. Other evidences showed that active autophagy appeared to increase the tendency to undergo apoptosis (37, 38).

Ras functions as a molecular switch linking receptor tyrosine kinase activation to downstream cytoplasmic or nuclear events through two major effectors: Raf and class I PI3-K (39, 40). Class I PI3-K are heterodimeric enzymes composed of a 110 – 120-kDa catalytic subunit, which associate with an adapter molecule of 85 kDa containing two SH2 domains. This regulatory p85 subunit allows PI3-K to move from the cytosol to the membrane where its p110 can bind to Ras, stimulating the activation of PI3-K (14).

Ras has a dual effect on autophagy; when it activates class I PI3-K, which activates the downstream target Akt, autophagy is inhibited, but when it activates the Raf $\rightarrow$ ERK cascade, autophagy is stimulated (41). In our previous study, we have demonstrated that inhibition of Ras activity enhanced oridonin-induced apoptosis (42), whereas here we found that inhibition of Ras by manumycin A facilitated oridonin-induced autophagy, indicating that the Ras signal pathway overlaps autophagy and apoptosis (Fig. 11). In addition, downregulated expression of Ras protein was enhanced by manumycin A preincubation in A431 cells that were treated with oridonin. Therefore, the autophagy-inducing effect here is due to the inhibition of the class I PI3-K pathway induced by the inactivation of Ras, presumably because of the decreased expression of Ras protein (42). However, our further studies will focus on the relationship of the proteins that lie downstream of Ras and possibly play a critical role in regulating autophagy and apoptosis.

Since class I PI3-K, downstream of Ras, usually links to AKT/PKB protein kinase in suppressing autophagy (43), we examined the protein expressions of Akt and Akt phosphorylation under manumycin A treatment.

Ras inactivation reinforced downeregulation of Akt phosphorylation without altering Akt expression in oridonin-treated A431 cells, suggesting that inactivation of AKT/PKB might participate in mediating the negative control of autophagy. Moreover, the introduction of PI3-K or Akt inhibitor reduced the autophagic level, while it increased the apoptotic ratio. All these results indicated that PI3-K plays an important role in both apoptosis- and autophagy-mediating pathways, functioning as a fork point of these two signaling pathways (11).

Mitochondrial permeability transition pore, which initiates the changes of MMP, plays a pivotal role in the initiation of apoptosis and autophagy (44). Once nutrition is deprived, a number of mitochondria spontaneously depolarize and enter the autophagic pathway. Under these conditions, elimination of dysfunctional mitochondria through autophagy could protect the cells from apoptosis. As the numbers of depolarized mitochondria increased in cells, $\Delta\psi$m would change, progressively leading to autophagy, apoptosis, and necrosis (13). In addition, bcl-2 inhibits apoptosis against various toxic stresses through stabilization of mitochondrial permeability transition pore (45). Here, the results of the MMP assay using flowcytometric analysis, morphological observation, and Western blotting showed that the $\Delta\psi$m was significantly lower in the manumycin A applied group than in the oridonin alone treatment group. Therefore, it can be concluded that on the one hand, augmented autophagy induced by inactivation of Ras might be at least linked with the downergulation of $\Delta\psi$m; and on the other hand, downregulation of Bcl-2 might contribute to the release of apoptosis inducer proteins such as cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria to cytoplasm and thus facilitate the subsequent apoptosis-executing signaling events.

Beclin-1, the first identified mammalian autophagy gene product, is a haploinsufficient tumor suppressor that was originally isolated as a Bcl-2-interacting protein (46 – 50). Beclin-1 expression in MCF-7 breast cancer cells increased the number of autophagic vesicles. Furthermore, Bcl-2-mediated inhibition of Beclin-1-induced autophagy is inhibited by either mutations in Bcl-2 that disrupt binding to Beclin-1 or by mutations in Beclin-1 that inhibit binding to Bcl-2 (48). In this study, after the cells were pretreated with manumycin A, the autophagic ratio was markedly increased while the expression of Beclin-1 was decreased. It may be speculated that on the one hand, Bcl-2 interacts with Beclin-1 all the time in A431 cells, which affect the autophagy-inducing effect of Beclin-1; and on the other hand, Beclin-1 has been regarded as a part of the PI3-K complex, which participates in the formation of autophagosome (35), so inactivation of Ras interrupts PI3-K activation, resulting in the blockage of Beclin-1 binding to PI3-K.

It is of note that Bcl-2 interferes with the functions of Beclin-1 during the execution of autophagy in malignant cells and thus contributes to tumorigenesis (43, 51). Here, expression of Bcl-2 was also downregulated by oridonin at 24 h; and this suppressive effect became more significant after manumycin A preincubation, consistent with the downregulation of MPT. At the same time, by treatment of cultured A431 cells with manumycin A alone, the expression level of Bcl-2 did not change compared with the control group. These results indicated that although downregulation of Bcl-2, which inhibits autophagy through disruption of Beclin-1 complex formation, did not facilitate Beclin-1-induced autophagy here, at least its downregulation partially contributed to the decreasing of $\Delta\psi$m. Therefore, Bcl-2 may function as an oncogenic product not only through blocking apoptosis but also through blocking autophagy.

Taken together, inhibition of class I PI3-K by inactivation of Ras markedly augmented oridonin-induced autophagy. However, Beclin-1, which has been reported to promote autophagy, did not participate in this autophagy process in A431 cells. In addition, the anti-apoptotic protein Bcl-2 was downregulated both in the apoptotic process and in the autophagic process, through which mitochondrial membrane potential was downregulated. All these results contribute to the initiation of autophagy of A431 cells. However, further studies must be performed to fully elucidate the relationship between Beclin-1 and Bcl-2 in the autophagy process.

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