Full Paper

Oridonin-Induced A431 Cell Apoptosis Partially Through Blockage of the Ras/Raf/ERK Signal Pathway

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Abstract. We have reported that oridonin, a diterpenoid isolated from the plant Rabdosia rubescens, had apoptosis-inducing activities in many cell lines (e.g., human melanoma A375-S2, human cervical cancer HeLa, human breast adenocarcinoma MCF-7, and murine fibrosarcoma L929). In this study, we further investigated signaling events involved in oridonin-induced apoptosis in human epidermoid carcinoma A431 cells. It was found that the total tyrosine kinase activity was inhibited and the protein expressions of epidermal growth factor receptor (EGFR) and phosphorylated EGFR were decreased in oridonin-induced A431 cell apoptosis. Expression of EGFR downstream effector proteins, Grb2, Ras, Raf-1, and extracellular signal-regulated kinase (ERK), was also downregulated by oridonin. Moreover, the oridonin-induced apoptosis was augmented by the Ras inhibitor manumycin A, Raf-1 inhibitor GW5074, or ERK inhibitor PD98059, suggesting that inactivation of Ras, Raf, or ERK participates in oridonin-induced apoptosis. Taken together, oridonin-induced apoptosis in A431 cells might be through blocking EGFR and its downstream Ras/Raf/ERK signal pathway.

Keywords: oridonin, tyrosine, apoptosis, epidermal growth factor receptor (EGFR)

Introduction

Various natural products have been reported to exert their antitumor effects by induction of cancer cell apoptosis (1, 2). Oridonin (Fig. 1), a diterpenoid isolated from Rabdosia rubescens, has various pharmacological and physiological effects. Our previous studies showed that oridonin possesses a variety of biological effects, including an anti-tumor effect, an anti-bacterial effect, and acts as a scavenger of active oxygen free radicals. It has been used for the treatment of human cancers, especially prostate carcinoma (3). Our previous study showed that oridonin exerts a potent apoptosis-inducing effect on many cell lines through caspases and mitogen-activated protein kinases (MAPKs)-dependent pathways (4). In this study, the epidermal growth factor receptor (EGFR) downstream signaling pathway in oridonin-induced A431 cell apoptosis was further investigated.

In the past decade, there has been rapid growth in the field of developing inhibitors of tyrosine kinases as potential cancer therapeutics (5). Understanding the role that these main signaling enzymes play in the proliferation and invasion of cancer has become important for the identification of new biological targets. Protein tyrosine kinases (PTKs) utilize ATP to phosphorylate specific tyrosine residues within the sequence of effector proteins and thereby mediate the transmission of mitogenic

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Fig. 1. Chemical structure of oridonin.
signals and numerous other cellular events, including cell proliferation, migration, differentiation, metabolism, and immune response (6).

PTKs can be divided into two classes: the transmembrane growth factor receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases. Src is one of the cytoplasmatic TKs and inhibition of its phosphorylation may suppress uncontrolled tumor cell growth. Besides Src, a family of enzymes that has attracted great interest has been the receptor tyrosine kinase, especially the EGFR (7). EGFR plays an important role in physiological processes such as cell proliferation, differentiation, and wound healing (8 – 11). The binding of EGF induces EGFR dimerization and autophosphorylation on tyrosine residues. Therefore, selective inhibition of receptor tyrosine kinase activity plays an important role in the field of cancer treatment. In this study, we used human epidermoid carcinoma A431 cells, which overexpress EGFR, as the source of tyrosine kinase.

It is clear that the binding of EGF to the extracellular domain of EGFR induces an increase in tyrosine kinase activity as well as the consequent phosphorylation and activation of its signal transduction components. The adaptor protein: growth factor receptor-bound protein 2 (Grb2), which links phosphoryrosines of RTKs to their downstream signaling pathway (12), plays an important role in coupling signals with small G-protein Ras downstream signaling pathway (12), plays an important role as well as the consequent phosphorylation and activation of its signal transduction components. The adaptor protein: growth factor receptor-bound protein 2 (Grb2), which links phosphoryrosines of RTKs to their downstream signaling pathway (12), plays an important role in coupling signals with small G-protein Ras through binding to proline-rich motifs on the guanine nucleotide releasing factor son-of-sevenless (Sos) (13, 14). Activated Ras enables it to bind to Raf-1 and recruit it from the cytosol to the cell membrane, where Raf-1 activation takes place. Activated Raf-1 phosphorylates and activates MEK [MAPK/ERK (extracellular signal-regulated kinase) kinase], which in turn phosphorylates and activates ERK. The resulting MAPK activation induces gene (ATF-2, Chop, c-jun, c-Myc, DPC-4, Elk-1, p53) transcription and cell proliferation (15, 16).

In the present study, we demonstrate that the EGFR tyrosine kinase pathway participates in oridonin-induced apoptosis. Among the EGFR downstream targets are Grb2, Ras, Raf, and ERK. Inhibitors of Ras, Raf, and ERK enhance oridonin-induced apoptosis in A431 cells. Therefore, the inhibition of its total tyrosine kinase activities or blockage of EGFR tyrosine phosphorylation is tightly involved in oridonin-induced apoptosis.

Materials and Methods

Chemicals and other materials

Oridonin was obtained from the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). The structure of oridonin was assigned by comparing the chemical and spectral data (1H-, 13C-NMR) with those reported in the literature (17). The purity of the oridonin was measured by HPLC and determined to be 99.4%. 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, propidium iodide (PI), and acridine orange (AO) were purchased from Sigma Chemical (St. Louis, MO, USA). Polyclonal antibodies against EGFR, phospho-EGFR, Grb2, Raf-1, Hsp90, ERK1/2, phospho-ERK1/2, and horseradish-peroxidase-conjugated secondary antibodies (goat-anti-rabbit, goat-anti-mouse, and bovine-anti-goat) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The tyrosine kinase activity assay kit was obtained from Chemicon International (Temecula, CA, USA). Human EGF was from Pepro Tech (Rocky Hill, NJ, USA).

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 fetal bovine serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China) containing 100 µg/ml streptomycin, 100 U/ml penicillin, and 0.03% L-glutamine and maintained at 37°C with 5% CO2 in a humidified atmosphere. The cells were preincubated for 1 h with specific inhibitors before being stimulated with oridonin. Manumycin A (Ras inhibitor), GW5074 (Raf-1 kinase inhibitor), and PD98059 (ERK inhibitor) were obtained from Calbiochem (La Jolla, CA, USA). The effective doses of manumycin A, GW5074, and PD98059 (18) were determined by preliminary studies and found to be comparable to those used in other studies.

DNA fragmentation assay

A431 cells (1 × 106) 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 mM Tris-HCl (pH 7.4), 10 mM 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, 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**

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) (19). The LDH released in the culture supernatant [designated as extracellular LDH (LDHe)] was used as an index of necrotic death, and the LDH present in the adherent viable cells was designated 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**

Apoptotic morphology was studied by staining the cells with the fluorescence, DNA-binding dye AO. Cells were harvested and washed three times with PBS after being incubated with 20 µM oridonin in the presence or absence of 10 µM GW5074 for 24 h, and then they were stained with 20 µg/ml AO (Sigma) for 15 min. After staining, the color and structure of the different cell types were observed under a fluorescence microscope (Olympus, Tokyo) (20).

**Observation of morphological changes**

A431 cells in Ham’s F-12 containing 10% FBS were seeded into 6-well culture plates and cultured for 24 h. Control cultures were treated with the vehicle (0.05% DMSO). The cells were preincubated with 10 µM PD98059 for 60 min, and then oridonin (20 µM) was added to the cell culture. After 24 h, the cellular morphology was observed by phase contrast microscopy (Leica, Wetzlar, Germany).

**Tyrosine kinase activity assay**

A431 cells were treated with lysis buffer [RIPA buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml phenylmethyl-sulfonylfluoride (PMSF), 1 µg/ml aprotinin, 2 µg/ml leupeptin, and 100 µM sodium vanadate] on ice for 10 min. The cell lysate was collected and centrifuged at 12,000 × g for 10 min. The supernatant (PTK) was first mixed with 5 × assay buffer (20 mM HEPES, 10 mM MgCl₂, 3 mM MnCl₂, 0.1 mM Na₂VO₃, and 1 mM DTT) and the peptide substrate (10 µg/ml) and then 10 µl of 5 × ATP/MgCl₂ solution was added. The reaction mixture was then incubated for 60 min at 30°C. Then the enzyme reaction was terminated by adding 10 µl of kinase inhibitor (120 mM EDTA). The reaction mixture was transferred to Streptavidin-coated wells and incubated at 37°C for 30 min. After the Streptavidin-coated wells were washed with wash buffer, blocking buffer solution was added. Diluted mouse anti-PY20-HRP (100 µl) was added to each reaction well and incubated for 1 h at room temperature on a shaking platform. The wells were then washed again with the wash buffer. After incubation with TMB substrate (100 µl) at room temperature for 15 min, stop solution (100 µl) was added into each well to stop the enzyme reaction. Then the absorbance of each microwell was read at 450 nm with an ELISA reader (TECAN SPECTRA, Wetzlar, Germany).

**Flow cytometric analysis (21)**

A431 cells (1 × 10⁶) were harvested and washed once in cold PBS. Cell pellets were fixed in 70% ethanol and washed in cold PBS. Then the pellets were suspended in 1 ml of PI solution containing 50 µg/ml of PI, 1 g/l RNase A, and 0.1% (w/v) Triton X-100 in 3.8 mM sodium citrate, followed by incubation on ice in the dark for 30 min. Samples were analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**Preparation for cytosolic extracts**

Lysates were prepared to examine the expression of EGFR, Grb2, Ras, Raf-1, Hsp90, and ERK. A431 cells were washed twice with PBS and then lysed in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton-X 100, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, and 1 mM PMSF (22). 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 was stored at ~80°C until analysis.

**Western blot analysis**

A431 cells were treated with various concentrations of oridonin or 20 µM oridonin for different time periods. Both adherent and floating cells were collected, and then Western blot analysis was carried out as previously
described with some modifications. Equal amounts of total protein were separated by 7.5% or 12% SDS polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (23). Proteins were detected using polyclonal antibody and visualized using anti-rabbit, anti-mouse, or anti-goat 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**

**Oridonin-induced apoptotic cell death in A431 cells**

Oridonin induced A431 cell death in a time- and dose-dependent manner (data not shown). To determine the features of A431 cell death induced by oridonin, flowcytometric analysis was performed (Fig. 2A). In the presence of 10 and 20 µM oridonin, the apoptotic ratio (the percentage of cells in sub-G₀/G₁ phase, a hallmark of apoptosis) increased 14.83% and 21.08% at 24 h, respectively, from the base line of the control (3.52%). To further characterize oridonin-induced A431 cell death, the ratio of LDH release from viable cells, floating dead cells, and the culture medium were compared (Fig. 2B). The ratio of apoptotic cells increased from 3.52% at 5 µM to 23.52% at 20 µM oridonin, but that of necrotic cells was still negligible in the presence of 20 µM. Consistent with the above results, activation of caspase-3, another hallmark of apoptosis, was also required for oridonin-induced apoptosis (Fig. 2C). The major cause of oridonin-induced A431 cell death was apoptosis.

**Fig. 2.** Oridonin-induced A431 cell death was characterized mainly by apoptosis. A: Flow cytometric analysis of oridonin-treated A431 cells. After treatment with different doses of oridonin for 24 h, the cells were washed by PBS and stained with PI followed by flowcytometric analysis. Arrows indicate cells at the sub-G₀/G₁ phase. The ratios of apoptosis are shown above the arrows. B: Cells treated with various doses of oridonin (5, 10, 20, 25, 30, or 40 µM) for 24 h were measured by LDH activity-based assay (diamond: apoptosis, square: necrosis). Data are each the mean ± S.D. (bars) from 3 independent experiments. C: A431 cells were incubated with 20 µM oridonin for different time periods. Cell lysates were separated by 12% SDS-PAGE, and procaspase-3 activated-caspase-3 protein expressions were detected by Western blot analysis. Con: medium, Ori: oridonin.
In addition, morphologic changes were observed by phase contrast microscopy (Fig. 9B-b) or by fluorescence microscopy of AO stained cells (Fig. 8B-b). A DNA ladder (a hallmark of apoptosis) was also found in the oridonin-treated A431 cells (Fig. 10, lane B). These results also demonstrated that oridonin caused apoptosis in A431 cells.

**Effect of oridonin and genistein on the total tyrosine kinase activities**

The total tyrosine kinase activities of the cellular proteins were measured by the tyrosine kinase activity assay kit with 20 μM oridoinin or genistein [a natural isoflavone product that has been shown to inhibit the tyrosine kinase activity and induce apoptotic cell death (24) in A431 cells]. Although the cells were pretreated with 15 nM EGF for 20 min, which led to a significant increase in the total tyrosine kinase activities as compared with the control, oridonin still exerted more significant inhibitory effects on the total tyrosine kinase activities than genistein (Fig. 3).

**EGF-stimulated EGFR tyrosine phosphorylation was inhibited by oridonin and genistein**

In order to determine whether oridonin or genistein might inhibit the EGF-augmented tyrosine phosphorylation, A431 cells were treated with EGF alone or in combination with 20 μM oridonin or genistein (Fig. 4). Incubation of the cells with 15 nM EGF for 20 min increased tyrosine phosphorylation of EGFR, as compared with the control (Fig. 4, lane 2). Consistent with the results of Fig. 3, oridonin at 20 μM greatly reduced EGFR or phosphorylated EGFR protein level (Fig. 4: lanes 5 and 6), but genistein at 20 μM slightly decreased the protein level of EGFR or phosphorylated EGFR at 24 h (Fig. 4: lanes 3 and 4).

**The effect of oridonin on the protein levels of Grb2 and Ras**

To ascertain whether the downregulated protein level of phosphorylated EGFR affects the recruitment of Grb2 in oridonin-induced apoptosis, A431 cells were treated with 20 μM oridonin for different time periods (Fig. 5A). It was found that the protein level of Grb2 was decreased with culturing time, suggesting that downregulation of Grb2 might be correlated with oridonin-induced inhibition of phosphorylated EGFR, which affected the binding of Grb2 to phosphorylated EGFR.

Since Grb2 is the key adaptor molecule that couples...
EGFR to the Ras/MAPK cascade (25), the protein level of Ras was detected by Western blot analysis (Fig. 5B). Oridonin time-dependently downregulated the expression of Ras, suggesting that downregulation of Grb2 by oridonin might affect membrane translocation of Ras, which was associated with Ras protein expression.

**Downregulation of Raf-1 and ERK in oridonin-treated A431 cells**

Once Ras is activated, subsequently, its downstream protein Raf-1 is activated and couples Ras to the MAPK cascade. Therefore, the activation of EGFR downstream signal molecules partly required the positive regulation by Raf-1 (26, 27). Western blot analysis showed that Raf-1 expression was also downregulated by oridonin administration (Fig. 6A).

The molecular chaperone Hsp90 is critical for maintaining normal cellular homeostasis, which functions in a selected range of key oncogene proteins, including ErbB2, Raf-1, Akt/PKB, and others (28). In order to investigate whether Hsp90 was involved in the downregulation of Raf-1, the expression of Hsp90 was also analyzed (Fig. 6B). Oridonin had no dose-and time-dependent effect on the expression of Hsp90, further indicating that downregulation of Raf-1 was not affected by Hsp90, but was associated with oridonin-induced growth inhibition.

A major substrate of activated Raf is MEK. Upon activation by Raf, MEK can in turn phosphorylate and activate ERK. Therefore, ERK activation is required for cellular transformation induced by oncogenic Ras and Raf (29). We found that after oridonin treatment, the

![Fig. 5. Effect of oridonin on Grb2 and Ras. The cells were incubated with 20 \( \mu \)M oridonin for different time periods (12, 24, 36, or 48 h). Expressions of Grb2 (A) and Ras (B) were detected by Western blot analysis. Con: medium. Triplicate experiments gave similar results. n = 3, mean ± S.D.

![Fig. 6. Effect of oridonin on Raf-1 and ERK. The cells were incubated with 20 \( \mu \)M oridonin for different time periods (12, 24, 36, or 48 h) or with various doses of oridonin (5, 20, 40, or 60 \( \mu \)M) for 24 h. Expressions of Raf-1 (A), Hsp90 (B), and ERK and phosphorylated ERK (C) were detected by immunoblotting.](image-url)
expression of phosphorylated ERK was decreased in a
time-dependent manner, while ERK expression did not
change, suggesting that oridonin-induced downregu-
lation of Raf-1 might block ERK-activation in A431 cells
(Fig. 6C).

The inhibitors of Ras, Raf, and ERK enhanced oridonin-
induced apoptosis in A431 cells

As the above results suggested, oridonin-induced
A431 cell apoptosis resulted in the inhibition of the total
tyrosine kinase activity and blocking EGFR phosphor-
ylation; and this apoptosis-inducing effect blocks the
expression of EGFR downstream proteins, including
Ras, Raf, and ERK. Therefore, one of the RTKs
pathways, such as the Ras/Raf/ERK pathway, might be
required in oridonin-induced A431 cell apoptosis.

The Ras inhibitor manumycin A at 10 µM enhanced
oridonin-induced A431 cell apoptosis. The ratio of
apoptotic cells was increased from 18.01% to 23.36% as
assessed by the LDH assay. This observation indicated

![Fig. 7. Effect of Ras on oridonin-treated A431 cells. The cells
were incubated with or without 10 µM Ras inhibitor (manumycin A:
mA) for 60 min before 20 µM oridonin was added. After 24 h, the
apoptotic ratio was measured by LDH. **P<0.01.]

![Fig. 8. The function of Raf-1 in oridonin-treated A431 cells. A: The
cells were incubated with 20 µM with or without 10 nM
Raf-1 inhibitor (GW5074) pretreatment; then at 24 h, the LDH activity was measured. B: Morphologic changes of A431 cells.
The cells were treated as above, and then the cells were stained with AO (20 µg/mL) and examined by fluorescence microscopy.
a: control, b: 20 µM oridonin, c: GW + oridonin. Arrows indicate fragmented nuclei (×200 magnification). Control: medium.
Triplicate experiments gave similar results. GW: Raf-1 inhibitor. n = 3, mean ± S.D. Significant difference between vehicle-
treated cells and oridonin-treated cells: **P<0.01. Bar = 20 µm.]

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the involvement of Ras protein inactivation in the apoptotic action of oridonin (Fig. 7), which was consistent with the result of Fig. 5B.

We next investigated the role of Raf-1 in oridonin-induced apoptosis by employing the well-known Raf-1 inhibitor GW5074 (Fig. 8A). Consistent with the result of the application of manumycin A, the number of apoptotic cell was increased when A431 cells were pretreated with 10 nM GW5074 prior to oridonin treatment. In addition, morphologic changes were observed by using AO staining (Fig. 8B). Control cells exhibited uniformly green fluorescence (Fig. 8B-a), whereas oridonin-induced apoptotic cells with condensed, fragmented chromatin were stained orange (Fig. 8B-b). Notably, pretreatment with GW5074 also reinforced the apoptotic alternation compared with the group treated by oridonin alone (Fig. 8B-c). Therefore, oridonin-induced A431 cell apoptosis might be partially through inducing inactivation of Raf-1 protein.

Next we further analyzed the effect of the ERK inhibitor PD98059 on the growth inhibition of oridonin-treated A431 cells (Fig. 9). As shown in Fig. 9A, the apoptotic ratio was also increased by pretreatment with 10 µM PD98059. Simultaneously, apoptotic bodies appeared (Fig. 9B-b), and pretreatment with 10 µM PD98059 induced more significant apoptosis compared with stimulating with 20 µM oridonin alone (Fig. 9B-c). This suggested that ERK-activation mediated A431 cell survival and its inactivation was involved in oridonin-induced growth inhibition.

In addition, DNA ladder formation, another hallmark of typical apoptosis, was also analyzed. Pretreatment with 10 nM GW5074, 10 µM manumycin A, or 10 µM PD98059 augmented oridonin-induced DNA fragmentation of A431 cells (Fig. 10). Inhibition of Ras, Raf-1, and ERK activity was required for oridonin-induced apoptosis in A431 cells.

![Fig. 9.](image)

**Fig. 9.** The protective effects of ERK on oridonin-stimulated A431 cells. A: The cells were incubated with 20 µM oridonin alone or 10 nM ERK inhibitor (PD98059) and oridonin for 24 h, and then the apoptotic ratio was measured by LDH. B: Morphologic changes of A431 cells were examined by phase contrast microscopy at 24 h in the absence (a) or presence (b) of 20 µM oridonin or the presence of both oridonin and PD98059 (c) (×200 magnification). Arrows indicate an apoptotic A431 cell displaying apoptotic bodies and membrane blebbing. Con: medium, PD: PD98059. n = 3, mean ± S.D. Significant difference between cells treated with vehicle and oridonin-treated cells: **P<0.01. Bar = 15 µm.
Discussion

The activation of PTKs, followed by the reversible phosphorylation of tyrosyl residues of cellular proteins, accounts for the control of many fundamental cellular events including proliferation, migration, morphogenesis, cytoskeletal changes, and gene expression (30). PTK inhibitors are a new class of pharmacological agents, which are currently utilized in clinical trials against various types of cancers.

In this study, we demonstrated that oridonin significantly inhibited the total tyrosine kinase activities and downregulated the expression of EGFR or phosphorylated EGFR when it induced A431 cell apoptosis. Moreover, both effects exerted by oridonin were more significant than those of genistein. Simultaneously, the blockage of EGFR tyrosine phosphorylation led to the downregulation of its downstream effector proteins, including Grb2, Ras, Raf, and ERK. It was further confirmed by inhibition of Ras, Raf-1, or ERK, respectively, using manumycin A, GW5074, or PD98059, which each significantly enhanced oridonin-induced apoptosis. These observations indicate that oridonin-induced blockage of EGFR tyrosine phosphorylation is tightly linked to the oridonin-mediated inhibitory effect on the total tyrosine kinase activity.

EGFR is a member of the ErbB-receptor family, which plays an important role in the proliferation, survival, migration, and differentiation of targeted cells after stimulation by many growth factors. This might be due to mediating various types of protein phosphorylations or dephosphorylations (31, 32). It was reported that the binding of EGF to the extracellular domain of EGFR induces an increase in tyrosine kinase activity as well as the consequent phosphorylation and activation of its signal transduction components (33). In our study, significant downregulation of phosphorylated EGFR occurred after treatment of A431 cells with 20 µM oridonin, although EGFR tyrosine phosphorylation was augmented after EGF-stimulation. These results indicate that oridonin-induced blockage of EGFR tyrosine phosphorylation is tightly linked to the oridonin-mediated inhibitory effect on the total tyrosine kinase activity.

Tyrosine autophosphorylation sites on EGFR provide a mechanism for the recognition and assembly of signaling complexes, functioning as binding sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains of a variety of signaling proteins (12). Therefore, activated EGFR become a platform for the recognition and recruitment of a specific complement of signaling proteins. One such signaling protein is Grb2, which is a cytosolic adaptor and contains a central SH2 domain flanked by two Src homology 3 (SH3) domains that allow it to constitutively associate with the prolinerich regions of the nucleotide exchange factor Sos (34). In this study, oridonin downregulated the expression of Grb2 in a time-dependent manner, suggesting that oridonin-induced downregulation of EGFR phosphorylation disrupted the recruitment of its downstream protein Grb2.

Once EGFR is activated by tyrosine phosphorylation, Grb2 links RTKs to the downstream signaling pathway. Therefore, Grb2 is essential for the Ras/Raf/ERK pathway (35). Binding of the two SH3 domains of Grb2 to Sos homologue protein results in the activation of Ras. Ras family members are anchored to the cytoplasmic face of the plasma membrane by carboxyl-terminal farnesylation. This localization to the inner leaflet brings Ras into close proximity with the Grb2-Sos complex, stimulating the exchange of guanosine diphosphate (GDP) bound to Ras with guanosine triphosphate (GTP) from the cytosol. This exchange activates Ras conformationally, allowing it to interact with a number of downstream effectors and thereby stimulating cell growth (27, 36, 37).

It was reported that Ras does not function unless it is attached to the inside of the cell membrane and inhibition of Ras activity might be related to the downregulation of Ras protein levels (29, 38). In the present study, it was found that the expression of Ras protein was decreased in oridonin-treated A431 cells. It might be hypothesized that oridonin-induced downregulation of...
EGFR phosphorylation disrupted phosphorylated EGFR interaction with Grb2, subsequently inhibiting Ras activation. Inactivation of Ras leads to inhibition of human carcinoma cell proliferation by blocking the Ras signal transduction pathway and reduced the expression levels of functional Ras (29). Manumycin A, an antibiotic, can inhibit Ras activity by preventing farnesylation and membrane translocation of Ras. In this study, manumycin A significantly enhanced oridonin-induced apoptosis in A431 cells, further indicating that Ras protein inactivation might be involved in the apoptotic action of oridonin.

Raf-1 is a cytoplasmic serine/threonine protein kinase that plays an important role in the transmission of signals leading to transcriptional activation and mitogenesis (39, 40). Protection from apoptosis is the physiologically relevant function of Raf-1 (41). Therefore, downregulation of Raf-1 protein expression might be due to oridonin-induced apoptosis. Inhibition of Raf-1 activity significantly increased oridonin-induced A431 cell apoptosis, indicating that oridonin-induced apoptosis is to some extent exerted through inhibition of Raf activity.

A number of groups demonstrated that the recruitment of Raf-1 by activated Ras implicated Raf-1 as the link between Ras and ERK (41). Activated ERK phosphorylates a number of cytoplasmic and nuclear targets, including transcription factors that mediate growth factor-regulated gene expression. Therefore, activation of ERK, which is a key component in the transduction of one of the signals leading to growth and transformation in many cell types, is mainly caused by Raf-1 (29, 42). Consistent with the downregulation of Raf-1, ERK phosphorylation was blocked in response to oridonin, suggesting that oridonin-induced decreased expression of Raf-1 might result in downregulation of phosphorylated ERK. Pretreatment of A431 cells with the ERK inhibitor PD98059 facilitated oridonin-induced apoptosis. Since it has been reported that downregulation of phosphorylated ERK protein expression level is consistent with inhibition of ERK activation (37), blockade of the function of ERK might be associated with oridonin-induced apoptosis.

Taken together, our present results suggest that oridonin-induced apoptosis is related to the inhibitory effect on the total tyrosine kinase activity and the blockage of EGFR phosphorylation in A431 cells. Moreover, inactivation of some proliferation-related signal proteins downstream of EGFR, including Ras, Raf-1, and ERK, is likely due to, at least in part, the apoptosis exerted by oridonin in A431 cells.

References

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