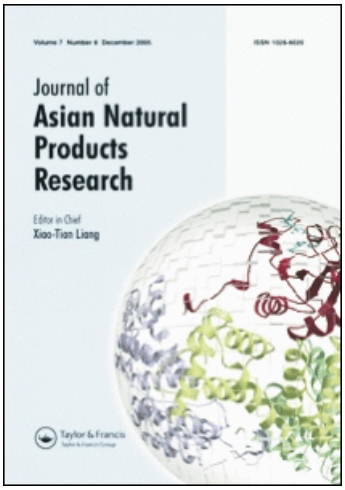

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Wang, Hong-Jun , Li, Dan , Yang, Fan-Yi , Tashiro, Shin-ichi , Onodera, Satoshi and Ikejima, Takashi(2008) 'Oridonin induces human melanoma A375-S2 cell death partially through inhibiting insulin-like growth factor 1 receptor signaling', Journal of Asian Natural Products Research, 10: 8, 777 — 788
To link to this Article: DOI: 10.1080/10286020802030918
URL: http://dx.doi.org/10.1080/10286020802030918

Journal of Asian Natural Products Research
Vol. 10, No. 8, August 2008, 777–788

Oridonin induces human melanoma A375-S2 cell death partially through inhibiting insulin-like growth factor 1 receptor signaling

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(*Received 29 July 2007; final version received 20 February 2008*)

Our previous studies indicated that oridonin, a diterpenoid isolated from *Rabdosia rubescens*, induced human melanoma A375-S2 cell apoptosis. In this study, we investigated whether the proapoptotic effect of oridonin on A375-S2 cells would depend on an interference with function of the insulin-like growth factor 1 (IGF-1) receptor, a plasma membrane receptor critical for the survival or antiapoptotic ability in melanoma cells. We found that IGF-1 receptor (IGF-1R) signaling was a potential survival pathway against a low concentration of 20 μmol/L oridonin-induced apoptosis in A375-S2 cells. The activation of Ras or its downstream effector p38 mitogen-activated protein kinase (p38 MAPK) was shown to be necessary for IGF-1-mediated protection, but the activation of phosphatidylinositol-3-OH kinase (PI3 kinase) or extracellular signal-regulated kinase (ERK) did not correlate with the regulation of survival. However, in the presence of 40 μmol/L (IC_{50} at 24 h) oridonin, A375-S2 cells could not be protected by IGF-1 from apoptosis, accompanied by a severe impairment of IGF-1R expression. Therefore, we concluded that the proapoptotic activity of oridonin was partially attributed to its repression of IGF-1R signaling. In addition, p53 was supposed to be a pivotal transducer of proapoptotic and survival signaling pathway in this system.

Keywords: oridonin; IGF-1R; IGF-1; A375-S2 cells

1. Introduction

Apoptosis, a form of altruistic suicide, is generally defined cytologically by violent membrane blebbing, nuclear condensation, endocytosis, and DNA degradation into oligonucleosome-sized fragments. It has become clear that apoptosis is the predominant mechanism by which cancer chemotherapeutic agents kill cells [1,2].

Oridonin (**1**) (Figure 1), an *ent*-kaurane diterpenoid isolated from *Rabdosia rubescens*, has various pharmacological and physiological effects such as autophagy induction, phagocytosis stimulation, and antitumor effects [3–4], and has been used for the treatment of human cancers [5]. Our previous studies showed that oridonin could induce human malignant melanoma A375-S2 cell apoptosis at different concentrations ranging from 8.56 to 68.7 μmol/L, via bax-regulation, caspase pathway activation, and the cytochrome *c*/caspase-9 apoptosome formation [6–8]. However, apoptosis is a closely regulated process and much less is known about the signaling mechanisms used by survival factors that inhibit the commitment to apoptosis.

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020802030918
http://www.informaworld.com

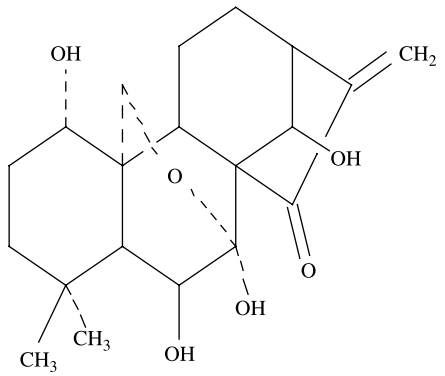

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Figure 1. Structure of oridonin.

Insulin-like growth factor 1 (IGF-1), a major survival factor in serum, was able to protect cells from apoptosis under a wide variety of circumstances [9–12]. IGF-1 elicits its responses through interaction with and activation of the IGF-1 receptor (IGF-1R). As is the case with other receptor tyrosine kinases, IGF-1 receptor is autophosphorylated in response to the ligand that activates its kinase activity. Receptor activation, in turn, results in the phosphorylation of insulin receptor substrate 1 (IRS-1) and the activation of cascades of kinases which include phosphatidylinositol-3-OH kinase (PI3 kinase) and mitogen-activated protein kinases (MAPKs). IGF-1R is expressed by all melanoma cells, and the expression increases with progression [13]. The down-regulation of IGF-1R with antisense oligonucleotides inhibits melanoma growth *in vivo* [14], suggesting that IGF-1 is one of the major factors in melanoma development and progression.

The goal of the work described here is to confirm whether the IGF-1R signaling is a potential survival or an antiapoptotic pathway in the process of oridonin-induced A375-S2 cell death, and whether the proapoptotic effect of oridonin depends on the interference with the expression or function of the IGF-1R or its downstream kinases.

2. Results and discussion

2.1. IGF-1 protected A375-S2 cells from a low concentration oridonin-induced growth inhibition

In the presence of IGF-1 or IGF-1R inhibitor tyrphostin AG1024, A375-S2 cells were incubated with 20 or 40 μmol/L oridonin for 24 h to examine whether the activity of IGF-1R affected oridonin-induced growth inhibition. The inhibitory ratio was assayed by MTT method. 12.7 nmol/L IGF-1 significantly attenuated 20 μmol/L oridonin-induced growth inhibition with a decrease by as much as 46% (Figure 2A). In contrast, IGF-1 did not induce protection in cells treated with 40 μmol/L (IC_{50} at 24 h) oridonin. IGF-1R inhibitor tyrphostin AG1024 remarkably enhanced 20 μmol/L oridonin-induced growth inhibition by 85%, but did not affect 40 μmol/L oridonin-induced growth inhibition. This result suggested that A375-S2 cell survival was dependent on the activity of IGF-1R.

DNA–agarose gel electrophoresis analysis was performed to determine the effect of IGF-1 on oridonin-induced apoptosis in A375-S2 cells. Small fragments appeared in preparations of DNA from A375-S2 cells 24 h after oridonin treatment. Apoptosis induced by 20 μmol/L, but not 40 μmol/L, oridonin was inhibited by 12.7 nmol/L IGF-1 (Figure 2B).

2.2. The expressions of the IGF-1R and its precursor proteins were impaired by oridonin in a dose-dependent manner

Marked expressions of IGF-1R precursor and IGF-1R proteins were detected in A375-S2 cells by Western blot analysis, and the expressions were severely impaired by 40 μmol/L oridonin and only slightly reduced by 20 μmol/L oridonin after treatment for 24 h. In addition, down-regulated expressions of IGF-1R and its precursor induced by 20 μmol/L, but not 40 μmol/L, oridonin were reversed by pretreatment with IGF-1 (Figure 3). Since the antiapoptotic signaling by IGF-1R depends on the receptor kinase activity, we examined whether oridonin could modulate the

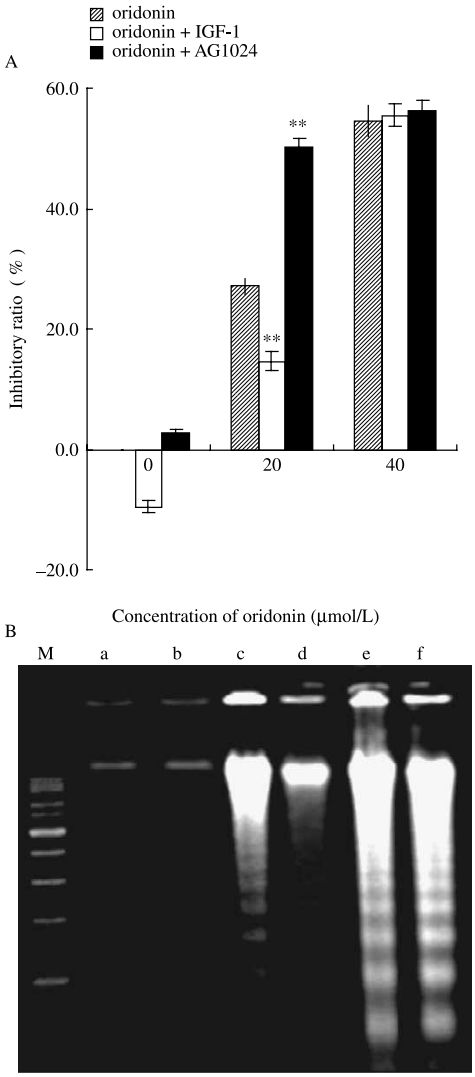

Figure 2. IGF-1 protected A375-S2 cells from 20 μmol/L, but not 40 μmol/L, oridonin-induced apoptosis. (A) After pretreatment with 12.7 nmol/L IGF-1 for 0.5 h or IGF-1R inhibitor tyrphostin AG1024 for 1 h, the cells were co-incubated with 20 or 40 μmol/L oridonin for another 24 h. The inhibitory ratio was assayed by MTT method. $n = 3$, mean ± SD, $^{**}p < 0.01$ *vs.* oridonin alone group. (B) The cells were treated with 12.7 nmol/L IGF-1 for 0.5 h prior to co-incubation with 20 or 40 μmol/L oridonin for 24 h. DNA degradation was examined by 2% agarose gel electrophoresis. Markers (lane M), medium (lane a), IGF-1 (lane b), 20 μmol/L oridonin (lane c), 20 μmol/L oridonin and IGF-1 (lane d), 40 μmol/L oridonin (lane e), and 40 μmol/L oridonin and IGF-1 (lane f). Triplicate experiments gave similar results.

phosphorylation of IGF-1R induced by IGF-1. The level of phosphorylated IGF-1R was up-regulated by IGF-1, and this up-regulation was almost totally blocked by 40 μmol/L oridonin and significantly reduced by 20 μmol/L oridonin. These results suggested that at least a part of the proapoptotic activity of oridonin was due to its repression of IGF-1R expression or activation.

2.3. Survival signaling pathways downstream of the IGF-1 receptor

Ras and PI3 kinase are important components of signal transduction by insulin family receptors. Hence, we considered the possible role of Ras or PI3 kinase in IGF-1-mediated protection from oridonin-induced growth inhibition in A375-S2 cells. For subsequent experiments, we chose to use the optimal concentration of 12.7 nmol/L IGF-1, so as to best detect the effects of agents that might enhance or inhibit the protective action of IGF-1.

In the presence of 10 μmol/L Ras inhibitor manumycin A, 20 or 40 μmol/L oridonin-induced growth inhibition was enhanced by 66 and 37% ($p < 0.01$), respectively (Figure 4A). IGF-1 reduced 20 μmol/L oridonin-induced growth inhibition by 27% ($p < 0.01$), while there was no significant change between 20 μmol/L oridonin + manumycin A and 20 μmol/L oridonin + manumycin A + IGF-1 groups ($p > 0.05$), suggesting that manumycin A completely blocked the protective effect of IGF-1 on 20 μmol/L oridonin-induced growth inhibition. By contrast, IGF-1 did not affect 40 μmol/L oridonin-induced growth inhibition (Figure 4A). These results indicated that the basal activity of Ras was necessary for cell survival, and that IGF-1 transmitted a cell survival signal through a Ras-dependent pathway.

As wortmannin, a specific inhibitor of the activation of PI3 kinase, had no effect on IGF-1-mediated protection (Figure 4B), the activation of PI3 kinase was not necessary in this process. Thus, the activation of Ras but not

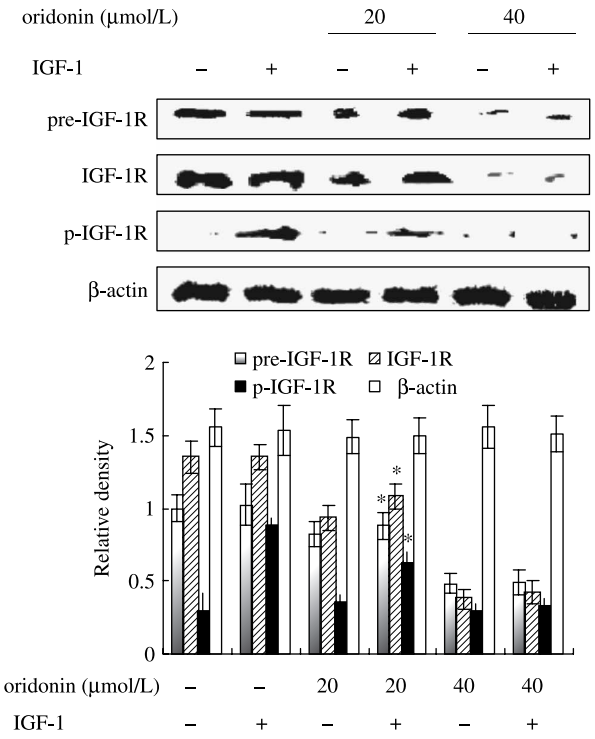

Figure 3. The effect of IGF-1 or different concentrations of oridonin on the protein levels of IGF-1R, IGF-1R precursor, and phosphorylated IGF-1R. The cells (3.0×10^6) were pretreated with 12.7 nmol/L IGF-1 for 0.5 h, followed by co-incubation with 20 or 40 μmol/L oridonin for 24 h. Cell lysates were separated by 8% SDS-PAGE and the protein levels of IGF-1R, IGF-1R precursor, and phosphorylated IGF-1R were detected by Western blot analysis. Three repeated experiments gave similar results. The density of protein bands was detected using the Bandscan 5.0 software (Glyko, Novato, CA, USA). *Significant difference vs. 20 μmol/L oridonin alone group.

PI3 kinase was required for IGF-1-mediated protection in A375-S2 cells.

2.4. Activation of p38 MAPK was necessary for protection

The MAPK superfamily has been postulated to be important in regulating apoptosis [15]. In a broad sense, the MAPK signaling pathways consist of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of the terminal kinases, p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK).

Since, the antiapoptotic signaling of IGF-1R was blocked by manumycin A, we examined whether ERK, the putative downstream target of Ras, was necessary to antagonize the oridonin-induced growth inhibition in A375-S2 cells. However, the results showed that the ERK phosphorylation inhibitor PD98059 had no detectable effect in this system (Figure 5A).

Both p38 inhibitor SB203580 and JNK inhibitor SP600125 remarkably enhanced the oridonin-induced growth inhibition (Figure 5B and C). SB203580 completely abolished IGF-1-mediated protection from 20 μmol/L oridonin-induced growth inhibition, since there was no significant difference between oridonin + SB203580 and oridonin + SB203580 + IGF-1 groups ($p > 0.05$) (Figure 5B). Comparing 20 μmol/L oridonin + IGF-1-treated cells (9% decrease in the inhibitory ratio

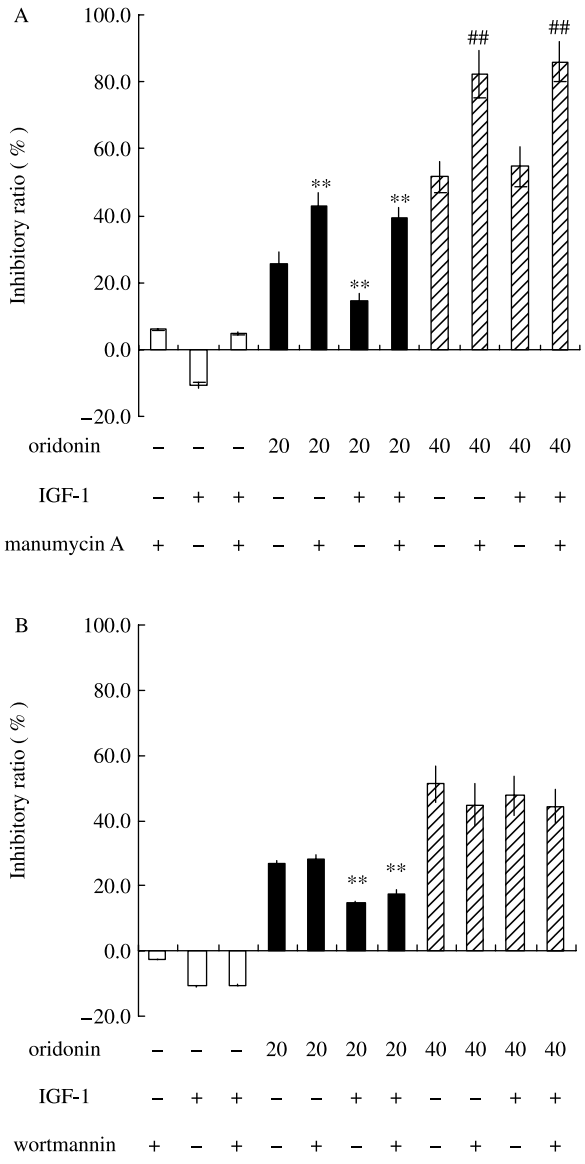

Figure 4. The activation of Ras but not PI3 kinase was necessary for IGF-1-mediated protection. After pretreatment with 10 μmol/L Ras inhibitor manumycin A (A) or 200 nmol/L PI3 kinase inhibitor wortmannin (B) for 1 h, the A375-S2 cells were then incubated in the presence of 12.7 nmol/L IGF-1 for 0.5 h, followed by treatment with 20 or 40 μmol/L oridonin for 24 h. The inhibitory ratio was measured by MTT method. $n = 3$, mean ± SD, $^{**}p < 0.01$ *vs.* 20 μmol/L oridonin alone group, $^{\#\#}p < 0.01$ *vs.* 40 μmol/L oridonin alone group.

over the 20 μmol/L oridonin-treated cells, $p < 0.01$) with 20 μmol/L oridonin + IGF-1 + SP600125-treated cells (11% decrease in the inhibitory ratio over the 20 μmol/L oridonin + SP600125-treated cells, $p < 0.01$), we speculated that JNK had no direct relation with the activity of IGF-1 receptor pathway in this experimental model (Figure 5C). The results showed that the basal activities of both p38 and JNK were necessary for A375-S2

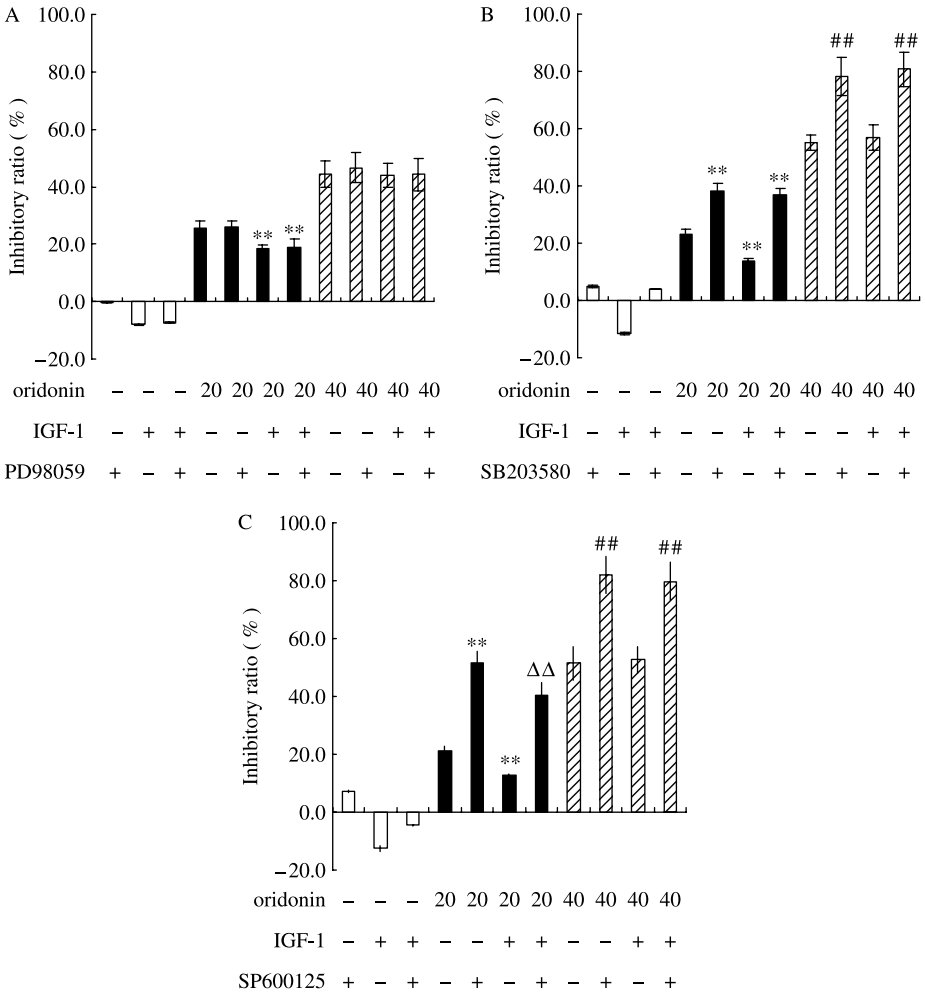

Figure 5. The effects of MAPK inhibitors on IGF-1-mediated protection. After pretreatment with 20 μmol/L ERK inhibitor PD98059 (A) or 20 μmol/L p38 MAPK inhibitor SB203580 (B) or 40 μmol/L JNK inhibitor SP600125 (C) for 1 h, the A375-S2 cells were then treated with 12.7 nmol/L IGF-1 for 0.5 h, followed by co-incubation with 20 or 40 μmol/L oridonin for 24 h. The inhibitory ratio was measured by MTT method. $n = 3$, mean ± SD. $^{**}p < 0.01$ *vs*. 20 μmol/L oridonin alone group, $^{\#\#}p < 0.01$ *vs*. 40 μmol/L oridonin alone group, $^{\Delta\Delta}p < 0.01$ *vs*. oridonin plus inhibitor group.

cell survival, while only p38 delivered a cell survival signal of IGF-1.

To further confirm the involvement of MAPK in IGF-1 receptor signaling, Western blot analysis was performed. It was shown that IGF-1 was able to promote p38 phosphorylation and that 20 or 40 μmol/L oridonin significantly inhibited the activation after 24 h treatment (Figure 6A). The protein level of JNK or its phosphorylation form was not affected in this process (Figure 6B), consistent with the results from growth inhibition analysis described above. Note that manumycin A or tyrphostin AG1024 abolished the up-regulation of phosphorylated p38 level induced by IGF-1, thus, p38 activation should be a downstream event of Ras or IGF-1R activation (Figure 6C).

2.5. Down-regulation of IGF-1 receptor expression in response to oridonin was dependent on p53 activity

Our previous report showed that p53 activation was required by oridonin-induced A375-S2 cell death [9]. When compared with the magnitude of p53 induction triggered by 40 μmol/L oridonin, the induction of p53 by 20 μmol/L oridonin was quite modest. The levels of p53 and phosphorylated p53 induced by 20 μmol/L oridonin were suppressed by IGF-1, indicating that p53 might be a downstream target of IGF-1R antiapoptotic signaling (Figure 7A).

Early reports demonstrated that p53 could repress IGF-1R gene transcription [16]; therefore, we investigated whether down-regulation of IGF-1 receptor expression in response to oridonin was dependent on the activation of p53. A375-S2 cells were pretreated with 8 μmol/L pifithrin-α, an inhibitor of p53, for 1 h, and then cultured with 20 or 40 μmol/L oridonin for another 24 h. In the presence of pifithrin-α, decreased expressions of IGF-1R and IGF-1R precursor were reversed (Figure 7B). These results suggested that p53 activation was related to down-regulations of IGF-1R and its precursor induced by oridonin.

2.6. Discussion

In this work, we found that IGF-1R signaling was a potential survival pathway against oridonin-induced human melanoma A375-S2 cell death, and that the proapoptotic activity of oridonin was partially attributed to its repression of IGF-1R signaling. We used A375-S2 cells treated with oridonin for 24 h at a sub-IC_{50} concentration of 20 μmol/L and an IC_{50} concentration of 40 μmol/L as a model system. IGF-1R signaling could perform antiapoptotic function in the presence of 20 μmol/L, but not 40 μmol/L, oridonin. The advantages of this system included the fact that it perfectly enclosed IGF-1-mediated survival signaling, and also simplified mechanism analysis of the optimal 40 μmol/L oridonin-induced IGF-1R signaling inhibition by removing confounding results due to irrelevant kinase expression changes.

For melanoma cells, IGF-1 is one of the most critical factors required for survival and growth. We detected the marked expression of IGF-1R in A375-S2 cells by Western blot analysis. Melanoma cells do not produce IGF-1, but they produce growth factors for stimulating fibroblasts and endothelial cells in the tumor stroma. The activated stromal fibroblasts can in turn secrete IGF-1 for paracrine stimulation. Thus, we chose to add exogenous IGF-1 to ascertain whether IGF-1R mediated protection in this experimental model. Like most signaling activations by tyrosine kinase receptors, antiapoptotic signaling by the IGF-1 receptor is dependent on the levels of receptor expression and activity. We found that 12.7 nmol/L IGF-1 significantly protected the A375-S2 cells from 20 μmol/L, but not 40 μmol/L, oridonin-induced growth inhibition or apoptosis, and protein levels of the IGF-1R, IGF-1R precursor, and phosphorylated IGF-1R proteins were severely impaired by 40 μmol/L oridonin and more slightly affected by 20 μmol/L oridonin. The dependency of A375-S2 cell survival on IGF-1R was confirmed by tyrphostin AG1024, a specific inhibitor of IGF-1R, which selectively inhibits IGF-1R autophosphorylation. Tyrphostin AG1024 significantly enhanced 20 but not 40 μmol/L oridonin-induced growth inhibition. Therefore, A375-S2 cell survival should be attributed to the activity of IGF-1R which could effectively activate a potential survival or antiapoptotic signaling, and the remarkably proapoptotic activity of 40 μmol/L oridonin was at least partially due to its repression of IGF-1R expression.

IGF-1 is a major survival factor in serum and prevents apoptosis in a number of cell types. The survival signaling pathways downstream of IGF-1R depend on the cell type and stress. Several studies were focused on the Ras and extracellular signal-regulated kinase (ERK) pathway in IGF-1-mediated cell survival, whereas other reports showed that PI3 kinase and Akt also mediated antiapoptotic signaling by IGF-1R [17].

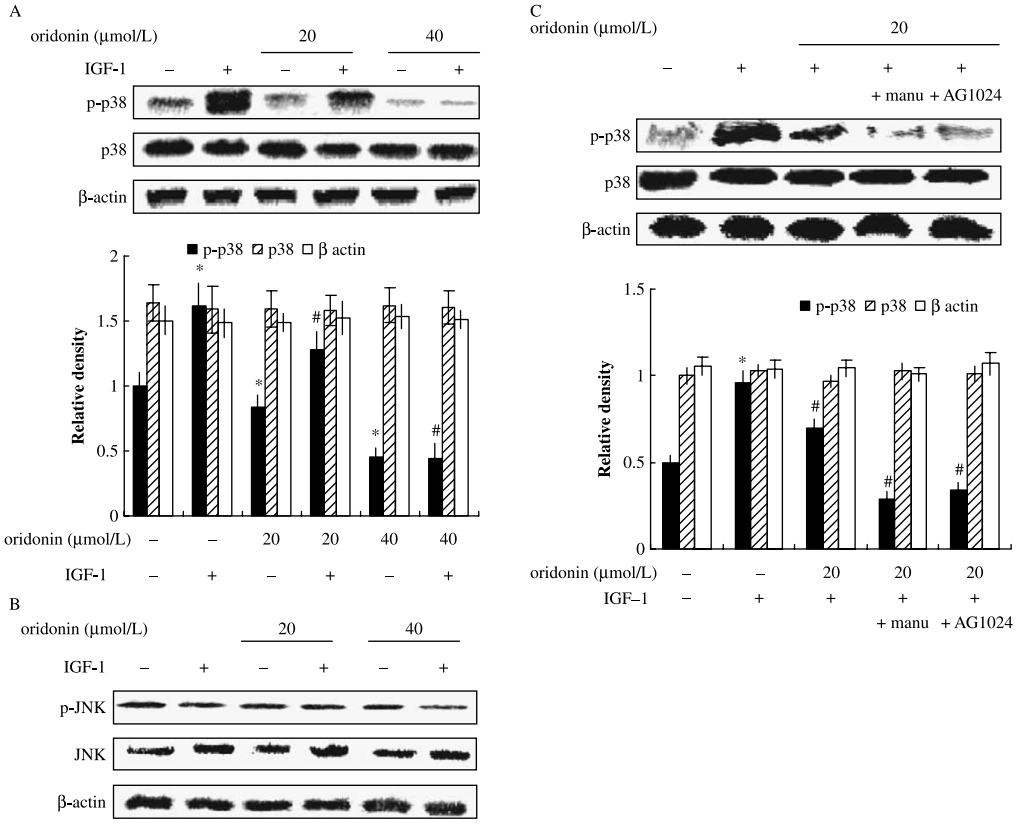

Figure 6. The effects of different concentrations of oridonin on the activation of p38 MAPK or JNK. The cells (3.0×10^6) were pretreated with IGF-1 for 0.5 h, followed by co-incubation with 20 or 40 μmol/L oridonin for 24 h. Cell lysates were separated by 10% SDS-PAGE, and p38, phosphorylated p38 (A), JNK and phosphorylated JNK (B) protein bands were detected by Western blot analysis. The cells (3.0×10^6) were pretreated with 10 μmol/L Ras inhibitor manumycin A (manu) or 1 μmol/L IGF-1R inhibitor tyrphostin AG1024 for 1 h, then co-treated with IGF-1 for 0.5 h, followed by co-incubation with 20 μmol/L oridonin for 24 h (C). p38, phosphorylated p38 protein levels were detected by the method described in (A). Three repeated experiments gave similar results. The density of protein bands was detected using the Bandscan 5.0 software (Glyko, Novato, CA, USA). *Significant difference *vs*. control group; #significant difference *vs*. IGF-1 alone group.

PI3 kinase is activated by both receptor tyrosine kinases (RTKs) and Ras, and in turn activates several downstream signaling pathways [18]. However, it appeared to be irrelevant to IGF-1-mediated protection in our model system. We then moved to determine whether the Ras/MAPK pathway played a survival role. The Ras/MAPK pathway, such as Ras-Raf-ERK, can be activated in response to the activations of various RTKs by their specific ligands. In addition to the ERK pathway, the JNK and p38 pathways are distinct but parallel MAPK cascades in mammalian cells. Once activated, MAPKs (ERK, JNK, and p38) activate a variety of transcription factors, including ELK and c-Jun, thus leading to changes in the expression of genes that play critical roles in cell survival, migration, and apoptosis.

It was found that IGF-1 transmitted a cell survival signal through the Ras-dependent pathway against oridonin-induced A375-S2 cell death, whereas ERK had no direct relation with the process, since PD98059, the specific inhibitor of MEK activation, had no effect on IGF-1-mediated protection. The activations of

both p38 MAPK and JNK by various stimuli have been correlated with apoptosis in many instances. However, basal activities of p38 and JNK were necessary for survival in oridonin-treated A375-S2 cells. JNK was then proved to have no direct relation with IGF-1-mediated protection in A375-S2 cells, whereas IGF-1 induced a marked up-regulation of phosphorylated p38 MAPK, and the effect was blocked by IGF-1R inhibitor tyrphostin AG1024 or Ras inhibitor manumycin A. Therefore, p38 MAPK activation was a downstream event of Ras or IGF-1R activation, and p38 MAPK was a necessary effector in IGF-1R signaling in this system. The result that inhibitors of Ras and p38 enhanced oridonin-induced growth inhibition suggests that the combination of oridonin and inhibitors of IGF-1R signaling is useful for cancer chemotherapy.

IGF-1R expression was down-regulated by oridonin, whereas p53 inhibitor pifithrin-α reversed these expressions. p53, as a tumor suppressor, was able to repress IGF-1R gene transcription. Pifithrin-α has been demonstrated to reversibly block p53-dependent transcriptional activation and apoptosis. Thus, IGF-1R down-regulation induced by oridonin is attributed to p53 activation. On the other hand, the up-regulation of p53 expression induced by 20 μmol/L oridonin was attenuated by IGF-1, therefore, it is supposed that p53 is a pivotal transducer of proapoptotic and survival signaling pathway in this system.

3. Experimental

3.1. Reagents

Oridonin was obtained from the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). The purity of oridonin was measured by HPLC and determined to be about 99%. Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The concentration of DMSO 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-diphenyltetrazolium bromide (MTT), RNase A, proteinase K, and 3,3-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma Chemical (St. Louis, MO, USA). Rabbit polyclonal antibodies against p38 MAPK, phosphorylated p38 MAPK, JNK, phosphorylated JNK, p53, phosphorylated p53, and horseradish peroxidase-conjugated secondary antibodies (goat antirabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against IGF-1Rβ (C-20), phosphorylated IGF-1R (TYR1131), and recombinant human insulin-like growth factor-1 (IGF-1) were purchased from Chemicon (Temecula, CA, USA). Manumycin A, SB203580, PD98059, and SP600125 were from Calbiochem (La Jolla, CA, USA). Wortmannin, tyrphostin AG1024, and pifithrin-α were obtained from Sigma Chemical.

3.2. Cell culture

Human melanoma A375-S2 (#CRL-1872) cells were obtained from American Type Culture Collection (ATCC, Manasas, VA, USA) and were cultured in Roswell Park Memorial Institute medium 1640 (RPMI 1640, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Dalian Biological Reagent Factory, Dalian, China), L-glutamine (2 mmol/L, Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. The cells in the exponential phase of growth were used in the experiments.

3.3. Growth inhibition assay

The growth inhibitory effect of oridonin on A375-S2 cells was measured by MTT assay. The cells were dispensed in 96-well flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells/well. After 24 h incubation, they were treated with various concentrations of oridonin, IGF-1 or inhibitors, and incubated further for the indicated time periods. To each well, 20 μl MTT solution (5.0 mg/ml) was added followed

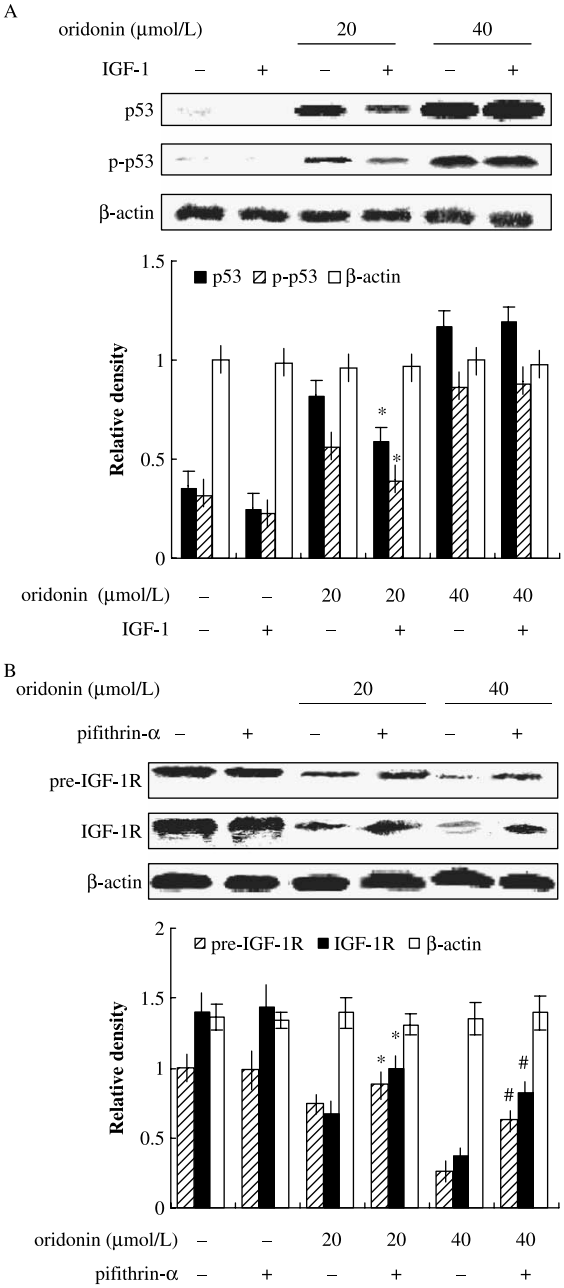

Figure 7. Down-regulation of IGF-1 receptor expression in response to oridonin was dependent on p53 activity. (A) The cells (3.0×10^6) were pretreated with IGF-1 for 0.5 h, followed by co-incubation with 20 or 40 μmol/L oridonin for 24 h. p53 and phosphorylated p53 protein levels was detected by Western blot analysis. (B) The cells (3.0×10^6) were pretreated with 8 μmol/L pifithrin-α for 1 h, followed by co-incubation with 20 or 40 μmol/L oridonin for 24 h. The protein levels of p53, phosphorylated p53, IGF-1R, and IGF-1R precursor were detected by Western blot analysis. Three repeated experiments gave similar results. The density of protein bands was detected using the Bandscan 5.0 software (Glyko, Novato, CA, USA). *Significant difference *vs.* 20 μmol/L oridonin alone group; #significant difference *vs.* 40 μmol/L oridonin alone group.

by 4 h incubation, and the resulting crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured with an ELISA reader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

$$\text{Inhibitory ratio}\,(\%) = (A_{490,\text{control}} - A_{490,\text{experiment}})/(A_{490,\text{control}} - A_{490,\text{blank}}) \times 100.$$

3.4. DNA fragmentation assay

A375-S2 cells (1×10^6 cells) were harvested and centrifuged at $150 \times$ g for 5 min, and then washed with PBS. The cell pellets were suspended in 10 mmol/L Tris (pH 7.4), 10 mmol/L EDTA (pH 8.0), and 0.5% Triton X-100, and kept at 4°C for 30 min. The lysate was centrifuged at $7200 \times g$ for 20 min. The supernatant was incubated with 20 mg/ml RNase A (2 μl) and 20 mg/ml proteinase K (2 μl) at 37°C for 1 h, and was stored in 0.5 mol/L NaCl (20 μl) and isopropyl alcohol (120 μl) at −20°C overnight; it was then centrifuged at $7200 \times g$ for 15 min. DNA was dissolved in TE buffer, pH 7.8 (10 mmol/L Tris–HCl, pH 7.4 and 1 mmol/L EDTA, pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 40 min and stained with 0.1 μg/ml ethidium bromide.

3.5. Western blot analysis

Both adherent and floating A375-S2 cells were harvested, washed twice with ice-cold PBS, and then lysed in lysis buffer (50 mmol/L Hepes (pH 7.4), 1% Triton X-100, 2 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L PMSF), supplemented with the proteinase inhibitors 10 μg/ml aprotinin, 10 μg/ml leupeptin and 100 μg/ml pepstatin at 4°C for 1 h. After $13{,}000 \times g$ centrifugation at 4°C for 15 min, the protein concentration was determined by a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of total proteins were separated by SDS-polyacrylamide gel eletrophoresis, and then electroblotted onto nitrocellulose membranes. The proteins were detected with indicated primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibody, and visualized by HRP substrate 3,3-diaminobenzidine tetrahydrochloride (DAB).

3.6. Statistical analyses

All data represent at least three independent experiments and are expressed as means ± SD. Statistical comparisons were made by Student's *t*-test. $P < 0.05$ were considered statistically significant.

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