Oridonin inhibits BxPC-3 cell growth through cell apoptosis

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Abstract

Oridonin, an ent-kaurene diterpenoid extracted from the traditional Chinese herb Rabdosia rubescens, has multiple biological and pharmaceutical functions and has been used clinically for many years. While the antitumor function of oridonin has been corroborated by numerous lines of evidence, its anticancer mechanism has not been well documented. In this study, the pancreatic cancer cell line BxPC-3 was used as a model to investigate a possible anticancer mechanism of oridonin through examining its effects on cell viability. The results showed that oridonin affected cell viability in a time- and dose-dependent manner. After exposure to different oridonin concentrations, growth rates and cell cycle arrest of BxPC-3 cells were significantly reduced compared with untreated cells, suggesting its effects on proliferation inhibition. Detailed signaling pathway analysis by western blot analysis revealed that low-dose oridonin treatment inhibited BxPC-3 cell proliferation by up-regulating p53 and down-regulating cyclin-dependent kinase 1 (CDK1), which led to cell cycle arrest in the G2/M phase. A high-dose oridonin not only arrested BxPC-3 cells in the G2/M phase but also induced cell accumulation in the S phase, presumably through γH2AX up-regulation and DNA damage. In addition, our results showed that a cell subpopulation was stained with propidium iodide after oridonin treatment. Protein quantification showed that cleaved poly(ADP-ribose) polymerase (PARP) expression was increased after a high-dose oridonin treatment, especially after long-term exposure. Accompanied by the increased level of deactivated PARP in BxPC-3 cells, the apoptosis initiators caspase-3 and caspase-7 expressions were also significantly increased, suggesting that caspase-mediated apoptosis contributed to cell death.

Key words: oridonin, pancreatic cancer, BxPC-3 cells, proliferation, apoptosis

Introduction

A large proportion of modern anticancer drugs are derived from plants [1]. Oridonin, a biologically active compound, is extracted from Rabdosia rubescens, which has been used for thousands of years in traditional Chinese medicine [2]. R. rubescens has been mainly used as an inflammatory disease therapy and to treat several types of tumors [3–5]. The effects of R. rubescens on these diseases are believed to be caused by the antibacterial and antimutagenic functions of oridonin [6–8], and the ent-kaurene structure of oridonin is thought to be the crucial domain for its activity [9,10]. Recently, in vitro studies have demonstrated that oridonin significantly inhibits the growth of cells derived from tumors [11–14]. In addition, in vivo
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Oridonin administration has also shown significant growth inhibition in some fast-growing tumors, including prostate carcinoma, non-small-cell lung cancers, acute myeloid leukemia, and glioblastoma multiforme [15–17]. Mechanistic studies revealed that these inhibitory effects were mostly due to oridonin toxicity, which affected cell mitosis and inhibited cell proliferation [18–20]. Some studies have shown that oridonin-induced cell cycle arrest and apoptosis through different signaling pathways. For example, it has been reported that oridonin-induced G2/M arrest and apoptosis in HepG2 cells through the mitogen-activated protein kinase and p53 signaling pathways [21], while it induced apoptosis in HeLa and U937 cells through the PI3k/Akt [22] and extracellular regulated protein kinases pathways [23], respectively. Oridonin appears to have different functions in different cell types, particularly in immortalized cells possessing different genetic mutations. Thus, predicting the effects of this drug on different tumor/cancer cells is difficult, especially in cancers with multiple mutations, such as pancreatic adenocarcinoma, which may be ameliorated by the harsh microenvironment.

The failure of cells to undergo apoptosis is one of the main mechanisms of tumor development. Hence, many drugs have been reported to inhibit tumor growth by inducing apoptosis through caspase cascade activation in tumor cells [24,25]. To our knowledge, no evidence has been published indicating that oridonin effectively inhibits pancreatic cancer growth, and the mechanisms underlying the effects of oridonin on pancreatic adenocarcinoma cells still remain poorly characterized.

Pancreatic cancer is an extremely aggressive cancer with a high incidence of relapse and drug-resistance after relapse [26,27]. Therefore, identifying drugs that show no cross-resistance with Gemcitabine and Cisplatin which were primarily used in pancreatic cancer treatment is an important research topic. Herein, we reported that oridonin can effectively inhibit cancer cell growth (BxPC-3 cells) derived from a pancreatic adenocarcinoma through cell cycle arrest and apoptosis induction, suggesting that oridonin is a new option for future pancreatic cancer treatment.

Materials and Methods

Cell culture

The human pancreatic cancer cell line BxPC-3 (ATCC® CRL1687™) was supplied by the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum, 2 M glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO₂.

Cell toxicity and proliferation assays

Cell toxicity was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St Louis, USA) according to the manufacturer’s instructions. In brief, BxPC-3 cells were cultured in triplicate in 96-well plates at a density of 8 × 10³ cells/well for 24 h. Then, the medium was replaced by 100 μl of culture medium containing different concentrations of oridonin (2, 4, 6, 8, 16, 32, or 64 μg/ml) (Tauto Biotech Company, Shanghai, China), and the cells were incubated for 12, 24, 36, 48, 60, or 72 h. Control cells were treated with medium containing 0.1% dimethyl sulfoxide (DMSO) only. After oridonin treatment, 20 μl of MTT solution (5 mg/ml) was added to each well, and the cells were incubated for 4 h at 37°C. The mixture of MTT and medium was then removed, and 150 μl of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Model 680; Bio-Rad, Hercules, USA), and the percentage of growth inhibition was calculated as follows:

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\text{inhibition ratio} \% = \frac{A_{570\text{control}} - A_{570\text{oridonin}}}{A_{570\text{control}}} \times 100\%.
\]

Cell proliferation was measured by counting cells after oridonin treatment. In brief, 2 × 10⁴ BxPC-3 cells were seeded in each well of 24-well plates and cultured for 24 h at 37°C, and then the medium was replaced by medium containing 8 or 32 μg/ml oridonin. At 12, 24, 36, 48, 60, and 72 h post-treatment, cells were harvested by trypsinization and counted with an automated cell counter (C10227; Countess, Carlsbad, USA).

Flow cytometry analysis of cell cycle progression and apoptosis

For flow cytometry analysis, BxPC-3 cells were cultured in T75 flasks until 70% confluence was reached, and then treated with 8 or 32 μg/ml oridonin for 36 h. Cells were then harvested by trypsinization and collected by centrifugation at 200 g for 5 min. Cell pellets were resuspended in 1 ml of phosphate-buffered saline (PBS) followed by dropwise addition of the cell suspension into 3 ml of ice-cold absolute ethanol. The nuclei were stained by adding 1 ml of DNA staining solution (Cell Cycle Staining Solution; MultiSciences Biotech, Hangzhou, China) to the cell suspension and incubated for 30 min at room temperature. The cell cycle was analyzed by flow cytometry in the presence of propidium iodide (PI). At least 10,000 cells were analyzed, and the data were analyzed on a FACScan flow cytometer using the Cell ModFIT software to determine cell subpopulations in each cell cycle phase.

For apoptosis analysis, BxPC-3 cells (5 × 10⁵ cells/well) in 6-well plates were treated with 8 or 32 μg/ml oridonin for 36 h. Cells were collected and stained with the Annexin V/PI apoptosis kit (MultiSciences Biotech) according to the manufacturer’s instructions. In brief, the cells were trypsinized and resuspended in 500 μl of 1x binding buffer, followed by addition of Annexin V-FTTC/PI into the binding buffer; then the cells were incubated in the dark for 5 min at room temperature. Annexin V-FTTC binding was analyzed by flow cytometry (Ex = 488 nm; Em = 530 nm) using a FITC signal detector (usually FL1), and PI staining was analyzed with the phycoerythrin emission signal detector (usually FL2). At least 10,000 cells were analyzed for each treatment.

Detection of morphological changes in BxPC-3 cells

BxPC-3 cells were cultured in 24-well plates at a density of 1 × 10⁵ cells/cm². After 24 h, cells were treated with 8 or 32 μg/ml oridonin for 36 h at 37°C with 5% CO₂. Then, cells were washed twice with prewarmed PBS and stained with Hoechst 33342/PI (MultiSciences Biotech) for 10 min at 37°C in the dark. Morphological alterations were observed and analyzed by immunofluorescence microscopy.

Protein analysis

BxPC-3 cells were cultured for 24 h in 6-well plates at a density of 3 × 10³ cells/cm². Then, BxPC-3 cells were treated with 8 or 32 μg/ml oridonin. After 12, 24, or 36 h of treatment, cells were rinsed twice with PBS and lysed with radio immunoprecipitation assay lysis buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate]. The protein concentrations of the cell lysates were measured with a bicinchoninic acid kit (PQ0011; MultiSciences
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Biotech). Lysate containing 20 μg of proteins was loaded onto a 12% SDS-polyacrylamide gel for electrophoresis, and proteins were transferred onto a polyvinylidene fluoride membrane. The membrane was blocked in blocking buffer (5% skim milk in tris-buffered saline and tween 20) for 1 h at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-γH2AX (ab26350; 1:1000 dilution; Abcam, Cambridge, UK); anti-CDK1 (ab18; 1:1000 dilution; Abcam); anti-p53 (ab26; 1:1000 dilution; Abcam); anti-caspase-7 (E22; 1:1000 dilution; Abcam); anti-poly(ADP-ribose) polymerase (PARP)-1 (p116/p85, E78; 1:1000 dilution; Abcam); anti-caspase-3, anti-caspase-8, and anti-caspase-9 (Cat.60-6663, 60-6663, and 96-2-2, respectively; 1:500 dilution; ebioscience, San Diego, USA); and anti-β-actin (Mab1443; 1:500 dilution; MultiSciences Biotech). Then, membrane was incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies: HRP-conjugated goat anti-mouse IgG (GAM007; MultiSciences Biotech), HRP-conjugated goat anti-rabbit IgG (GAR007; MultiSciences Biotech), or HRP-conjugated goat anti-rat IgG (GRT007, MultiSciences Biotech). Immunoblots were developed with the enhanced chemiluminescence western blot substrate kit (Pierce, Lakewood, USA) using the OmegaLum G imaging system. Protein quantification was normalized to β-actin expression. The ImageJ software was used to quantify band intensities.

Statistical analysis
The data were expressed as the mean ± SD. Statistical comparisons were performed with the SPSS 17.0 software (univariate analysis of variance). P-values < 0.05 were considered significant.

Results
Oridonin affected cell viability and inhibited cell proliferation
At very low doses (2 and 4 μg/ml), oridonin showed no inhibitory effects on BxPC-3 cells (Fig. 1). The MTT assay revealed no significant difference in cell viability between the negative control and very low-dose treatments (Fig. 1A). Compared with the control cells and cells treated with very low doses of oridonin, the cells treated with 8 μg/ml oridonin showed a slight decrease of cell viability (the inhibition rate increased). This inhibitory effect was observed after 36 h of treatment (inhibition ratio 17.22% ± 4.40%, P < 0.05), which was similar at 72 h post-treatment. Nevertheless, a significant inhibitory effect was observed at 24 h post-treatment with 16 μg/ml oridonin, and the inhibition ratio continued to increase until the highest inhibition ratio achieved at 72 h, which was doubled compared with that induced by 8 μg/ml oridonin (36.58% ± 5.10% vs. 17.22% ± 4.40%; P < 0.01). At 12 h post-treatment, the cells treated with 16 μg/ml oridonin did not show any significant difference in viability compared with cells treated with 8 μg/ml or a very low dose of oridonin (4.81% ± 3.80% vs. 3.20% ± 3.10%; P > 0.05). However, high doses (32 and 64 μg/ml) of oridonin produced significant toxicity to the cells even after a short exposure. After 12 h of treatment, cell viability decreased by 19.22% ± 3.10% and 25.80% ± 2.90% for cells treated with 32 and 64 μg/ml oridonin, respectively. The inhibition ratio increased and peaked at 36 h, and an ~60% decrease in viability was observed in cells treated with both 32 and 64 μg/ml oridonin. Further exposure to drugs up to 72 h did not significantly increase the inhibition ratio. There was no significant difference between the 32 and 64 μg/ml-treated cells.

Similar to the toxic effects of oridonin, 8 μg/ml oridonin produced a slight proliferation inhibition of BxPC-3 cells, whereas 32 μg/ml oridonin almost completely inhibited cell proliferation (Fig. 1B). After 72 h of treatment, the total number of control cells reached 8.5 × 10^5, whereas the total number of cells treated with 8 μg/ml oridonin was 6.4 × 10^5 (70% of the control cell number). The total number of cells slightly increased (2.9 × 10^5) when treated with 32 μg/ml oridonin. Compared with the DMSO control and 8 μg/ml oridonin conditions, 32 μg/ml oridonin significantly inhibited cell proliferation.

Oridonin induced cell cycle arrest
From the dose-response curves in the cell viability and proliferation assays, 8 μg/ml oridonin and 32 μg/ml oridonin were selected for cell cycle progression analysis. BxPC-3 cells were cultured for 36 h in the presence of 8 or 32 μg/ml oridonin, and the cell cycle phases were assessed by flow cytometry. As shown in Fig. 2, exposing BxPC-3 cells to oridonin resulted in cell accumulation in the G2/M phase. The percentage of cells in the G2/M phase increased to 27.26% ± 3.85% in cells treated with 8 μg/ml oridonin and to 38.67% ± 2.75% in cells treated with 32 μg/ml oridonin, which were much larger than that (21.54% ± 2.73%) in the DMSO-treated cells (P < 0.05). In addition, the percentage of cells in S phase significantly increased to 37.25% ± 2.28% in cells treated with 32 μg/ml oridonin compared with 18.78% ± 1.82% in the control cells (P < 0.01) (Fig. 2A). Nonetheless, unlike cells treated with 32 μg/ml oridonin that showed increased subpopulations of G2/M and S phase cells, the cells treated with 8 μg/ml oridonin only showed cell accumulation in the G2/M phase. The mean percentage of cells in the S phase in this treatment slightly increased compared with the control cells. However, the difference was not statistically significant.

Oridonin regulated γH2AX, p53, and CDK1 expressions
The regulatory effects of oridonin on proteins involved in cell cycle progression, including γH2AX (phosphorylated histone H2AX), p53, and CDK1, were verified by western blot analysis (Fig. 3). The expression levels of γH2AX, p53, and CDK1 in control and BxPC-3 cells treated with 8 or 32 μg/ml oridonin were quantified. The results showed that the levels of these proteins post-oridonin treatment were significantly altered. γH2AX expression was significantly up-regulated by 8 μg/ml oridonin in a time-dependent manner (Fig. 3A). Protein quantification revealed a 1.4- and 2.7-fold increase after 24 and 36 h of treatment, respectively. Similar results were observed in cells treated with 32 μg/ml oridonin, with an earlier onset after 12 h of treatment. After 24 h of treatment, γH2AX expression peaked in cells treated with 32 μg/ml oridonin. Protein quantification revealed a 5.38-fold increase compared with the control, which was followed by a slight decrease (to a 3.72-fold increase). Similar trends were observed for p53 expression in these cells (Fig. 3B). However, the up-regulation of p53 after oridonin treatment was not as dramatic as the γH2AX expression (at 24 h, 1.22- and 1.38-fold increase in p53 expression after 8 and 32 μg/ml oridonin treatment, respectively). Interestingly, p53 expression continued to increase at 36 h (1.65-fold increase), even in the cells treated with 32 μg/ml oridonin. Contrary to the up-regulation of γH2AX and p53 after oridonin treatment, CDK1 expression was decreased, especially in cells treated with 32 μg/ml oridonin. CDK1 expression was slightly decreased by 10% in cells treated with 8 μg/ml oridonin for 36 h. However, CDK1 expression in cells treated with 32 μg/ml oridonin was significantly reduced at 12 h (78% of the control), and a minimum expression level was reached after 36 h (55% of the control) (Fig. 3C).

Oridonin induced apoptosis in BxPC-3 cells
To determine how oridonin affects cell viability, a method to distinguish apoptosis from necrosis was used. After this treatment, the
cells were stained with Hoechst 33342 and PI. While a homogeneous and weak blue fluorescence was observed in healthy cells, apoptotic cells stained with Hoechst 33342 produced bright blue fluorescence in the nuclei because of the condensed chromatin. Necrotic and late apoptotic cells stained with PI displayed red fluorescence. This analysis revealed that the number of PI positive cells with condensed chromatin significantly increased after 36 h of 8 or 32 µg/ml oridonin treatment (Fig. 4A). Early apoptosis in BxPC-3 cells was investigated through membrane phosphatidylserine detection with Annexin V-FITC staining, and the percentages of cells in early and late apoptosis were measured using Annexin V-FITC/PI double staining and flow cytometry. The results showed that the percentages of early apoptotic BxPC-3 cells induced by 8 or 32 µg/ml oridonin were significantly increased compared with control cells (18.3% and 54.8% vs. 2.5%, respectively; \( P < 0.05 \)) (D4 in the histograms) (Fig. 4B,C).

Oridonin induced apoptosis through caspase signaling pathways
To elucidate the molecular mechanism of oridonin-induced apoptosis in BxPC-3 cells, the pivotal initiators and executors of apoptosis, i.e. caspases-3, -7, -8, and -9 and PARP, were investigated by western blot analysis. Compared with the DMSO control, oridonin treatment significantly altered the expression of cleaved caspases-3, -7, and PARP (Fig. 5); however, the expression of the uncleaved protein forms remained unchanged. The significant up-regulation of cleaved caspase-3 was first observed after 24 h of treatment with 8 µg/ml oridonin...
(10.5-fold increase), and the protein levels were similar at 36 h post-treatment. In contrast to the unchanged levels of cleaved caspase-3 in cells treated for 12 h with 8 µg/ml oridonin, an earlier up-regulation of cleaved caspase-3 was observed in cells treated with a high dose of oridonin. Protein quantification revealed that cleaved caspase-3 increased by 25 folds after 12 h of treatment with 32 µg/ml oridonin, which had a 2-fold increase observed at 24 h in cells treated with 8 µg/ml oridonin. In the cells treated with 32 µg/ml oridonin, cleaved caspase-3 peaked after 36 h of treatment, and protein quantification results indicated a 43.8-fold increase compared with control cells. In contrast to the accumulation of cleaved caspase-3 in BxPC-3 cells after oridonin treatment, caspase-8 levels were significantly down-regulated after oridonin treatment. Compared with the DMSO control, 8 µg/ml oridonin decreased caspase-8 expression by 30% after 12 h of treatment, and over time, the oridonin treatment did not alter protein expression levels. However, higher dose further decreased caspase-8 level. The caspase-8 level was decreased by at least 50% of the control levels after treatment with 32 µg/ml oridonin for 24 and 36 h. In contrast to the abovementioned proteins, caspase-7 and PARP showed distinct expression patterns after oridonin treatment. After 12 h of...
Figure 4. Oridonin-induced apoptosis in BxPC-3 cells  BxPC-3 cells were treated with oridonin (8 and 32 µg/ml) for 36 h. (A) The change of fluorescence after treating BxPC-3 cells with 8 and 32 µg/ml oridonin for 36 h. (B) A histogram showing cells stained with Annexin V-FITC/PI and measured by flow cytometry. (C) A histogram showing the percentage of cells in different cell cycle phases. The data are expressed as the mean ± SD (**P < 0.01). D1, detection error; D2, late-phase apoptotic and necrotic-phase cells; D3, healthy cells; D4, early-phase apoptotic cells. Ori, oridonin; Con, control.
treatment, cleaved caspase-7 expression was significantly increased in cells treated with 8 or 32 µg/ml oridonin, with 12- or 29-fold increases, respectively, when compared with the controls, whereas PARP expression was increased only in the 32 µg/ml-treated cells (3.79-fold increase; \( P < 0.05 \)). After 24 h of treatment with 32 µg/ml oridonin, cleaved caspase-7 and PARP expression levels peaked (71.56- and 23.84-fold increase, respectively, compared with controls; \( P < 0.01 \)), which was followed by a decrease at 36 h (12.48 and 15.32 folds for caspase-7 and PARP, respectively).

**Discussion**

The anticancer drugs currently available have distinct mechanisms of action, but their main role is to slow and halt the growth and spread of cancer cells, mainly by damaging the DNA of the affected cells, inhibiting the synthesis of new DNA strands, stopping mitosis by breaking down the mitotic spindles or inducing apoptosis. For example, Gemcitabine inhibits DNA replication, whereas Cisplatin interferes with cell division by crosslinking DNA; treatment with either drug results in apoptosis. In this study, we investigated the effects of oridonin on the pancreatic cell line BxPC-3, and our results clearly demonstrated that oridonin can effectively inhibit BxPC-3 cell proliferation.

Cell proliferation requires functionally intact mitochondria, without which cells cease to aerobically respire and quickly die. In the MTT assay, the DMSO treatment was not toxic to BxPC-3 cells, and BxPC-3 cells proliferated robustly (Fig. 1). The total number of cells tripled after 72 h in the presence of DMSO, so the inhibitory effect of DMSO was negligible (Fig. 1A). In contrast, oridonin significantly inhibited cell metabolism, especially when the oridonin
concentrations were higher than 8 µg/ml. The inhibition ratio was significantly increased in cells treated with 32 µg/ml oridonin, and almost all cell division ceased after 36 h of treatment, suggesting that the inhibitory effect was in dose-dependent manner. Although the mechanism is still unclear, it is plausible that, in this assay, mitochondria were affected by the oridonin concentrations, which weakened cell metabolism and inhibited cell proliferation but did not cause cell death, because we did not observe any obvious cell loss in the oridonin-treated cells (Fig. 1B). Thus, we could not rule out the possibility that oridonin-treated cells were undergoing apoptosis or initiation of apoptosis after treatment because mitochondrial damage is one pathway that induces apoptosis.

When BxPC-3 cells were cultured in oridonin-containing medium, the number of cells with bright boundaries was significantly increased (data not shown). This observation is presumably because of the functional damage to the P-glycoprotein membrane pump in apoptotic cells, which leads to a discharge failure of Hoechst 33342. This discharge usually occurs in cells in the early stages of apoptosis [28]. During apoptosis, the chromatin condenses into compact patches against the nuclear envelope. Consistent with this process, we observed an increased number of cells with condensed nuclei in oridonin-treated cells (data not shown), suggesting that oridonin might initiate apoptosis.

The cells accumulated not only in the G2/M phase but also in the S phase when treated with a high dose of oridonin (Fig. 2). We investigated the proteins that regulated cell cycle progression including CDK1 by western blot analysis. Our results suggested that S-phase arrest occurred because of the deactivation of cyclin-dependent kinases or DNA damage caused by oridonin toxicity. In support of this possibility, the increased levels of γH2AX, a prominent indicator of DNA double-strand breaks (DSB) [29,30], were found to be accompanied by decreased CDK1 (cdc2) levels in BxPC-3 cells after oridonin treatment. Recent studies demonstrated that DNA damage caused S-phase arrest [31], and the DSB repair would occur at the stage after γH2AX expression. Our results showed a significant increase in γH2AX expression after oridonin treatment, suggesting an acute response to DNA damage (Fig. 3). Following this acute response, a slight decrease in γH2AX expression in BxPC-3 cells was observed at 36 h, suggesting perturbed DSB repair in these cells after a long-term exposure to a high dose of oridonin.

CDK1 plays a pivotal role in cell cycle progression in the G2/M phase, and its down-regulation arrests the cell cycle in the G2 phase. However, CDK1 transcription is normally stable throughout the cell cycle. A significant decrease in CDK1 expression usually indicates dysregulated transcription because CDK1 transcription is only modulated under pathological conditions [32]. Thus, the G2/M arrest in BxPC-3 cells could be a direct consequence of dysregulated CDK1 transcription, but the mechanism is still unclear. To address this question, we assessed whether p53 expression was associated with CDK1 expression because p53 negatively regulated CDK1 expression [33]. Our results revealed a dose- and time-dependent significant increase of p53 expression levels and a decrease of CDK1 expression after oridonin treatment, suggesting an association of p53 with CDK1 expression (Fig. 3). p53 is a protein with multiple important regulatory functions in the cell cycle and thus acts as a tumor suppressor. p53 becomes activated in response to DNA damage, and under such conditions, the half-life of the p53 protein is drastically increased, which leads to a rapid accumulation of p53 in stressed cells. The significantly increased expression levels of p53 in oridonin-treated cells indicate that oridonin is a stressor that induces p53 accumulation.

CDK1 down-regulation and p53 up-regulation may result in extensive apoptosis [32,34,35]. To clarify the mechanism of oridonin in inhibiting cell proliferation in BxPC-3 cells, western blot analysis was used to investigate the caspase levels after oridonin treatment. Caspases are a family of cysteine proteases that play key roles in apoptosis [36,37]. They include the initiator caspases-2, -8, and -9, as well as the effector caspases-3, -6, and -7 [38]. In oridonin-treated cells, the pro-active forms of caspases-8 and -9 were significantly reduced, implying an elevation of active caspases-8 and -9. This elevation sequentially activated caspases-3 and -7, and resulted in the accumulation of cleaved caspases-3 and -7. This accumulation ultimately led to cellular apoptosis [39,40]. With the increase of oridonin concentrations and exposure time, the cellular levels of cleaved caspase-3 increased. Interestingly, the cleaved caspase-7 levels plunged after a period of increase.

Caspases-3 and -7 are the inactivators of PARP, which is activated when single-strand DNA breaks occur [41,42]. After PARP is activated, it synthesizes a poly(ADP-ribose) chain (PAR) as a signal for other DNA-repairing enzymes, such as DNA ligase III and DNA polymerase β. When DNA damage is extensive, the energy would be retrieved from other cells where repairing the damage is feasible and PARP is inactivated. In this study, cleaved PARP was found to be significantly increased in the cells treated with 8 µg/ml oridonin for 24 h and in the cells treated with 32 µg/ml oridonin for 12 h (Fig. 5), indicating that extensive DNA damage occurred after oridonin treatment. Cleaved PARP expression was decreased at 36 h at both 8 and 32 µg/ml oridonin-treated conditions, indicating the inactivation of PARP because of extensive damage to genomic DNA. PARP also has the ability to induce apoptosis through the production of PAR, which stimulates the mitochondria to release apoptosis-inducing factor [43]. We could not distinguish whether the apoptosis was induced by caspases or by PAR because the mechanism of PAR-induced apoptosis appears to be caspase-independent [44]. Both of these pathways may be involved in oridonin-induced apoptosis.

Our findings indicated that DNA damage and caspase activation are the two main events that mediate oridonin-induced apoptosis in BxPC-3 cells. Based on the results of this study, the mechanisms by which oridonin affects BxPC-3 cells are summarized in Fig. 6.

In conclusion, this study proposed a new mechanism underlying oridonin-induced proliferation inhibition in cancer cells derived from pancreatic adenocarcinoma. Oridonin significantly inhibits the proliferation of BxPC-3 cells in a dose- and time-dependent manner by arresting cells in both the S and G2/M phases and regulating key factors for apoptosis induction. Our results demonstrated that BxPC-3 cells can be lethally damaged by a high concentration of oridonin.
oridonin or a long exposure to a low dose of oridonin, which affects cellular metabolism and DNA repair through different pathways. Proliferation inhibition through multiple pathways is superior to single pathway inhibition because cells arrested in the S and G2/M phases are more sensitive to Doxorubicin and Gencitabine than cells arrested in the G1 phase [30,31]. This investigation may produce new drug combinations for clinical therapies, especially for pancreatic cancers.

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