RESEARCH ARTICLE

Downregulation of Cdk1 and CyclinB1 Expression Contributes to Oridonin-induced Cell Cycle Arrest at G_2/M Phase and Growth Inhibition in SGC-7901 Gastric Cancer Cells

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Abstract

Background: Oridonin isolated from *Rabdosia rubescens*, a plant used to treat cancer in Chinese folk medicine, is one of the most important antitumor active ingredients. Previous studies have shown that oridonin has anti-tumor activities *in vivo* and *in vitro*, but little is known about cell cycle effects of oridonin in gastric cancer. Materials and Methods: MTT assay was adopted to detect the proliferation inhibition of SGC-7901 cells, the cell cycle was assessed by flow cytometry and protein expression by Western blotting. Results: Oridonin could inhibit SGC-7901 cell proliferation, the IC_{50} being 15.6 μM, and blocked SGC-7901 cell cycling in the G_2/M phase. The agent also decreased the protein expression of cyclinB1 and CDK1. Conclusions: Oridonin may inhibit SGC-7901 growth and block the cells in the G_2/M phase by decreasing Cdk1 and cyclinB1 proteins.

Keywords: Oridonin - human gastric cancer cells SGC-7901 - cyclinB1 - Cdk1 - cell cycle

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Introduction

Gastric cancer is one of the most common malignant tumors worldwide, with 989,600 new diagnosed cases (8% of the total malignancy) and 738,000 deaths annually (10% of total cancer deaths)(Jemal et al., 2011). Over 70% of new cases and deaths occur in developing countries. The highest incidence rates are in Eastern Asia (Kamangar et al., 2006), Eastern Europe, and South America (Jemal et al., 2011). Half of all gastric cancer patients are from Eastern Asia (463, 000 gastric cancer patients in China alone), and approximately two thirds of all cases occur in developing countries (Choi et al., 2014). Therefore, the development of novel approaches and effective anticancer strategies is critically needed for prolonged survival of stomach cancer.

Rabdosia rubescens are used in Chinese folk medicine for treatment of esophageal cancer in Taihang Mountains area of China for a long time. In 1970s, Research from Chinese researcher showed *Rabdosia rubescens* had better effect in the treatment of Gush door cancer, liver cancer and esophagus cancer, and oridonin was one of the most important antitumor active ingredient of *Rabdosia rubescens*.

Oridonin, molecular formula $C_{20}H_{28}O_6$ (Figure 1), is a diterpenoid compound. Previous studies have shown that oridonin has anti-tumor activities *in vivo* and *in vitro* (Zhou et al., 2007; Lou et al., 2009; Wang et al., 2013), and oridonin inhibited proliferation of cancer cells by inducing autophagic pathways (Ye et al., 2012a; 2012b; Yu et al., 2012; Zang et al., 2012; Liu et al., 2013), by inducing apoptosis through the mitochondrial pathway (Li et al., 2008; Zhu et al., 2013) or Fas/FasL death signal pathway (Liu et al., 2006), or by arresting the cell cycle on G_0/G_1 phase in human breast adenocarcinoma MDA-MB-231 cell (Hsieh et al., 2005), but G_2/M phase in human breast adenocarcinoma MCF-7 cell (Cui et al., 2007; Zhang et al., 2013), human liver hepatocellular carcinoma HepG2 cell (Wang et al., 2010), Human Pancreatic Carcinoma PANC-1 cell (Qi et al., 2012), murine fibroblast L929 cells (Cheng et al., 2009), Human Laryngeal Carcinoma Cells (Kang et al., 2010). However, little was known about the cell cycle arrest of oridonin on gastric cancer. Therefore, this paper explored the proliferation inhibition, cell cycle arrest and cell cycle protein expression induced by oridonin on human gastric cancer SGC-7901 cell.

Materials and Methods

Material

Oridonin (>98%) was purchased from National Institutes for Food and Drug Control in China. hydroxycamptothecin (HCPT) was provided by Shanghai Longxiang Biological Medicine Development Co. Ltd. in China, Ltd. MTT (3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from the

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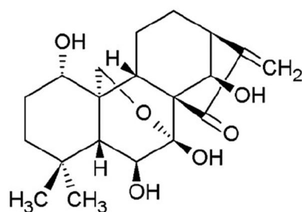

Figure 1. Structure of Oridonin

United States Molecular Probe company. RPMI-1640 medium was purchased from GIBCO Company. Dimethyl sulfoxide (DMSO), trypsin, and propidium bromide (PI) were purchased from Sigma. Rabbit polyclonal anti-Cyclin B1 antibody, anti Cdk1/Cdc2 rabbit polyclonal antibody and alkaline phosphatase-labeled goat anti-rabbit IgG were provided by Beyotime institute of Biotechnology in China.

Cell culture

Human gastric cancer SGC-7901 cell line was obtained from Institute of Tumor Research of Harbin Medicine University (Harbin, China). Cells were cultured in RPMI 1640 medium (Gibco, 31800-022) supplemented with 10% (v/v) fetal bovine serum (Gibco, 10099-141), 100 U/mL penicillin, 100μg/mL streptomycin and 1 mM L-glutamine at 37°C in an atmosphere of 5% CO_2. The medium was renewed two or three times/week. Cells in logarithmic growth phase were used for further experiments.

Cell Viability Assay

Cell viability was measured by the MTT assay, which was based on the conversion of MTT to formazan crystals by mitochonfrial dehydrogenases (Mosmann, 1983). Briefly, SGC-7901 cells were plated at a density of 1×10^3 cells/well in 96-well plate, which was in 100μL RPMI 1640 medium containing 10% (v/v) fetal bovine serum for 24h incubation. After 24h, 100 μl oridonin solution in different concentrations were added into the wells and adjusted the final concentrations to 3.125, 6.25, 12.5, 25, 50 μmol/L, respectively. Similarly, negative group was added into PRMI 1640 and positive control group was HCPT. At the end of 72h incubation, the medium were discarded and 100μL of MTT stock solution (1mg/mL) were added to each well and the plates were further incubated. Four hours later, DMSO (150μL) was added to each well to solubilize the water-insoluble purple formazan crystals. The amount of MTT-formazan is directly proportional to the number of living cells and was determined by measuring the optical density (OD) at 570nm using microplate reader (Bio-Rad). The percentage of cytotoxic activity compared to the untreated cells was determined using the following equation:

$$\text{Cell inhibitory rate (\%)} = \frac{\text{OD of control cells - OD of treated cells}}{\text{OD of control cells}} \times 100$$

Flow cytometry assay

1 ml/well (1×10^5 cells /ml) SGC-7901 cell suspension was added into 6-well plate and cultured for 24h at 37°C in an atmosphere of 5% CO_2. After 24h, oridonin in different concentrations were added into the wells and cultured for 48h. Then, 70% cold ethanol fixed the collected cells in 4°C overnight. Finally, the cells were treated with 50μM PI in 37°C for 0.5h and detected by flow cytometry (excitation wavelength 488nm, emission wavelength of 525nm)(Choi et al., 2007).

Table 1. Doses Inducing 50% Cell Growth Inhibition(IC_{50}) of Oridonin Against Human Gastric Cancer SGC-7901 Cell

Groups	IC_{50} (μM)
HCPT	32.98
Oridonin	15.64

*Cells were treated with different concentration of Oridonin and HCPT for 72hours. Viability was quantitated by MTT assay.

Table 2. Effect of Oridonin on Cell Cycle of SGC-7901 Cell

Groups	concentration (μmol·L-1)	G_0/G_1	S	G_2/M
Control	-	59.98±1.05	32.54±0.59	7.48±0.46
HCPT	10.00	48.14±0.71	35.11±1.96	16.75±2.47**
Oridonin	0.375	55.99±1.63	30.15±1.06	13.87±0.57**
Oridonin	0.75	53.14±1.10	32.62±0.49	14.24±0.62**
Oridonin	1.50	53.16±1.20	31.84±1.92	14.98±0.69**

Cells were treated with different concentration of Oridonin for 72 h. Cell cycle was quantitated by flow cytometer.p<0.05, **p<0.01 *vs* Control

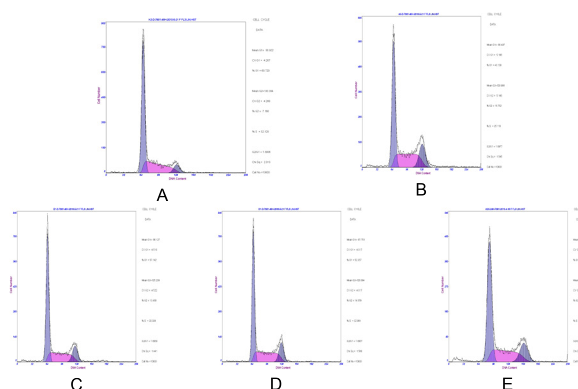

Figure 2. Effect of Oridonin on Cell Cycle of SGC-7901. cells were treated with different concentrations of oridonin (0.375, 0.75, and 1.5μmol/L) for 48h, **(A)**control group, **(B)** 10μmol/L HCPT, **(C)** 0.375μmol/L Oridonin, **(D)** 0.75 μmol/L Oridonin, **(E)** 1.5μmol/L Oridonin

Western blot assay

5 ml/well (2×10^5 cells/ml) SGC-7901 suspensions were added into 150 ml flask, cultured for 24h. Then, oridonin in different concentrations were added into the flasks and cultured for 48h. After 48h, the cells were collected, and proteins were extracted with lysis buffer [50mM Tris-Cl, pH 8.0, 120mM NaCl, 50mM NaF, 200μM sodium vanadate, 0.5% NP-40, 10 mM phenylmethylsulfonyl fluoride (PMSF), 2μg/mL aprotinin 0.2 μL, 10μg/mL Leupeptin 10μL], and then the cells were centrifuged at 12000×g for 10min at 4°C, the supernatant was saved in -80°C. The protein concentration of the supernatant was detected by Bradford assay. The protein samples were separated by SDS-PAGE and then transferred to the NC membrane with TBST containing 5% skim milk at room temperature for 2h, and the membranes were incubated with the primary antibodies overnight at 4°C. Next day, TBST solution sufficiently washed the membrane for 10

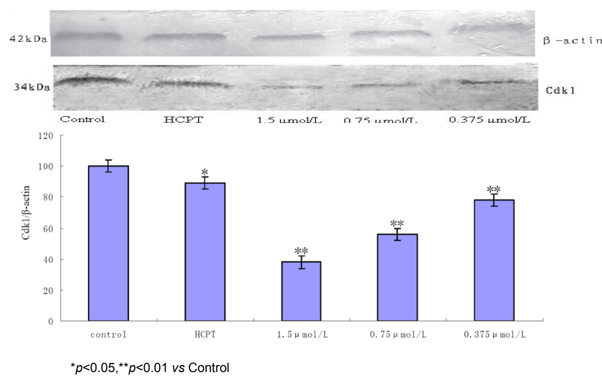

Figure 3. Effect of Oridonin on the Expression of Cdk1 in SGC-7901

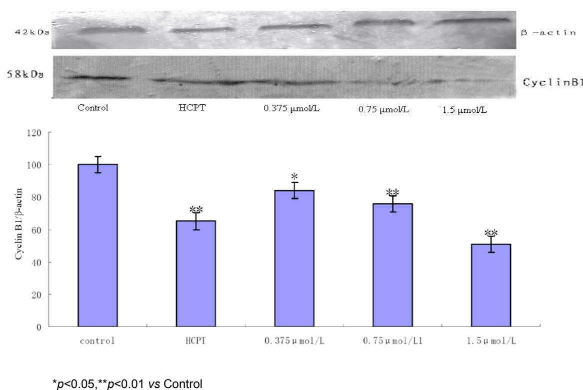

Figure 4. Effect of Oridonin on the Expression of CyclinB1 in SGC-7901

min, 3 times and then shaken with alkaline phosphatase-labeled secondary antibodies and incubated for 2 h at room temperature. After washing unbound second antibody, TBST shaken the membranes 3 times for 10 min. Gel imaging analysis system detected the protein signals of NC membrane.

Statistical analysis

All the data was represented as the mean±SD. Statistical significance was calculated using student's t-test. P-values of 5% or less were considered statistically significant.

Results

Effect of Oridonin on SGC-7901 Cell Viability

In ordor to evaluate the effect of oridonin on proliferation of the SGC-7901 cell, the cells were treated with different concentrations of oridonin for 72h, the impact of oridonin on cell viability was quantitated by MTT assay. The results showed that oridonin could inhibit the proliferation of SGC-7901 cell, and the IC_{50} of oridonin was 15.64μM. The results were shown in Table 1.

Effect of oridonin on SGC-7901 cell cycle

To study roles of oridonin in cell cycle arrest, oridonin was used to test cell cycle on SGC-7901 cell line. The SGC-7901 cells were treated with concentrations of 0.375, 0.75, 1.5μmol/L oridonin for 48h. After treatment, the cells were harvested, and the cell cycle arrest was tested by flow cytometre (Figure 2). Quantitative analysis showed that proportion of G_2/M phase increased from 7.48%±0.46% at control group to 13.87%±0.57%, 14.24%±0.62%, 14.98%±0.69% when the cells were treated with the concentrations of 0.375, 0.75, 1.5μmol/L oridonin, respectively (Table 2). The results showed that oridonin could arrest the SGC-7901 cells in G_2/M phase.

Effect of oridonin on Cdk1 and CyclinB1 protein expression

To determine whether the Cdk1 protein and CyclinB1 protein are involved in the cell cycle arrest effects of oridonin on SGC-7901 cells, Western blot assay was applied. As shown in Figure 3 and Figure 4, after 48h of the SGC-7901 cells being exposed to oridonin for different concentrations (0.375, 0.75, 1.5μmol/L), the expression levels of Cdk1 protein and Cyclin B1 protein in SGC-7901 decreased (p<0.01), compared with negative group (show in Figure 3 and Figure 4).

Discussion

Gastric cancer is one of the high incidence of tumor. Establishment of effective therapies for stomach cancer is very important for cancer research (Hu et al., 2012). Although there are a lot of therapeutic options available for patients with tumor, the efficacy is not curative. Cytotoxicity is believed as one of the major goals of cancer chemotherapy. It is thought that arrestting the cell cycle of cancer is important factor in the development of cancer cell (Alabsi et al., 2012; Yang et al., 2013). Therefore, it appears that exploiting the cell cycle blocker is good idea about anti-cancer drug discovery.

A lot of antitumor drugs were derived from plants or traditional medicine, which were used to treat the disease as different folk medicine for a long time, so new drug discovery from herbs is more likely to succeed (Shin et al., 2012; Huang et al., 2013). *Rabdosia rubescens* has been used as an herbal remedy for various ailments including cancer in China, and oridonin is one of the anti-cancer components of *Rabdosia rubescens*. The paper was initiated with the purpose of evaluating the action of oridonin on human stomach cancer.

The experimental results show that oridonin had good cytotoxicity against the SGC-7901 cells based on the high IC_{50} value as similar as hydroxycamptothecine (Table 1). Mang documents show that oridonin could block MCF-10A, HepG2, laryngeal carcinoma and other tumor cell cycles (Hsieh et al., 2005; Kang et al., 2010; Wang et al., 2010). However, there is little report about the SGC-7901. So, this paper explored SGC-7901 from the perspective of cell cycle. The result showed that oridonin in different concentrations could block the SGC-7901 cells in G_2 /M phase.

Cell cycle involves four sequential phases that go from quiescence (G_0 phase) to proliferation (G_1, S, G_2, and M phases), by which all living things reproduce. In all eukaryotic cells, including human cells, cell cycling is driven by sequential activation of cyclin-dependent kinases (CDKs) and its cofactor cyclins (Diaz-Moralli et al., 2013). Among the countless elements taking part in this process, the sequential activation of heterodimeric

CDK-cyclin complexes (cyclins and their counterpart cyclin-dependent kinases (CDKs) has been described as the key regulatory events. Cyclin-dependent kinases (CDKs) are a family of mammalian heterodimeric serine/threonine protein kinases composed of two subunits, the catalytic one known as CDK and the regulatory one known as cyclin (Shapiro, 2006; Malumbres and Barbacid, 2007). The kinase activity of CDKs is tightly regulated by the binding to cyclins, the activating subunits which are expressed in an oscillatory way, the binding to negative regulators (CDK inhibitors, CKI) and phosphorylation/dephosphorylation events (Manchado et al., 2012). Progression through each cell cycle phase and transition from one phase to the next are monitored by sensor mechanisms, called checkpoints, which maintain the accurate sequence of events (Hartwell and Weinert, 1989).

CDK1-cyclin B complexes are essential for initiating mitosis and can phosphorylate a broad spectrum of proteins involved in regulatory and structural processes required for mitosis such as nuclear envelope breakdown, chromosomal condensation, fragmentation of the Golgi apparatus, formation of the spindle and attachment of chromosomes to it (Malumbres and Barbacid, 2005; Mahadevan et al., 2011). Exiting mitosis requires the inactivation of CDK1-cyclin B, which is carried out by the ubiquitin-dependent proteolysis of B-type cyclins by the APC/C-CDC20 complex (Harper et al., 2002; Peters, 2002).

So, cell cycle proteins (cyclinsB1) and cyclin-dependent protein kinase (Cdk1) were closely related with G_2/M phase. CyclinB1, as the key factor to switch on mitosis, could compose compound with Cdk1 to adjust the G_2/M phase.

Our study showed that oridonin arrested the SGC-7901 cell cycle in G_2/M phase, To explore the relation of G_2/M phase arrestion to CDK1and cyclin B1, we tested the effect of oridonin on Cdk1 and Cyclin B1 proteins. The results showed that oridonin could significantly decreased the expression of Cdk1 and Cyclin B1 proteins in SGC-7901 cells, which would led to a significant reduction in the formation of CDK1-cyclin B complexes, and then block the cells in G_2/M phase.

The above analysis showed that oridonin blocked SGC-7901 cells in G_2/M phase by down-regulating the protein expressions of CDK1, CyclinB1.

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