Biomedicine & Pharmacotherapy 106 (2018) 175–182

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Oridonin enhances the cytotoxicity of 5-FU in renal carcinoma cells by inducting necroptotic death

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ARTICLE INFO

Keywords:
Oridonin
5-FU
Necroptosis
Renal cell carcinoma

ABSTRACT

Background: 5-fluorouracil (5-FU) is widely used for the treatment of renal carcinoma. However, drug resistance remains the reason for failure of chemotherapy. Oridonin, extracted from Chinese herb medicine, displays anti-tumor effect in several types of cancer. Whether oridonin could enhance the effect of 5-FU in renal carcinoma has not been studied.

Methods: 786-O cells were used in the current study. Cell death was measured by MTT assay or live- and dead-cell staining assay. Glutathione (GSH) level was examined by ELISA. Necroptosis was identified by protein levels of receptors interaction protein-1 (RIP-1) and RIP-3, lactate dehydrogenase (LDH) and high mobility group box-1 protein (HMGB1) release, and poly [ADP-ribose] polymerase-1 (Parp-1) activity. Using a xenograft assay in nude mice, we tested the anti-tumor effects of the oridonin combined with 5-FU.

Results: 5-FU only induced apoptosis in 786-O cells. Oridonin activated both apoptosis and necroptosis in 786-O cells. Oridonin-induced necroptosis was reversed by addition of GSH or its precursor*N*-acetylcysteine (NAC). Oridonin-induced necroptosis was associated by activated JNK, p38, and ERK in 786-O cells, which were abolished by GSH or NAC treatment. However, JNK, p38, and ERK inhibitors showed no effect on oridonin induced-cell death. GSH or NAC treatment partly abolished the synergistic effects of oridonin and 5-FU on cell death. Oridonin enhanced the cytotoxicity of 5-FU both *in vitro* and *in vivo*.

Conclusion: Oridonin enhances the cytotoxicity of 5-FU in renal cancer cells partially through inducing necroptosis, providing evidence of using necroptosis inducers in combination with chemotherapeutic agents for cancer treatment.

1. Introduction

Renal cell carcinoma (RCC) is the most prevalent malignant neoplasm of the kidney, accounting for 3% of all malignancies and nearly 100,000 patients dying each year [1]. Although surgical resection together with 5-fluorouracil (5-FU) can effectively improve the prognosis, drug resistance limits the therapeutic effect of 5-FU. Thus, exploring new drugs to overcome 5-FU resistance or enhance the antitumor effect on RCC becomes very important.

Oridonin, a (7,20-epoxy-*ent*-kaurane) diterpenoid extracted from *Rabdosia rubescens* [2,3], which is an active ingredient of commonly used herb in traditional Chinese medicine. Oridonin has been used for treatment of several types of carcinomas, including lung cancer, colorectal cancer, and hepatocarcinoma [4–7], via promoting apoptosis. Oridonin triggered apoptotic program in HSC-T6 cells via intracellular glutathione (GSH) depletion and such an effect was inhibited by GSH supplement [8]. Importantly, previous data, including ours, indicated that GSH depletion was also involved in cell necroptosis [9,10], suggesting that oridonin could be a necroptotic inducer.

Necroptosis is a necrotic-like cell death that characterized by loss of cell membrane integrity, reduction of mitochondrial membrane potential as well as increasing high mobility group box-1 protein (HMGB1) release and poly [ADP-ribose] polymerase-1 (Parp-1) activity [11,12]. Incomplete necroptosis is reportedly involved in the mechanism of drug resistance [13]. Unlike traditional cell necrosis that cannot be controlled, necroptosis can be reversed or alleviated by a chemical compound necrostatin-1 [14] or receptor interaction protein-1 (RIP-1) deletion [15]. Thus, targeting necroptosis could become a

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https://doi.org/10.1016/j.biopha.2018.06.111
Received 15 March 2018; Received in revised form 19 June 2018; Accepted 19 June 2018

new approach to overcome drug resistant in cancer chemotherapy [16], and necroptosis inducers such as shiconin, granulysin and smac mimetic have been shown to effectively potentiate the antitumor effect of chemotherapeutic drugs [17–19]. In the current study, we found that oridonin induced-necroptosis lowered the dosage of 5-FU used in the treatment of renal cancer cells, and potentiated the anti-tumor effect of 5-FU at the same time.

2. Methods

2.1. Cell culture

Human renal carcinoma cell line 786-O obtained from American Type Culture Collection was grown in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin and streptomycin (Beyotime Biotechnology, China) at 37 °C with 5% CO_2. The human podocyte cell line was cultured in RPMI 1640-based medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 g/L of sodium bicarbonate ($NaHCO_3$), insulin-transferrin-selenium (ITS) supplement (Sigma-Aldrich), and 100 U/ml penicillin and streptomycin, and plated on a collagen pre-coated plate (Sigma-Aldrich, USA) at 37 °C with 5% CO_2. Before stimulation with 5-FU or oridonin (Sigma-Aldrich, USA), the culture medium was replaced with serum-free DMEM for 12 h.

2.2. Cell viability assay

Podocyte and 786-O cells were plated at a density of 5×10^3/per well on a 96-well plate. After cell attachment and serum starvation, different concentrations of 5-FU (25, 50 and 100 μM) with or without oridonin (40 μM) were added and then incubated for 24 or 48 h. After the appropriate treatment, cells were incubated with 5 mg mL^{-1} MTT at 37 °C, 5% CO_2 for 4 h, then 200 μL DMSO was added to dissolve the crystal. Absorbance was measured at a wavelength of 490 nm with a plate reader (Tecan Infinite m200, Mannedorf, Switzerland).

2.3. LDH assay

786-O cells were treated with 10, 20 or 40 μM oridonin for 24 h, then cell culture medium was collected. LDH could catalyze the synthesis of pyruvic acid from lactic acid and then to form 2,4-dinitrophenyl-hydrazine, which showed brownish red color. After the reaction, the absorbance was read at wavelength of 450 nm.

2.4. Live and dead-cell staining

As we have demonstrated previously, after drug treatment, cells were washed by PBS for 3 times and then stained by the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen, USA) according to the manufacturer's instructions. The live and dead cells were detected and acquired under a fluorescence microscope, live cells appeared as green whereas dead cells as red.

2.5. Measurement of intracellular GSH

Intracellular level of GSH was measured by using a total GSH assay kit (Beyotime Biotech, China) according to the manufacturer's instructions. Cells were lysed in the protein removal solution S provided in the kit, and then samples were centrifuged at 10,000× *g* for 10 min at 4 °C. The supernatant was treated with Ellman's reagent (DTNB), GSH reductase enzyme and NADPH. The absorbance values were measured at a wavelength of 412 nm by a microplate reader.

2.6. Elisa assay

After drug treatment, cell culture medium was collected and HMGB1 concentration was determined using ELISA 96 well Assay Kits (Cloud-Clone Corp., USA). The culture medium was centrifuged at 10,000× *g* for 10 min at 4 °C, then 100 μL of supernatant was added to each well, and then processed according to the manufacturer's protocol. Finally, the plate was read for absorbance at 450 nm on a microplate spectrophotometer and samples were compared to the obtained standard curve.

2.7. Parp-1 activity assay

Parp-1 activity in 786-O cells was determined using a universal colorimetric Parp-1 assay kit (Trevigen, USA). After drug treatment, cells were lysed in Parp lysis buffer, which contained protease inhibitor. The homogenate was centrifuged at 16,500 × *g* for 15 min at 4 °C, and protein (50 μg) from each sample was added into a 96-well plate coated with histones and biotinylated poly ADP-ribose, and then Parp cocktail was also added for 60 min at room temperature. Next, the supernatant was aspirated and then the diluted with Strep-HRP was added for another 60 min at room temperature and then followed by TACS-Sapphire treatment for 15 min in the dark, then 0.2 M HCl was added to stop the reaction and the plate was read at wavelength of 450 nm.

2.8. Western blot analysis

Total protein was extracted from 786-O cells in lysis buffer. Protein concentrations were determined by using a BCA Protein Assay kit (Thermo Fisher, USA). Protein (50 μg) was subjected to SDS–PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore, USA), followed by incubation with the corresponding primary antibodies overnight at 4 °C. After incubation with HRP-conjugated secondary antibodies (Sigma-Aldrich, USA), chemiluminescence was determined using the Protein Simple Fluor Chem E image acquisition system. Membranes were re-probed with GAPDH to verity loading consistency.

2.9. Mouse xenograft assay and anti-tumor interventions

Six-week-old nude mice were obtained from the animal center of Dalian Medical University and were maintained under specific pathogen free conditions. Mouse care and use protocols were approved by the Animal Committee of Dalian Medical University in accordance with national guidelines. 786-O cells (1×10^6) were subcutaneously injected into the right flank. After transplantation, body weight and tumor size were measured every two days. To test the effects of 5-FU or oridonin on tumor growth, mice were randomly assigned into 4 groups and received the following treatment via the lateral tail vein once every 3 day for 32 days after one week transplantation. (1) saline; (2) oridonin (20 mg/kg); (3) 5-FU (25 mg/kg); (4) oridonin (20 mg/kg) combined with 5-FU (12.5 mg/kg). Tumor size was measured with calipers in two perpendicular diameters without the knowledge of the treatment groups. Tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

2.10. Data analysis

Data are presented as mean ± SEM. Significance was determined by using one-way ANOVA, followed by Tukey's post test. $P < 0.05$ was considered as significant.

3. Results

3.1. 5-FU inhibited cell proliferation in renal cancer cells by inducing apoptosis

To identify the cytotoxic effect of 5-FU in renal cancer cells, 786-O