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Original Articles

A new oridonin analog suppresses triple-negative breast cancer cells and tumor growth via the induction of death receptor 5

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ABSTRACT

Triple-negative breast cancer (TNBC) remains the leading cause of death among women with breast cancer worldwide. Oridonin is a natural anti-cancer compound that is isolated from the traditional Chinese herb *Rabdosia rubescens*. However, the antitumor efficacies of oridonin in the treatments of TNBC and other cancers are far from ideal. In this study, we investigated a series of newly designed oridonin analogs in terms of their actions against HCC1806 and HCC1937 TNBC cell lines and identified CYD-6-28, which significantly inhibits cancer cell proliferation and induces G2/M-phase cell cycle arrest and apoptosis. CYD-6-28 induces the expression of p21 and the cleavage of caspase-3, -7, -8 and PARP and inhibits the expression levels of Cyclin D1, $FLIP_L$ and XIAP. CYD-6-28 also inhibits the activations of STAT3 and AKT and induces the activation of ERK. We demonstrated that CYD-6-28 induces apoptosis at least partially by inducing the expression of death receptor 5 (DR5). Finally, CYD-6-28 significantly suppresses HCC1806 xenograft tumor growth in nude mice at 5 mg/kg without affecting body weight. Taken together, these results indicate that CYD-6-28 has the potential to be developed as a therapeutic agent to treat TNBC.

Introduction

Breast cancer is the most common life-threatening cancer among women worldwide. Breast cancer is the sixth leading cause of cancer death in Chinese women [1]. Presently, surgery, radiation therapy and chemotherapy are the most common treatments for breast cancer patients. Estrogen receptor (ER) α, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) triple-negative breast cancer (TNBC) is a subtype of breast cancer that accounts for approximately 15% of diagnosed breast cancers [2]. TNBC does not respond to endocrine therapy or other available targeted therapies [3,4]. Therefore, more effective and safer anti-cancer drugs are urgently needed for the treatment of TNBC.

Oridonin is an *ent*-kaurene diterpenoid that is isolated from the traditional Chinese herb *Rabdosia rubescens*. The use of *Rabdosia* in traditional Chinese medicine has a long history, and this herb has mainly been used as an anti-bacterial, anti-inflammatory and anti-angiogenic agent [5]. In 1977, the standard extract of this plant was first recorded in the China Pharmacopoeia [6]. Beginning in the mid-1970s, oridonin has drawn attention for its anticancer activity [7–10]. A few clinical studies have reported that the crude extract of *Rabdosia* may improve survival rates, hamper the growth of tumors and relieve the pain, especially in the treatment of esophageal and gastric cancers [11,12]. The anticancer mechanisms are mainly associated with the induction of cell cycle arrest, apoptosis and autophagy, the production of intracellular reactive oxygen species formation (ROS) and the regulation of telomerase [13–15]. Due to its important bioactivities, low toxicity and attractive antitumor profile, oridonin has become a promising candidate compound for cancer therapy. However, the potential of oridonin has been hampered by its limited efficacy and drug-like properties, such as aqueous solubility and rapid plasma clearance [16,17]. It is imperative to develop oridonin analogs with optimized drug properties and enhanced anticancer activities particularly for TNBC. Previously reported oridonin analogs have mainly been derived from the 1-*O* and 14-*O* derivatives [18–23]. We recently designed and generated a compound library of novel oridonin derivatives via convergent and stereo-controlled synthesis with the aid of NMR, mass

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spectrometry and X-ray techniques. These structure-based drug design approaches include efficient protection of group-free heterocyclization, ring A-based α, β-unsaturated enone formation, and novel scaffold constructions [24,25].

In this study, we investigated the activities of series of these oridonin analogs against HCC1806 and HCC1937 TNBC cell lines and identified the promising anticancer drug candidate CYD-6-28 in which a thiazole ring is fused at C-1 and C-2 of the A-ring to yield superior aqueous solubility and better cell permeability. CYD-6-28 inhibits the proliferation of TNBC and induces cell cycle arrest at the G2/M phase and apoptosis. Intriguingly, CYD-6-28 induces the expression of death receptor 5 (DR5) and thereby leads to apoptosis. This study presents a novel oridonin derivative that exists outside of the repertoire of compounds found in nature and exhibits greatly improved anticancer efficacy and a unique mechanism of suppression of TNBC.

Materials and methods

Compounds and cell lines

A series of newly designed oridonin analogs including CYD-6-28 was synthesized in Dr. Zhou's laboratory following our previously published methods. The chemical structures (CYD-6-28 is shown in Fig. 1A) were fully characterized by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrum (HRMS) analyses. The purities of the compounds were determined by analytical HPLC. The biologically evaluated compounds were >98% pure. Doxorubicin was purchased from Sangon Biotec (Shanghai, China). Oridonin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All cell lines were purchased from the American Type Culture Collection (ATCC). HCC1806 and HCC1937 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. These cell lines were validated by the STR analysis (Kunming Cell Bank, Kunming Institute of Zoology, Chinese Academy of Sciences). All media were purchased from Hyclone (Logan, UT, USA).

Antibodies

The anti-PARP, $FLIP_L$, XIAP, p21, pSTAT3, STAT3, pAKT, AKT, Cyclin D1, caspase 3, 7, and 8, and cleaved caspase 3, 7, and 8 antibodies were obtained from the Cell Signaling Company (Danvers, MA, USA). The anti-pERK, ERK and GAPDH antibodies were from Santa Cruz (Santa Cruz, CA, USA). The anti-DR5 antibody was from ProSci (Poway, CA, USA).

In vitro *cytotoxicity assays*

In vitro cytotoxicity was quantified with the sulforhodamine B (SRB, Sigma) assay. Briefly, 7×10^3 cells per well were plated in 96-well plates and treated with doxorubicin, oridonin, oridonin analogs including CYD-6-28, or DMSO at various concentrations for different times. The cells were fixed with 100 μL cold 10% trichloroacetic acid at 4 °C overnight and washed 5 times with deionized water. The cells were stained with 100 μL 0.4% (W/V) SRB in 1% acetic acid for 5 min. The plates were washed 5 times with 1% acetic acid and dried. Finally, 100 μL 10 mM Tris base was added to each well. The absorbance of each well was determined with a microplate reader (Epoch, Bio-Tek) at a wavelength of 530 nm.

DNA synthesis assays

DNA synthesis was measured with Click-iT® EdU Imaging Kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The cells were treated with the compounds and then incubated with EdU for 4 h. The cells were fixed and stained for DAPI (blue). The proportions of cells that incorporated EdU were determined with fluorescence microscopy. Ten random fields were observed, and the total numbers of cells and EdU-positive cells in each sample were counted. The proportion of EdU-positive cells (%) was taken as the EdU-positive cell number/the total cell number.

Cell cycle analysis

Cancer cells were seeded in six-well plates and treated with CYD-6-28 or DMSO at various concentrations for 24 h. The cells were harvested, washed twice with 1 × PBS and resuspended in 200 μL of 1 × PBS. The cells were fixed in 5 mL of ice-cold 70% ethanol at 4 °C overnight and stained with 100 μL propidium iodide (PI) buffer (0.1 mg/mL PI, Sigma-Aldrich), 0.6% NP-40, 1 mg/mL RNase A) in the dark for 30 min at 37 °C. The cells were then analyzed with Accuri™ C6 flow cytometry (BD Biosciences).

Apoptosis analysis

Cancer cells were incubated with CYD-6-28 or DMSO at different concentrations for 24 h. The cells were harvested, washed twice with cold 1 × PBS and resuspended in 100 μL binding buffer at a density of 2×10^6 cells/mL. The cells were then stained with 5 μL Annexin-V and 5 μL PI for 15 min in the dark at room temperature and subjected to analysis via Accuri™ C6 flow cytometry.

Western blotting (WB) analysis

Cancer cells were treated with CYD-6-28 or DMSO at different concentrations for 24 h or different times. The cells were washed with cold 1 × PBS and harvested in lysis buffer with a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) for 30 min on ice. The protein samples were centrifuged at 13,000 *g* for 10 min at 4 °C. The total protein concentrations were determined with DC™ Protein Assays (Bio-Rad, Hercules, CA, USA). Equal amounts of the protein samples were subjected to SDS-PAGE electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% nonfat milk and incubated with primary antibodies (1000 × dilution) overnight at 4 °C followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratory, West Grove, PA). Finally, the membranes were incubated with Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Shelton, CT) and then viewed on an ImageQuant LAS4000 Biomolecular imager (GE Healthcare, UK).

Knockdown of DR5

The small interfering RNA (siRNA) duplexes for the control and the DR5 gene have previously been described [26]. The siRNAs were synthesized by Ribobio (Guangzhou, China) and transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The silencing effects were evaluated by WB.

Tumorigenicity assays

Five- to six-week-old female nude mice were purchased from Hunan SJA Laboratory Animal Co., Ltd. The mice were maintained in a barrier unit with a 12 h light–dark switch. Freshly harvested HCC1806 cells (1×10^6 cells per mouse, resuspended in 100 μL of PBS) were injected subcutaneously near the third mammary fat pad of the mice, and two xenografts were established in an individual mouse. The mice were then randomly assigned to one of two groups (n = 5). When the tumor volumes reached nearly 80 mm^3, the mice were treated with 5 mg/kg of CYD-6-28 or vehicle via daily intraperitoneal injections. CYD-6-28 was dissolved in 50% DMSO with 50% polyethylene glycol for *in vivo* administration. The body weights and tumor volumes were measured twice per week, and the tumor volumes were calculated according to the formula $V = 0.5 \times L \times W^2$, where L = length (mm) and W = width (mm). The animal protocol was approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences.

Statistical analysis

The statistical analyses were performed with one-way analyses of variance for comparisons of multiple variables, and Student's tests were used to compare pairs of variables. Results were considered statistically significant at the level of $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$. The graphs were generated with GraphPad Prism version 5 (Graph Software, San Diego, CA). The results are presented as the means ± the standard deviations of three independent experiments.

Results

We investigated the growth inhibiting activities of a series of new oridonin analogs (2.5 μM) against the human TNBC cell lines HCC1806 and HCC1937 (Supplemental Fig. S1A). Of the 21 tested compounds, approximately half of the compounds appeared to have significant effects. CYD-6-28 (Fig. 1A) appeared to be one of the most potent anti-cancer compounds according to the half-maximal inhibitory concentration (IC_{50}). Importantly, CYD-6-28 is a nitrogen-enriched heterocyclic compound in which a thiazole ring is fused at C-1 and C-2 of the oridonin A-ring, which leads to superior aqueous solubility and improved cell permeability [25]. Given the strong effects of CYD-6-28, we tested this compound against four TNBC cell lines and found that CYD-6-28 dose dependently inhibited the growth of the TNBC lines (Supplemental Fig. S1B) with an IC_{50} of approximately 1–4 μM. A battery of further *in vitro* and *in vivo* anticancer studies of the drug candidate CYD-6-28 was performed.

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