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OP16, a novel *ent*-kaurene diterpenoid, potentiates the antitumor effect of rapamycin by inhibiting rapamycin-induced feedback activation of Akt signaling in esophageal squamous cell carcinoma

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ABSTRACT

Hyperactivation of mTOR signaling pathway has been viewed as a significant molecular pathogenesis of cancer. However, inhibition of mTOR by rapamycin and its analogs could induce numerous negative feedback loops to attenuate their therapeutic efficacy. As a traditional Chinese herbal medicine, Rabdosia rubescens has been used to treat esophageal squamous cell carcinoma (ESCC) for hundreds of years, and its major effective component is oridonin. Here we reported that OP16, a novel analog of oridonin, showed potent inhibition of cell proliferation and Akt phosphorylation in ESCC cells. The combination of OP16 and rapamycin possesses synergistic anti-proliferative and pro-apoptotic effects both in ESCC cells and ESCC xenografts, and no obvious adverse effect was observed *in vivo*. Mechanistic analysis revealed that OP16 could inhibit rapamycin-induced Akt activation through the p70S6K-mediated negative feedback loops, and the combination of OP16 and rapamycin was more effective in activating caspase-dependent apoptotic signaling cascade. This study supports the combined use of OP16 with rapamycin as a feasible and effective therapeutic approach for future treatment of ESCC.

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1. Introduction

Esophageal cancers are prominent worldwide and cause the deaths of over 400,000 people annually [1]. Generally, esophageal cancer is classified by histopathology as adenocarcinoma (EAC) or squamous cell carcinoma (ESCC) [2]. ESCC is one of the most common and aggressive cancers in some areas including China, South America and Western Europe [1,3]. Despite improvements in therapeutic strategies and surgical techniques, the 5-year survival rate of patients with ESCC remains dismal [4], which drives us to explore novel pharmacological strategies for its treatment.

Mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that regulates various cellular processes, including cell growth, proliferation, differentiation, metabolism and autophagy [5]. mTOR comprises two distinct multi-protein complexes designated mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which exerts different but related functions [6]. mTORC1 regulates translation initiation, protein synthesis and cell cycle progression by phosphorylating p70 ribosomal S6 kinase 1 (p70S6K) and 4E-binding protein 1 (4E-BP1), and mTORC2 regulates cell survival and metabolism through phosphorylating Akt [7,8]. Hyperactivation of mTOR signaling pathway has been viewed as a significant molecular pathogenesis of cancer [9], including ESCC [10,11].

Rapamycin and its analogs (rapalogs) such as RAD001 and CCI-779 are highly selective mTORC1 allosteric inhibitors with broad antitumor effects, and have been approved by FDA for the treatment of various tumor types [12–14]. Unfortunately, the efficacy of these rapalogs as broad-based monotherapy for the treatment of cancer patients has not been as promising as initially expected [15]. Accumulating evidences have demonstrated that rapalogs

Abbreviations: Akt, protein kinase B; DMSO, dimethyl sulfoxide; ESCC, esophageal squamous cell carcinoma; FBS, fetal bovine serum; IC_{50}, half maximal inhibitory concentration; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3 kinase; Rapa, rapamycin; Rapalogs, rapamycin and its analogs; Rictor, rapamycin-insensitive companion of mTOR.

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could induce numerous negative feedback loops emanating from p70S6K to PI3K or mTORC2, which dramatically lead to reactivation of PI3K/Akt signaling pathway, thus attenuating anticancer effects of rapalogs [16–18]. Therefore, combined inhibition of PI3K or mTORC2 together with mTORC1 may be an alternative way to circumvent the rapalogs-induced feedback loops [19].

Natural compound has been serving as an invaluable source for medicine discovery. In the past centuries, Rabdosia rubescens has been used as a traditional Chinese herbal medicine to treat ESCC which is one of the most frequently diagnosed carcinoma in China, and has been listed in the Chinese Pharmacopeia [20]. Oridonin, an *ent*-kaurene diterpenoid extracted from Rabdosia rubescens, was reported to exert extensive antineoplastic effects by targeting cell cycle arrest, apoptotic and autophagic pathways [21,22]. However, some characteristics of oridonin have hindered its further clinical applications, like the relatively moderate therapeutic efficacy, limited aqueous solubility and low structural stability [23]. OP16, a novel analog of oridonin, was designed and synthesized to overcome these disadvantages. In this study, the antitumor effects of OP16 alone or combined with rapamycin in ESCC were investigated both *in vitro* and *in vivo*, and the molecular mechanisms underlying the synergistic antitumor activity were detected.

2. Materials and methods

2.1. Reagents and antibodies

Rapamycin was purchased from Sigma-Aldrich (USA). Primary antibodies against p-Akt (Ser473) (D9E), p-Akt (Thr308) (244F9), Akt (pan) (C67E7), p-p70S6Kinase (Thr389) (1A5), p70S6Kinase (49D7), PI3 Kinase p85α (6G10), PI3 Kinase p110α (C73F8), p-Rictor (Thr1135) (D30A3), Rictor (D16H9), Bax (D2E11), Bcl-2 (124), cleaved caspase-3 (Asp175) (5A1E), cleaved caspase-9 (Asp330) (D2D4), GAPDH (D16H11) and HRP-linked secondary antibodies were purchased from Cell Signaling Technology (USA).

2.2. Synthetic pathway of compound OP16

Compound OP16 is a 20-acetal derivative of JDA which is an *ent*-kaurene diterpenoid extracted from Rabdosia rubescens produced in Henan province of China. Compound JDA and its derivatives including OP16 had been patented in China and the United Stated [24,25]. The synthetic pathway of OP16 is outlined in Fig. 1A. In brief, JDA and 1-propanol were resolved in anhydrous chloroform and stirred in room temperature. Then one drop of strong sulfuric acid was added. After stirring for 1–2 h, the system was washed twice using saturated Na_2CO_3 and the chloroform layer was dried by anhydrous Na_2SO_4. After all the organic solvent was distilled, the residual solid was recrystallized using ethanol. And the target compound OP16, a white solid or power, was obtained. The purity of OP16 was determined to be >99% by HPLC.

2.3. Cell lines and experimental animals

Five ESCC cell lines (EC9706, TE-1, ECa109, KYSE790 and KYSE450) were obtained from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). EC9706 and TE-1 are poorly differentiated cell lines, and ECa109, KYSE790 and KYSE450 are well differentiated cell lines. Cells were cultured in RPMI-1640 medium (Biological Industries, Israel) containing 10% FBS (Biological Industries, Israel) at a CO_2 incubator with saturated humidity, 5% CO_2 and 37 °C, as described before [26].

All animal studies were approved by the Ethics Committee of Zhengzhou University, and were performed in compliance with the NIH Guide for Care and Use of Laboratory Animals. Forty female athymic BALB/c nude mice (SPF grade, 6 weeks old; Silikejingda Experimental Animal Ltd., China) were used in this study. All animals were housed in individually ventilated cage at a room temperature of 20 ± 2 °C, with 50 ± 10% relative humidity and a 12 h light/dark regime. All animals were provided with normal diet and purified water ad libitum, and acclimatized to the laboratory environments for at least 7 days before the experiments.

2.4. Cell proliferation assay

Cell proliferation was analyzed using Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, China) according to manufacturer's instructions. In brief, the cells were seeded in a 96-well flat-bottomed plate at a density of 6×10^3 cells per well, and cultured at 37 °C for 24 h. Subsequently, the cells were treated with OP16 and rapamycin alone or in combination for 48 h. After 10 μL CCK-8 reagent was added to each well, the cells were incubated at 37 °C for 4 h and then the absorbance was finally determined at 450 nm using a microplate reader (Bio-Rad Laboratories, USA). The combination index (CI) was calculated by CompuSyn software (ComboSyn Inc., USA).

2.5. Cell apoptosis assay

Cell apoptosis was measured by the Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech, China) according to manufacturer's instructions. In brief, cells treated with OP16 (10 μM) and rapamycin (50 nM) alone or in combination for 48 h were harvested and rinsed twice with cold phosphate-buffered saline (PBS). Subsequently, cells were resuspended in 500 μL binding buffer containing 5 μL Annexin V-FITC staining solution and incubated in the dark for 15 min, and then 5 μL PI staining solution was added to the cell suspension before analysis by a BD Accuri™ C6 flow cytometer (Becton Dickinson, USA).

2.6. Colony formation assay

EC9706 cells were seeded in 6-well plates at a density of 1500 cells per well, and cultured at 37 °C for 24 h. Subsequently, the cells were treated with OP16 (10 μM) and rapamycin (50 nM) alone or in combination for 12 h, and then the cells were cultured for an additional 10 days. Culture medium was changed every 3 days. At the end of experiment, the colonies were fixed with methanol, stained with crystal violet (Beyotime Institute of Biotechnology, China) and counted using ImageJ software (NIH, USA).

2.7. In vivo studies of antitumor effect and toxicity

EC9706 cells were harvested, rinsed, and resuspended in PBS, and cell resuspension of 200 μL (containing 4×10^6 cells) was inoculated subcutaneously into the right forelimb of each BALB/c nude mouse (SPF grade, 6 weeks old). When the tumor volume reached 60–80 mm^3 [27], the animals were randomly divided into four groups of ten animals each: control group (saline, 0.2 mL/kg/2 day, intravenous injection), OP16 group (20 mg/kg/2 days, intravenous injection), rapamycin group (50 μg/kg/2 days, intraperitoneal injection) and OP16 + rapamycin group (OP16: 20 mg/kg/2 day, intravenous injection; rapamycin: 50 μg/kg/2 days, intraperitoneal injection). During treatment, the tumor volume and body weight of each animal were measured every day. Tumor volume was calculated as: tumor volume (mm^3) = (longest diameter) × (shortest diameter)^2/2.

After being treated for 2 weeks, blood samples were collected from mice orbit and stabilized by EDTA to measure the routine haematological parameters using an automatic hematology

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