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Brevilin A induces apoptosis and autophagy of colon adenocarcinoma cell CT26 via mitochondrial pathway and PI3K/AKT/mTOR inactivation

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ABSTRACT

Brevilin A is a sesquiterpene lactone isolated from *Centipeda minima* and possesses inhibitory effects on proliferation of various tumor cells. In this study, Brevilin A inhibitory effect on proliferation and its molecular mechanism of action were investigated both *in vivo* and *in vitro* in colon adenocarcinoma CT26 cells. The results indicated that the inhibitory effect of Brevilin A in CT26 proliferation was dose-dependent and this effect was due to apoptosis. Furthermore, Brevilin A increased ROS levels, decreased mitochondrial membrane potential (MMP) and induced apoptosis of CT26 cell in a dose-dependent manner. Apoptosis induced by Brevilin A was higher than that induced by adriamycin under the same dose. Cleaved-caspase-8, cleaved-caspase-9 and cleaved-caspase-3 were up-regulated after Brevilin A treatment, together with an increase of Bax protein expression, while Bcl-2 was reduced. Further investigation revealed that Brevilin A inhibited the phosphorylation of PI3K, AKT and mTOR and promoted the expressions of autophagy-related proteins LC3-II, Beclin1 and Atg5 and consequent formation of autophagosomes, whereas 3-methyladenine (3-MA), a type III PI3K inhibitor, inhibited autophagosomes formation induced by Brevilin A. *In vivo* investigation suggested that Brevilin A significantly inhibited the growth of CT26 tumor compared to adriamycin and concurrently promoted the expressions of LC3-II and cleaved-caspase-3 in tumor tissues. Our results demonstrated that the anti-tumor activity of Brevilin A was mainly achieved by the induction of cell apoptosis and autophagy, suggesting a promising potential as antitumor drug against colon adenocarcinoma.

1. Introduction

Colon adenocarcinoma is one of the most common tumors, which occurs on the lining of human colon and its morbidity and mortality ranked third and fourth, respectively [1]. Surgery, radiotherapy and chemotherapy are currently the major treatments but show disadvantages such as high toxicity, severe side-effects and high relapse rate. Since antitumor drugs derived from natural products exhibit great advantages including multi-targets, low toxicity, slight side-effect and lower chance to generate drug resistance, more researchers focus on searching more and better new antitumor drugs from natural products and Chinese traditional herbs.

Centipeda minima is a Chinese traditional herbs used for relieving stuffy nose, cough and asthma [2]. It has been reported that extracts from *Centipeda minima* possess antibacterial [3], antiallergic [4] and antitumor [5] activities. Brevilin A is a sesquiterpene lactone firstly extracted from *Centipeda minima* and displays inhibitory effects on proliferation of various tumor cells [6–8]. Previous research demonstrated that Brevilin A induced the increase of ROS levels, decrease the mitochondrial membrane potential (MMP), activate caspase-3/7 as well as induce cell apoptosis *via* inhibiting the activation of NF-κB in HL60 cell [9]. Additionally, Brevilin A led to the dissociation and degradation of Skp2 by binding to Skp1, resulting in lung cancer cell A549 restriction in G2/M phase [10]. Other research showed that Brevilin A was an inhibitor for both STAT3 signal and JAK signal [11]. In this study, we found that Brevilin A represses the proliferation of 5 different types of tumor cells and the greatest inhibitory effect was achieved against colon adenocarcinoma CT26 cells. Thus, further studies both *in vivo* and *in vitro* were performed on the inhibitory effects and mechanism of Brevilin A against CT26 colon adenocarcinoma.

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2. Materials and methods

2.1. Chemicals

Brevilin A (purity > 96%, HPLC and spectral analysis) was isolated and purified from *Centipeda minima* in our lab, adriamycin was purchased from Shenzhen Main Luck Pharmaceuticals Inc.

2.2. Animals

6–8 week BALB/c male mice (25 ± 2 g) were purchased from Hubei Research Center of Laboratory Animals. All animals were fed in SPF lab of Experimental Animal Center of Hubei University of Chinese Medicine. All animal experiments were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Committee on the Ethics of Animal Experiments of Hubei University of Chinese Medicine.

2.3. Cell culture and treatment

The following cell strains were used and purchased from China Centre for Type Culture Collection: human lung adenocarcinoma cell line A549, human liver carcinoma cell line HepG2, human cervical cancer cell line Hela, human melanoma cell line A875 and mouse colon adenocarcinoma cell line CT26. All cells were cultured in DMEM (Gibco, USA) medium containing 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5% CO_2. Cells were treated with Brevilin A at the concentrations of 1, 2, 4, 6, 8, 10 μg/ml for different times.

2.4. Cell viability assay

CT26 cells were seeded into 96-well plates at the concentration of 8×10^3 cells/well/200 μl and treated by different concentrations of Brevilin A as indicated above. Cell Counting Kit-8 (CCK8) (Engreen Biosystem, China) was added into each well and then incubated for 4 h at 37 °C. The absorbance was measured at 450 nm by Model 680 microplate reader (Bio-Rad, USA).

2.5. ROS measurement

The level of intracellular ROS was quantified using a fluorescent probe 2′, 7′-dichloro-cluorescein diacetate (DCFH-DA) (Beyotime, China). Cells were seeded in 6-well plates (5×10^5 cells/well) and treated with Brevilin A at the concentrations of 1, 2, 4 μg/ml and adriamycin 2 μg/ml for 48 h, cells were harvested and incubated with 10 μM DCFH-DA for 20 min at 37 °C in the dark. After incubation, the cells were washed three times PBS and analyzed by flow cytometry.

2.6. Mitochondrial membrane potential analysis

The mitochondrial transmembrane potential was analyzed by flow cytometry using the mitochondrial membrane potential assay kit with JC-1 (Beyotime, China), Cells were seeded in 6-well plates (5×10^5 cells/well) and treated with Brevilin A at the concentrations of 1, 2, 4 μg/ml and adriamycin 2 μg/ml for 48 h, cells were incubated with JC-1 for 20 min at 37 °C. After incubation, the cells were washed three times PBS and analyzed by flow cytometry.

2.7. Apoptosis analysis

CT26 cells were seeded into 12-well plate at the concentration of 1×10^5 cells/ml and treated with Brevilin A at the concentrations of 1, 2, 4 μg/ml and adriamycin 2 μg/ml for 72 h. Next, cells were collected and stained using Annexin V-FITC/PI kit (KeyGEN Biotech, China) according to the manufacturer's instructions and analyzed using a flow cytometer with Cell Quest Software (FACSCalibur, BectonDickinson, USA).

2.8. Western blot analysis

CT26 cells were seeded into 6-well plate at the concentration of 1×10^6 cells/ml and treated as mentioned above for 48 h. Total protein extraction was performed using RIPA lysis Kit (Beyotime, China) containing 1 mM phenylmethanesulfonyl fluoride (Beyotime) and BCA protein assay kit (Takara, Japan) was used for quantitative analysis. An equal amount of protein samples was loaded on SDS-PAGE for electrophoresis and subsequently transferred to nitrocellulose membranes (Amersham Biosciences, UK). The membrane was blocked in TBST with 5% skim milk for 2 h at room temperature followed by an overnight incubation with primary antibodies diluted 1:1000 in TBST at 4 °C. The antibodies used were the following: anti-cleaved-caspase-8, anti-cleaved-caspase-9, anti-cleaved-caspase-3, anti-Bax, anti-Bcl-2, anti-p-PI3K, anti-p-AKT, anti-p-mTOR, anti-LC3-II, anti-Beclin1 and anti-Atg5 (Cell Signaling Technology, USA). Then the membrane was washed by TBST for three times followed by incubation with HRP anti-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature. The ECL detection reagent (Bio-Rad) was use for signal detection, according to the manufacturer's instructions.

2.9. Confocal microscopy

CT26 cells were seeded into 6-well plate at the concentration of 1×10^6 cells/ml and treated with 4 μg/ml Brevilin A, or 100 nM rapamycin or Brevilin A + 10 mM 3-MA for 48 h. Fixation was achieved using 4% paraformaldehyde for 20 min followed by cell membrane permeation using 0.1% Triton X-100 for 30 min. After washing in PBS, slides were blocked in 5% BSA for 1 h and subsequently incubated overnight in Alexa Fluor® 555 Conjugated LC3-II XP® Rabbit mAb (Cell Signaling Technology) at 4 °C. The signal was analyzed using a confocal laser scanning microscope system (Leica TCS SP2, Germany) after staining with DAPI for 5 min.

2.10. Murine model

Tumors were implanted by injection of 1.5×10^6 cells/150 μl CT26 cell suspension into the subcutaneous tissue of the right back of the mice. Mice were then randomly divided into three groups (n = 10). When the tumors volume reached about 40 mm^3, 5 mg/kg Brevilin A, 5 mg/kg adriamycin and PBS were peritumorally injected into CT26-bearing mice every day for 14 times and mice weight was recorded every day. Mice were sacrificed 24 h after the last drug injection. The solid tumor sizes were measured every day, and the tumor volumes and tumor growth inhibition rate were calculated by the following formula:

Tumor volume (mm^3) = (length × $width^2$)/2, where the length and width are given in mm.

Tumor inhibition rate (%) = [(Average tumor volume of the control group − average tumor volume of the test group)/average tumor volume of the control group] × 100

2.11. Immunohistochemistry

Tumor tissues from different groups were fixed by 4% paraformaldehyde and embedded in paraffin. Then the tissues were cut into 4 μm sections followed by dewaxing, antigen retrieval, blocking with 3% BSA (Sigma, USA) for 30 min and incubation overnight in anti-cleaved-caspase-3 and anti-LC3-II (Cell Signaling Technology) at 4 °C. Subsequently, slices were washed for three times followed by incubation with HRP anti-conjugated secondary antibody (Cell Signaling Technology) for 1 h, and visualized with 3, 3′-diaminobenzidin. Images