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Full Length Article

Steroidal Saponin *Ypsilandra thibetica* Induces Apoptosis in Colorectal Carcinoma Cells through the Mitochondrion-Dependent Reactive Oxygen Species Pathway

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Abstract

The whole plant of *Ypsilandra thibetica* Franch, a perennial herb, has the officinal functions in heat-clearing, detoxifying and relieving congeation. YB16, one of the steroidal saponin in *Y. thibetic*, exerts anticancer activity in prostate cancer and lung cancer. However, the bioactivity and underlying mechanisms of YB16 in colorectal carcinoma (CRC) are not understood. In the present study, by MTT, apoptosis, mitochondrial membrane potential levels and intercellular reactive oxygen species (ROS) assays, YB16 inhibited the proliferation of cells and induced apoptosis in CRC HCT116 and HCT8 cells. Meanwhile, YB16 provoked mitochondrial membrane potential depolarizing in a concentration-dependent manner. Further analysis showed that YB16 induced apoptosis *via* stimulating the production of intercellular ROS and the antioxidant NAC reversed the apoptosis in HCT116 and HCT8 after YB16 treatment. These datas demonstrated that YB16 induced the apoptosis in CRC cell lines *via* mitochondrial-dependent ROS pathway and YB16 might be a potential anticancer drug to CRC.

Key words: YB16; Apoptosis; Colorectal carcinoma; Mitochondrion; ROS
Abbreviations: CRC, colorectal carcinoma; ROS, reactive oxygen species; TCM, traditional Chinese medicine; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; HRP, horseradish peroxidase; AO, Acridine orange; JC-1, fluorescence tetraethyl benzimidazoly carbocyanineiodide; DCFH-DA, dichlorofluorescin diacetate; PARP, cleaved-poly (ADP-ribose) polymerase.

Introduction

Colorectal carcinoma (CRC) is one of the most occurring epithelial malignant cancers around the world (Fidler *et al.*, 2018; Pantoja *et al.*, 2018). At present, the main treatments of CRC are surgery, radiation, chemotherapy and traditional Chinese medicine (TCM) treatment (Fidler *et al.*, 2018). The TCM, used for thousands of years in clinical, is the hotspot on the researches because of the fewer side effects, life quality improvement, immune function improvement and prolongation of survival (Wang *et al.*, 2018; Wu *et al.*, 2018).

Traditional medical treatments involving phytotherapy have played important roles for people throughout history and modern medicine owes success to these practices. *Ypsilandra* is a small genus including only six herbaceous plant species and distributed in southwesten China and Myanmar. *Ypsilandra thibetica* Franch (Family Liliaceae), a perennial herb, is especially in Sichuan and Yunnan Provinces and the whole plant has medicinal functions, including heat-clearing, detoxifying and relieving congeation, which is used as the TCM to cure hydrops, scrofula and other symptoms (Wang *et al.*, 2018; Wu *et al.*, 2018). The plant materials of *Ypsilandra* were collected from Emei Mountain, Sichuan Province, China. YB16, one of the steroidal saponins in *Y. thibetica* (Fig. 1A), was isolated and kindly donated by research fellow Zhang (Xia *et al.*, 2016a). Emerging evidence indicated that the extract of *Y. thibetica* inhibits the growth of prostate cancer, lung cancer and other cancer cells by decreasing the mitochondrial membrane potential, generating intercellular reactive oxygen species (ROS) (Xia *et al.*, 2013; Xie *et al.*, 2013). However, the bioavailability and molecular mechanisms of YB16 in CRC have not been confirmed. In the present study, for the first time I had been studied that YB16 exerts the functions in induction apoptosis, suppressing cell proliferation, together with decreasing the mitochondrial membrane potential and ROS generation in human CRC cells. Therefore, YB16 can be a potential anticancer drug.

Materials and Methods

Cell Cultures

In present studies, the human CRC cells HCT116 and HCT8

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were kindly donated from professor Du at School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China. Cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 (GIBCO-BRL, USA), combined with 10% fetal bovine serum (GIBCO-BRL) in a humidified atmosphere at 37°C with 5% CO_2.

Cellular Viability Assay

The cellular viability assay was detected by MTT (Merck Millipore EA, Germany) (Zhao *et al.,* 2018) on the basis of the manufacturer's instruction. The absorbances of every well were measured in a microplate at 490 nm. The IC_{50}s were calculated by SPSS and the map of cell survival was performed by GraphPadPrism.

Western Blot

In brief, the total protein in the cells was collected in RIPA lysis buffer and the equal quantity of proteins (50 μg/sample) was electrophoretically separated by SDS-PAGE. And then, it was blotted on PVDF membranes. The membranes were probed with corresponding primary antibodies (1:1000 in volume; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Members were incubated with a horseradish peroxidase (HRP)-conjugated anti-IgG (1:5000 in volume; Bioworld Technology, MN). After washed for 30 min by PBST, they were exposed to automatic exposure machine Tanon by chemiluminescence (ECL) kit to detect the target protein.

Acridine Orange (AO) Staining

After cultured in 12-well plates overnight, HCT116 and HCT8 (3×10^5/mL) were treated with YB16 for 24 h. Cells were stained by 10 mL of 30 mg/l AO (Beyotime Biotechnology, Shanghai, China) after discarding the supernatant. The fluorescence was detected under a fluorescence microscope (NIKON Eclipse 55i, M.A., U.S.A.).

JC-1 Staining for Mitochondrial Membrane Potential (MMP)

The MMP was analyzed by JC-1 assay kit (Abcam, Cambridge, UK) according to the manufacturer's instruction. HCT116 and HCT8 cells were plated into 12-well plates for 24 h treatment, and cells were exposed to JC-1 dye (1 μM). Finally, cells were imaged by the fluorescent microscope.

ROS Assay

The intracellular ROS were measured by flow cytometry analysis of dichlorofluorescin diacetate (DCFH-DA) fluorescence. Cells were incubated for 15 min at 37°C with 5μM DCFH-DA. Cells were then trypsinized, centrifuged and re-suspended in cold PBS and kept on ice, protected from light. The fluorescence value was measured on the FL-1 channel of flow cytometry.

Statistical Analysis

All experiments included triplicates samples. The representative results were exhibited as mean ± SD. The statistical analysis was operated by SPSS 13.0. Student's t-test was applied to two group comparisons and the 1-way analysis of variance (ANOVA) was used on multi group comparisons. Statistical significance * was identified as p value <0.05.

Results

YB16 Inhibits the Cell Proliferation

To reveal the effect of YB16 in CRC cell lines, first time the cell proliferation was examined by MTT. HCT116 and HCT8 cells were handled with YB16 at the concentration gradients (0.234, 0.469, 0.938, 1.875, 3.750, 7.500, 15.000 and 30.000 μM, respectively) and cell viability was examined after 48 h treatment. The results showed that YB16 significantly inhibited HCT116 and HCT8 cell proliferations in the concentration-dependent way (Fig. 1B). The IC_{50} of HCT116 to YB16 was (1.452 ± 0.012) μM and the IC_{50} of HCT8 to YB16 was (5.471 ± 0.011) μM. Therefore, 0.5, 1, 2 μM YB16 were used to treat with HCT116 and 1, 3, 6 μM YB16 with HCT8 for the following experiments.

YB16 Induces the Cell Apoptosis

To further prove the inhibitory ability of YB16 on CRC cell lines, the apoptosis alteration in HCT116 and HCT8 cells were analyzed. After treatment of YB16, the cells were captured by microscope, the images showed that the cells shrink gradually and die according to the concentration of YB16 increases (Fig. 2A). The apoptosis markers Bax, cleaved- caspase 3, cleaved- poly (ADP-ribose) polymerase (PARP), and Bcl-2 were detected by WB. The results indicated that the cleaved-PARP, cleaved-caspase 3 and Bax expressions were upregulated as the concentration of YB16 increased (Fig. 2B). Furthermore, the AO staining showed that after YB16 treatment, the apoptosis features rose in HCT116 and HCT8 cell lines. These results declared that YB16 lead to cell apoptosis in HCT116 and HCT8 (Fig. 2C).

YB16 Decreases the Cells Mitochondrial Membrane Potential

JC-1 was used to detect the change of mitochondrial membrane potential to evaluate whether YB16-induced CRC apoptosis is related to the mitochondrial pathway. In present study, HCT116 and HCT8 were treated with YB16

for 24 h and then stained with JC-1. By comparing to the control cells, the cell lines treated with YB16 displayed significant green fluorescence (Fig. 3A). Moreover, the green fluorescence intensity ratio dramatically increased at a concentration-dependent way (Fig. 3B and C). These results showed that YB16 generated a significant decrease in mitochondrial membrane potential on HCT116 and HCT8 cells.

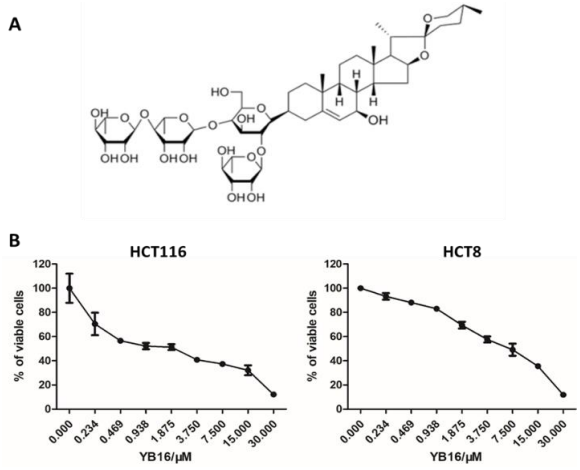

Fig. 1: (A) The structure of YB16 ($C_{15}H_{82}O_{20}$); (**B**) YB16 inhibition of HCT116 and HCT8 cell proliferation

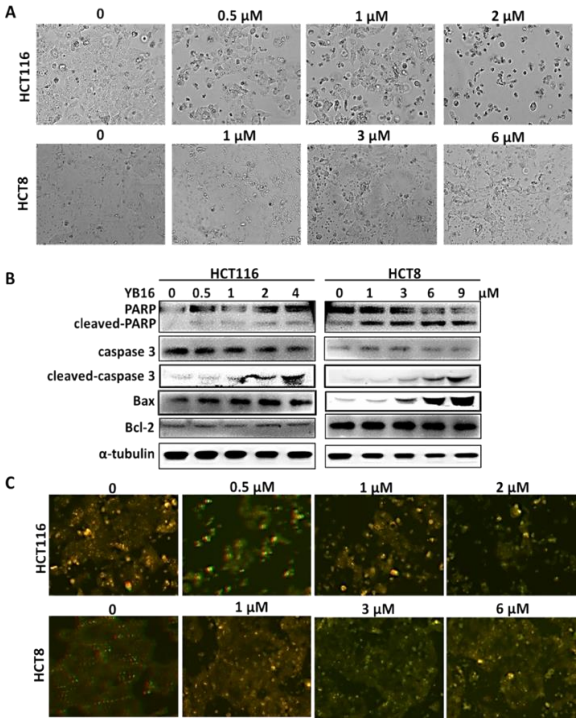

Fig. 2: YB16 induces apoptosis of HCT116 and HCT8. (**A**) Representative images of cells in control and YB16-treated. (**B**) Expression of cell death related proteins in HCT116 and HCT8 cells were detected by western blot. (**C**) Fluorescent micrographs of acridine orange (AO) stained HCT116 and HCT8 cells after treatment with YB16

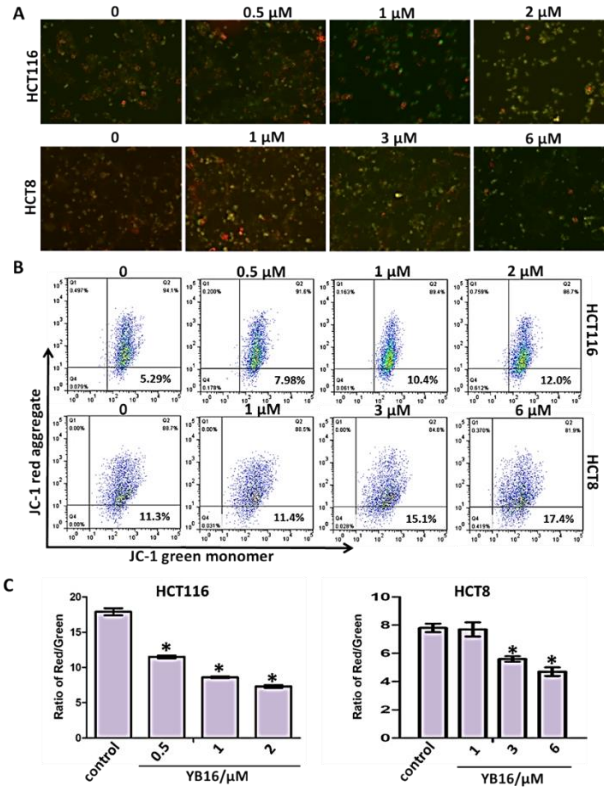

Fig. 3: The effect of YB16 on HCT116 and HCT8 cells mitochondrial membrane potential. (**A**) Fluorescent micrographs (×100) of JC-1 stained. (**B**) HCT116 and HCT8 cells were stained with DCFH-DA and analyzed by flow cytometry after treatment of YB16. (**C**) The ratio of JC-1 aggregates and monomers were calculated in triplicates by flow cytometry analysis. *$P < 0.05$

YB16 Induces Apoptosis through ROS Generation

In order to resolve whether YB16 induced the generation of ROS, HCT116 and HCT8 cells were stained with DCFH-DA for flow cytometry analysis after the YB16 treatment. The ROS levels showed that YB16 induced ROS generation in a concentration-dependent manner in CRC cells (Fig. 4A). Furthermore, the generation of ROS caused by YB16 was reversed by NAC, the inhibitor of ROS. More importantly, NAC also reversed the upregulated apoptotic markers induced by YB16 (Fig. 4B). Therefore, these results demonstrated that YB16 induced the apoptosis of CRC cells through ROS generation.

Discussion

The steroidal saponins YB16 was isolated and purified from *Y. thibetica* possess significant anticancer activities in many pharmacological studies (Si *et al.*, 2014; Xia *et al.*, 2016b). However, both cellular and molecular mechanisms of YB16 bioactivities in CRC cell lines are ambiguous. The present

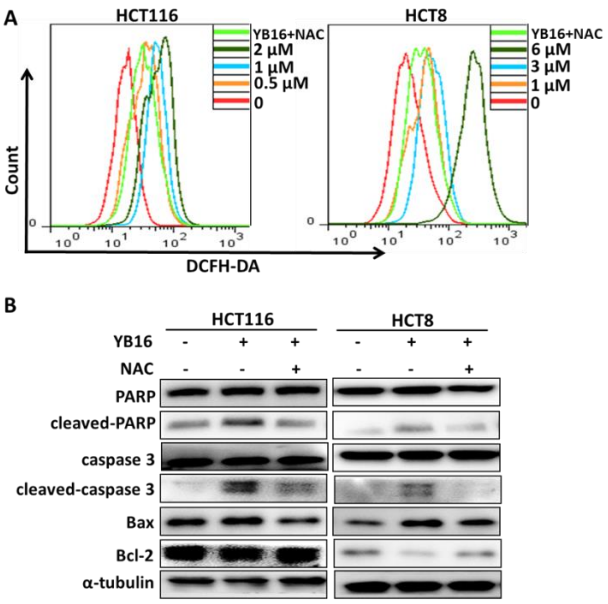

Fig. 4: The effect of YB16 on HCT116 and HCT8 cells ROS. HCT116 and HCT8 cells were treated with YB16 alone or with 1 mM NAC for 24 h. (**A**) The generation of ROS in HCT116 and HCT8 was evaluated by flow cytometry. (**B**) The expression of cell death related proteins were detected by western blot assay

study found that YB16 significantly inhibited the proliferation of HCT116 and HCT8 CRC cells in a dose-dependent manner. Among HCT116 and HCT8, HCT116 was more sensitive to YB16 treatment than HCT8, demonstrating that YB16 may exhibit a relatively selective antitumor effect.

Considerable studies have showed that the natural compounds isolated from TCM triggered cell apoptosis and then induced cells death of cancer cells (Han *et al.*, 2018; Lu *et al.*, 2018; Ye *et al.*, 2018). In present study, YB16 significantly exhibited strong cytotoxicity against HCT116 and HCT8. The apoptosis led by cytotoxicity is executed to the caspase proteases cascading activation (Hadj-Moussa *et al.*, 2018), which leads to the executioner caspase 3 and PARP activation (Morris *et al.*, 2018).

The apoptotic marker proteins were significantly increased and the apoptosis features also rose after YB16 treatment. Moreover, the apoptotic pathways are including death receptors and mitochondrial pathways (Sifuentes-Franco *et al.*, 2018; Tao *et al.*, 2018). Both death receptors and mitochondrial pathways are two important pathways which can initiate apoptotic responses (Ashkenazi and Dixit, 1998; Orrenius, 2004). In present study, YB16 decreased the red-to-green fluorescence intensity ratio of JC-1, demonstrating the decreased ΔΨm and the mitochondrial membrane depolarization. As a result, it was confirmed that YB16 induced a mitochondrial pathway-mediated apoptosis to HCT116 and HCT8 cells. Furthermore, the intrinsic apoptotic signaling pathways are especially susceptible to ROS and various chemotherapeutic medicines exert antitumor effects by inducing the ROS generation (Bolduc *et al.*, 2018; Reshetnikov *et al.*, 2018). In present study, the production of intercellular ROS increased significantly in HCT116 and HCT8 cell lines treated by YB16 in a dose-dependent way, while the ROS productions were inhibited by the antioxidant NAC, the apoptosis in HCT116 and HCT8 was significantly decreased. The results illuminated that YB16-induced apoptosis in HCT116 and HCT8 cell lines were closely associated with the generation of ROS.

Conclusion

The present study suggested that YB16 induced apoptotic cell death in HCT116 and HCT8 cell lines *via* triggering mitochondrial-mediated ROS generation pathways. These studies provide an insight into both the functions and molecular mechanisms of YB16 caused apoptosis in CRC cells. Hence, YB16 might become a new promising compound for the treatment of CRC.

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