

Original Research Article

Bergenin suppresses the growth of colorectal cancer cells by inhibiting PI3K/AKT/mTOR signaling pathway

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Abstract

Purpose: To investigate anticancer effects of bergenin on human colorectal cancer cell lines.

Methods: Human colorectal adenocarcinoma cell line HCT116 was treated with various concentrations of bergenin for 24 and 48 h. Cell viability, apoptosis, cell cycle arrest and reactive oxygen species (ROS) level were analyzed by MTT, flow cytometry and fluorescent dye assays, respectively. DNA damage-associated protein expressions were analyzed by Western blotting.

Results: Bergenin significantly suppressed the viability of HCT116 cells. Moreover, bergenin induced cells to accumulate in G1 phase and resulted in DNA breaks in HCT116 cells. It also led to marked accumulation of intracellular reactive oxygen species (ROS), a breaker of DNA strand in HCT116 cells. Interestingly, bergenin inhibited PI3K/AKT/mTOR pathway.

Conclusion: Bergenin effectively suppresses the growth of colorectal adenocarcinoma by inducing generation of intracellular ROS, DNA damage and consequent G1 phase arrest via inhibition of PI3K/AKT/mTOR pathway.

Keywords: Bergenin, Colorectal cancer, DNA damage, Cell cycle arrest, PI3K/AKT/mTOR

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related death, with between one and two million new cases being diagnosed every year, and its incidence has been increasing year by year [1]. Modifiable risk factors for CRC relating to lifestyle include smoking, physical activity habits, overweight, obesity and alcohol consumption. Chemotherapy and surgery are the most common treatment for CRC [2]. With the development and application of a new generation of chemotherapy and molecular-targeted drugs, the effects of CRC

treatment have improved, but are still unsatisfactory. In addition, the toxic side effects of chemotherapy drugs and the failure of chemotherapy due to drug resistance are some of the drawbacks of clinical treatment [3].

Bergenin, an active constituent of the herb of *Bergenia purpurascens* (Hook. f. et Thoms.) Engl. has been widely used as an active anti-inflammatory ingredient, which can inhibit chronic bronchitis, and attenuates chronic gastritis clinically [4,5]. Moreover, studies have reported that bergenin has various bioactivities, such as hepatoprotective [6], neuroprotective [7] and antioxidant properties [8]. Despite bergenin being

known for its multifaceted activities, the anti-colorectal carcinoma activity has not been studied.

The present study was designed to explore its efficiency in inhibition of cell growth in human colorectal carcinoma cells. In view of the effects on the activation of PI3K/AKT/mTOR pathway, the underlying mechanisms of how bergenin increases DNA damage and cell cycle arrest were explored.

EXPERIMENTAL

Chemicals and reagents

Bergenin (C₁₄H₁₆O₉, MW: 328.27, purity ≥ 98 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bergenin was dissolved in dimethylsulfoxide (DMSO) as stock solution of 0.1 M, stored at -20 °C, and freshly diluted with RPMI-1640 medium (Gibco, Carlsbad, CA, USA) to the final concentration used in the study. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide] and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich. Antibodies against p-AKT (Ser473), total AKT, p-mTOR (Ser2448), total mTOR and GAPDH were purchased from Bioworld Technology, Inc. (Louis Park, MN, USA). p-H2AX (Ser139), H2AX antibodies were purchased from EnoGene Biotech (Nanjing, China). Other chemical products used were of analytical grade.

Cell culture

Human colorectal adenocarcinoma HCT116 cell line was obtained from American Type Culture Collection (Bethesda, MD, USA). Cells were cultured in RPMI-1640 medium (Gibco), supplemented with 10 % fetal bovine serum (Gibco), 100 U/mL benzyl penicillin and 100 mg/mL streptomycin. Cells were cultured in a humidified environment with 5 % CO₂ at 37 °C.

Cell viability assay

Cell viability was measured using the colorimetric MTT assay as described previously [9,10]. The cells were incubated overnight and then treated with various concentrations of bergenin, and incubated for 24 and 48 h. Subsequently, 20 µL of MTT solution (5 mg/mL) was added to each well. The plate was incubated at 37 °C in a 5 % CO₂ atmosphere for 4 h, the supernatants were removed and 150 µL/well DMSO was added then placed in an orbital shaker for 5 min, and the absorbance was recorded at 570 nm with a Model 1500 Multiskan spectrum microplate reader (Thermo, Waltham, MA, USA). Data were

analyzed from three independent experiments and then normalized to the absorbance of the wells containing media only (0 %) and untreated cells (100 %).

Apoptosis analysis

HCT116 cells were treated with various concentrations of bergenin and were harvested by trypsinization and then washed twice with PBS. Cells were stained with Annexin V-FITC and PI, and then analyzed with a FACSCalibur flow cytometer using Cell Quest software (BD Bioscience, San Jose, CA, USA). Results were analyzed using Flowjo 7.6 software (Treestar, Ashland, OR, USA).

Cell cycle analysis

HCT116 cells were treated with various concentrations of bergenin and were harvested by trypsinization, fixed with 70 % ethanol and stored at -20 °C for at least 1 day. Following fixation, cells were subjected to a PBS wash and then stained with DNA staining solution comprising 2.5 mg/mL propidium iodide (PI) and 50 mg/mL RNaseA in PBS. Samples were incubated at 37 °C for 30 min away from light and then analyzed on a FACSCalibur flow cytometer (BD Bioscience) [9]. Results were analyzed using Flowjo 7.6 software (Treestar).

Western blotting

Whole cell lysates from HCT116 cells were prepared by suspending cells in NP40 buffer. The equal concentration of protein lysate of all the samples was separated on 8 % SDS-PAGE gel and further transferred to nitrocellulose membranes. The membranes were blocked with 5 % nonfat milk for 2 h, and incubated overnight at 4 °C with specific primary antibodies against p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-H2AX (Ser139), H2AX and GAPDH, and then incubated with IRDye-conjugated secondary antibody for 1 h at 37 °C. Detection was performed by the Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, MT, USA).

Measurement of ROS level

The production of cellular reactive oxygen species (ROS) was detected using fluorescent dye, 2, 7-dichlorofluoresceindiacetate (DCFH-DA, Beyotime Institute of Biotechnology, China) as previously described [11]. In brief, HCT116 cells were pretreated with/without NAC for 2 h and then exposed to bergenin for 12 h. Then cells were collected and incubated with 10 µM DCFH-DA attenuated with serum-free medium

for 30 min at 37 °C in the dark. After washing with serum-free medium, ROS level was measured by intensity of the fluorescence produced on a Model 1500 Multiskan spectrum microplate reader (Thermo).

Statistical analysis

Data are presented as mean \pm SEM ($n = 3$). Data sets were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test between control and experimental groups. $P < 0.05$ was considered significant.

RESULTS

Effect of bergenin on growth of human colon cancer cells

At first, we examined the effect of bergenin at different concentrations on cell viability. As shown in Figure 1, after 24 and 48 h of treatment, bergenin markedly inhibited the cell growth in HCT116 cells in a concentration-dependent manner.

Effect of bergenin on cell apoptosis in colon cancer cells

We next evaluated apoptosis in HCT116 cells after treatment with 3, 10 and 30 μM bergenin for 24 h, the percentage of total apoptosis cells were $2.53 \pm 0.51\%$, $5.21\% \pm 0.93\%$, $8.56 \pm 1.02\%$ and $14.87 \pm 1.35\%$, respectively (Figure 2).

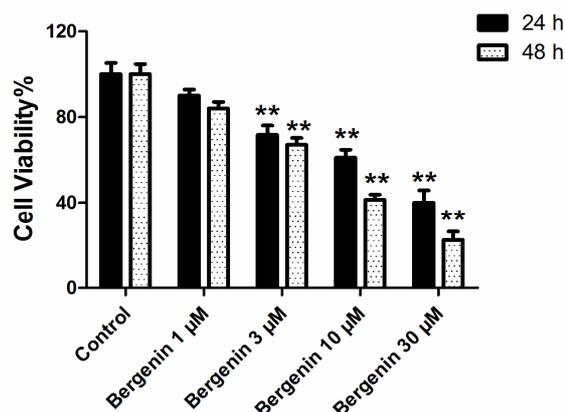


Figure 1: Effect of bergenin on the growth of human colon cancer cells. HCT116 cells were incubated with various concentrations of bergenin (1, 3, 10, 30 μM) for 24 and 48 h, and cell viability was analyzed by MTT assay. Data were presented as means \pm SEM of three independent experiments; * $p < 0.05$, ** $p < 0.01$ vs control

Effect of bergenin on cell cycle in colon cancer cells

Because of the significant inhibitory effect on cell viability and growth, we estimated the effect of bergenin on the distribution of cells in the cell cycle. As shown in Figure 3, bergenin (3, 10, 30 μM) treatment led to an increase in the number of cells in the G1 phase with decreased percentage of cells in the S phase at 24 h. The

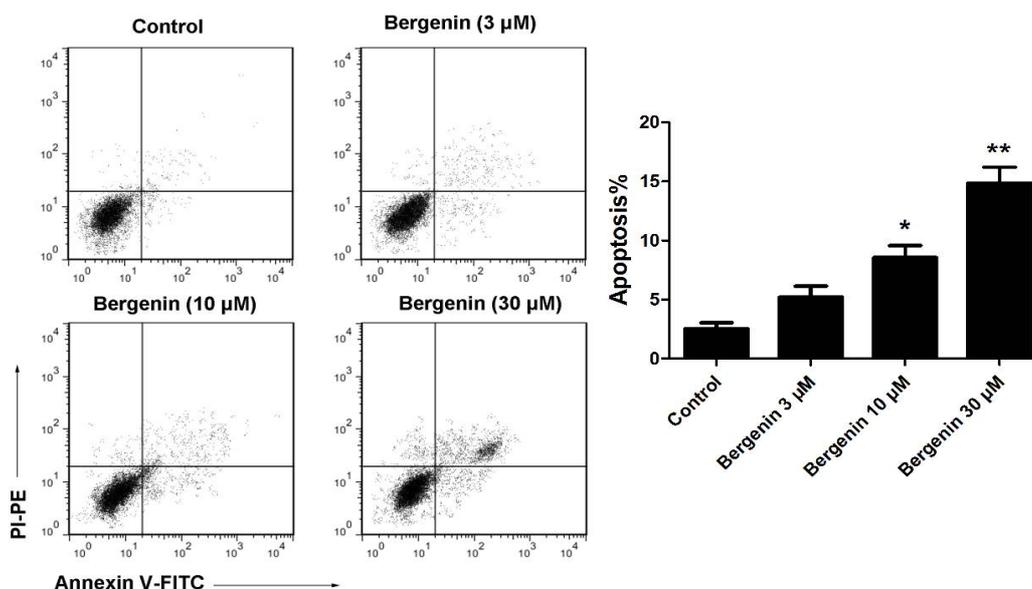


Figure 2: Effect of bergenin on cell apoptosis in colon cancer cells. HCT116 cells were incubated with various concentrations of bergenin (3, 10, 30 μM) for 24 h, and cell apoptosis was stained with Annexin V/PI followed by flow cytometry. The percentages of cells in apoptosis were represented. Data were presented as means \pm SEM of three independent experiments; * $p < 0.05$, ** $p < 0.01$ vs control

data showed that the number of cells in the G1 phase was 1.5-fold and 2-fold at 10 and 30 μM at 24 h, respectively.

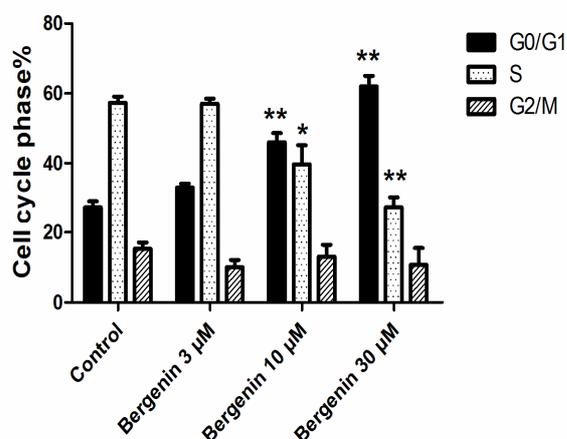


Figure 3: Effect of bergenin on the cell cycle in colon cancer cells. HCT116 cells were incubated with various concentrations of bergenin (3, 10, 30 μM) for 24 h, and the cell cycle was analyzed by flow cytometry after staining with propidium iodide. Cells in G0/G1, S and G2/M phases were quantified and presented. Data were presented as means \pm SEM of three independent experiments; * p < 0.05, ** p < 0.01 vs control

Effect of bergenin on DNA damage

We investigated the phosphorylation of histone variant H2AX at Ser139, an indicator of DNA break. As shown in Figure 4, bergenin markedly increased the level of phosphorylated H2AX at Ser139 in a concentration-dependent manner. The results indicated that bergenin might lead to DNA damage in HCT116 cells.

Effect of bergenin on ROS level

To investigate the effect of bergenin on ROS generation, we detected the levels of ROS in bergenin-treated cells. It was shown that bergenin (10 and 30 μM) treatment induced a substantial increase in ROS levels in HCT116 cells at 24 h. In addition, NAC (a ROS scavenger) markedly decreased the accumulation of ROS induced by bergenin (Figure 5).

Effect of bergenin on activation PI3K/AKT/mTOR pathway

The canonical PI3K/AKT/mTOR signaling pathway has emerged as a critical regulator pathway of cell proliferation [1].

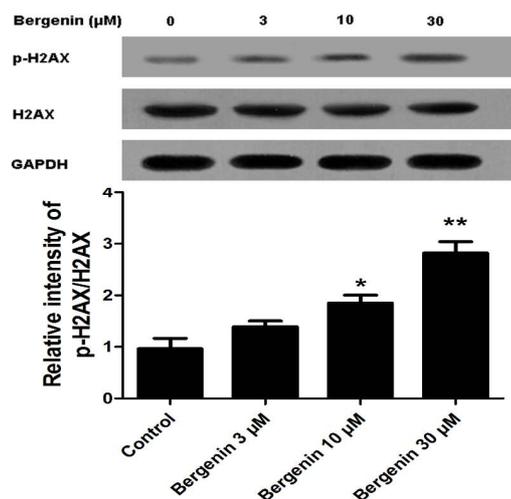


Figure 4: Effect of bergenin on DNA damage. HCT116 cells were incubated with various concentrations of bergenin (3, 10, 30 μM) for 24 h. Cell were harvested and lysed, and the levels of p-H2AX, H2AX and GAPDH were analyzed by immunoblotting. Densitometry analysis of immunoblotting was also shown. Data were presented as means \pm SEM of three independent experiments * p < 0.05, ** p < 0.01 vs control

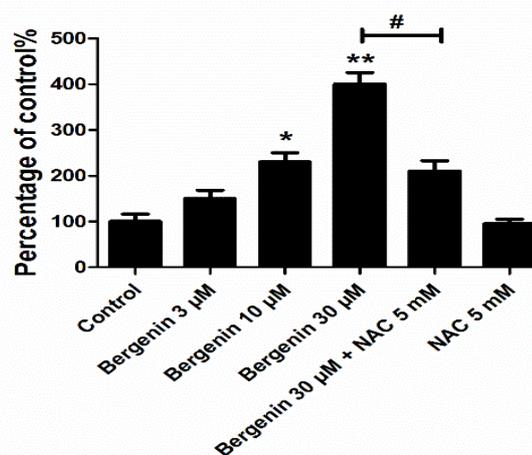


Figure 5: Effect of bergenin on ROS level. HCT116 cells were pretreated with/without NAC for 2 h and then exposed to various concentrations of bergenin (3, 10, 30 μM) for 12 h. ROS level was tested with fluorescence probe DCFH2-DA. Data were presented as means \pm SEM of three independent experiments; * p < 0.05, ** p < 0.01 vs control. # p < 0.05 vs the indicated

Our former data showed that bergenin could down-regulate cell viability and growth, thus we tested the effect of bergenin on PI3K/AKT/mTOR signaling pathway. Western blot analysis showed that bergenin markedly decreased the phosphorylation levels of AKT and mTOR, while it had no significant effect on the total levels of AKT and mTOR in a concentration-dependent manner (Figure 6).

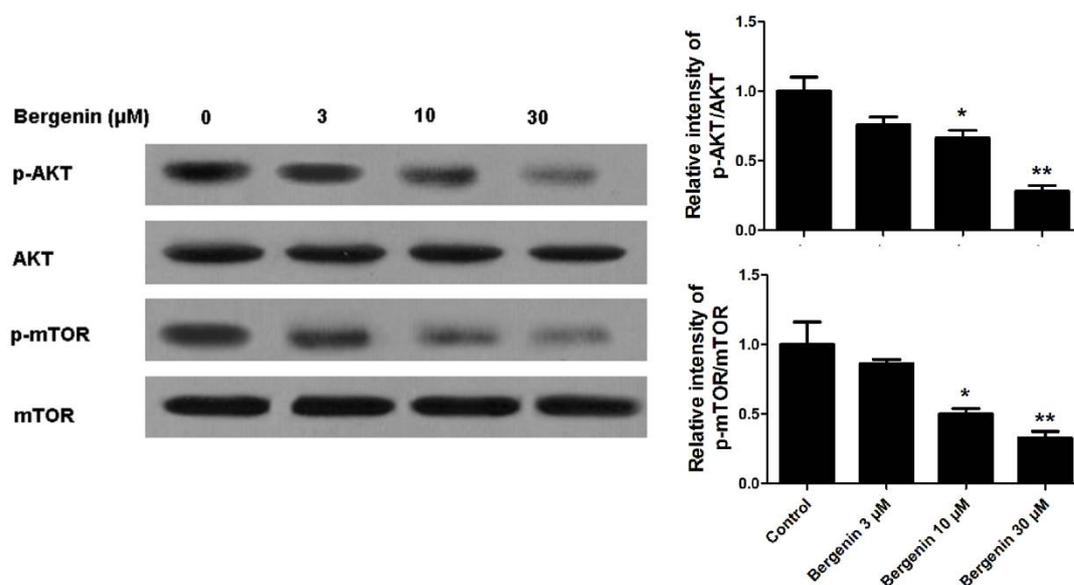


Figure 6: Effect of bergenin on the activation PI3K/AKT/mTOR pathway. HCT116 cells were incubated with various concentrations of bergenin (3, 10, 30 μ M) for 24 h. Cells were harvested and lysed, and the levels of p-AKT, AKT, p-mTOR and mTOR were analyzed by immunoblotting. Densitometry analysis of immunoblotting was also shown. Data were presented as means \pm SEM of three independent experiments; * p < 0.05, ** p < 0.01 vs control

DISCUSSION

CRC is one of the most common causes of cancer mortality. Traditional Chinese medicines have been recognized as new source of anticancer drugs as well as new chemotherapy adjuvants that enhance efficacy and diminish side effects of chemotherapeutic agent. In this study, bergenin showed significant inhibitory effect on the growth of HCT116 cells. Bergenin induced ROS-mediated DNA damage, which resulted in G1 phase arrest and inhibited the activation of PI3K/AKT/mTOR pathway.

Bergenin could significantly inhibit HCT116 cell proliferation and growth in a concentration- and time-dependent manner, which demonstrated that bergenin was a potent cytotoxic compound. Cell cycle is the essential mechanism by which all cells reproduce. New links between alterations in the cell cycle regulatory machinery and tumorigenesis are being constantly reported and virtually all molecular species involved in regulating cell proliferation described in the literature have been related to malignant transformation [12]. Thus, we assumed that bergenin-induced cell growth inhibition was due to cell cycle arrest. We found that bergenin induced concentration-dependent G1 phase cell cycle arrest in HCT116 cells. In advanced CRC, cancer cells usually become resistant to apoptosis [13]. Thus, identification that bergenin that can induce apoptosis in CRC cells is highly desirable. The results showed bergenin

treatment for 24 h in HCT116 cells led to an elevated percentage of apoptotic cells.

Many drugs in use disrupt genome integrity by causing DNA strand breaks, and consequently block cell proliferation mainly by suppressing factors that enable cells to proceed from one cell cycle phase to the next through checkpoints in the cell division cycle [14]. Then, we hypothesized that bergenin induced DNA damage, leading to G1 cell cycle arrest and apoptosis. The data showed that bergenin induced DNA damage in HCT116 cells in a concentration-dependent manner as confirmed by the increased level of p-H2AX (Ser139), the biomarker for DNA damage.

Accumulative data have indicated that induction of DNA damage by high levels of ROS can induce cell cycle arrest and apoptosis [15,16]. In normal cells, ROS are at low levels, which are important for the regulation of normal cell proliferation. However, cancer cells, which exhibit much higher levels of ROS close to the threshold of cytotoxicity, are susceptible to ROS-generating agents [17,18]. In this study, bergenin led to a marked increase in ROS levels in HCT116 cells.

PI3K/AKT/mTOR pathway is a particularly important pathway that has also been involved in regulating cell proliferation, cell cycle progression, apoptosis and morphogenesis in different organs [19,20]. Earlier work established

that PI3K/AKT/mTOR signaling pathway plays a critical role in the development of human cancer including colorectal cancer [21]. Thus, to suppress the excessive activation of PI3K/AKT/mTOR signaling pathway is a key therapeutic method of colorectal cancer. Our data demonstrated that bergenin could markedly inhibit the levels of p-AKT and p-mTOR in HCT116 cells.

CONCLUSION

The findings demonstrate that cell growth inhibition and G1 phase arrest occur in colorectal carcinoma cells HCT116 exposed to bergenin. Bergenin efficiently induces the generation of intracellular ROS, DNA damage and consequent G1 phase arrest and inhibition of activation of PI3K/AKT/mTOR pathway. These results provide new insights into the anti-cancer mechanisms of bergenin, which may be helpful in the potential development of bergenin into a promising therapeutic agent against colorectal carcinoma.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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