Ponicidin suppresses HT29 cell growth via the induction of G1 cell cycle arrest and apoptosis

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Abstract. Ponicidin is a diterpenoid extracted from the Chinese herb *Isodon adenolomus*, which has been reported as a therapeutic cytotoxic drug that may be used to treat various types of human cancer. The present study aimed to determine the antitumor effects of ponicidin, and to investigate its underlying mechanisms in colorectal cancer. The HT29 colorectal cancer cell line was used to detect the cytotoxicity of various doses of ponicidin. Cell proliferation was measured using a Cell Counting kit-8 assay. Cell cycle and apoptosis analyses were performed using flow cytometry and fluorescent microscopy. Western blot analysis was used to measure the expression levels of apoptosis-associated proteins following treatment with ponicidin. Treatment with ponicidin significantly suppressed HT29 cell growth by inducing G1 cell cycle arrest and apoptosis. The AKT and MEK signaling pathways were also suppressed by ponicidin; however, the p38 signaling pathway was significantly activated. The expression levels of caspase 3 and Bax protein were markedly upregulated following treatment with ponicidin. These results suggest that ponicidin exerts significant antitumor effects via the induction of cell cycle arrest and apoptosis in colorectal cells. In conclusion, ponicidin acted as an inducer of apoptosis, and may be used as a therapeutic cytotoxic drug to treat human cancer, including colorectal cancer.

Introduction

Ponicidin is a natural ent-kaurane diterpenoid, which is extracted from the traditional Chinese herb *Isodon adenolomus* (1,2). Ponicidin has been shown to possess antibacterial properties, as well as anti-inflammatory and anti-viral regulatory functions (3). Recent studies have reported that ponicidin exerts antitumor effects, and may function as a potential cytotoxic drug for the treatment of hepatocellular cancer, lung cancer, and monocytic leukemia (4-7). Zhang *et al* (4) reported that ponicidin exerts marked anti-proliferative effects on hepatocellular cancer cells by inducing apoptosis, via the suppression of survivin and Bcl-2 expression, and the activation of Bax expression (4). Liu *et al* (7) reported the anti-proliferative effects of ponicidin on leukemia cells *in vitro*, via the down-regulation of survivin and Bcl-2 expression, thus inducing potent apoptosis. Furthermore, Zhao *et al* detected the cytotoxic effects of ponicidin in lung cancer; ponicidin was able to disrupt the mitochondrial membrane potential, and trigger the activation of caspases-3, -8 and -9 (2). These previous reports suggest that ponicidin may serve as a novel cytotoxic drug for the treatment of various types of cancer.

Colorectal cancer is the third most frequently diagnosed type of cancer, and the second leading cause of cancer-associated mortality worldwide (8). Leucovorin and fluorouracil combined with or without oxaliplatin are considered the first-line treatment in advanced colorectal cancer (9). However, after numerous rounds of treatment, the development of chemoresistance is inevitable, and the treatment of metastatic colorectal cancer remains to be improved (10). The usage of ponicidin for the treatment of colorectal cancer has not yet been investigated. In order to determine whether ponicidin has therapeutic potential in colorectal cancer, the present study aimed to determine the effects of various doses of ponicidin on cell proliferation and apoptosis *in vitro,* using the HT29 colorectal cancer cell line. In addition, the underlying molecular mechanisms of ponicidin in cancer cells were investigated.

Materials and methods

Reagents and cell culture. Ponicidin was isolated from *Isodon adenolomus* (Kunming Institute of Botany, Kunming, China) as described previously (2). The HT29 human colorectal cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), in a humidified incubator containing 5% CO_2 at 37˚C. *In vitro* experiments were conducted in triplicate at 70% cell confluence.

Cell proliferation assay. A total of $1x10^3$ HT29 cells were seeded in 96-well plates and treated with various doses of ponicidin (0, 10, 20 and 50 μg/ml), for various time-points

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(6, 12, 24 and 48 h). After incubation, the medium was removed and cell proliferation was measured using the Cell Counting kit (CCK)-8 assay kit (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). CCK-8 solution (150 μl) was added to each well and incubated for 2 h. Subsequently, the absorbance was measured at 450 nm using a microplate reader (HTX; Biotek, Beijing, China).

Cell cycle and apoptosis analyses. For the cell cycle analysis, flow cytometric measurements of DNA content were detected in 70% ethanol-fixed HT29 cells using propidium iodide (PI). The fixed cells (10^6 cells/ml) were washed with phosphate-buffered saline (PBS), treated with 200 μg/ml RNase A (Sigma-Aldrich) for 30 min at room temperature, and stained with 50 μg/ml PI (Sigma-Aldrich). Measurements were made using a flow cytometer (BD Influx; BD Biosciences, Franklin Lakes, NJ, USA).

For the apoptosis assay, fluorescein isothiocyanate (FITC)-conjugated Annexin-V and PI were used to detect apoptotic cells. Trypsinized cells (10^6 cells/ml; Sigma-Aldrich) were washed twice with PBS and resuspended in binding buffer containing Annexin-V-FITC and PI (BD Biosciences). The cells were incubated at room temperature for 15 min. The samples were analyzed using a flow cytometer (BD Biosciences).

Hoechst 33342 staining. Hoechst 33342 staining was used to observe the apoptotic morphology of the cells. HT29 cells (10^6 cells/ml), untreated or treated with ponicidin, were fixed with 4% formaldehyde in PBS for 10 min. The cells were subsequently stained with 10 μg/ml Hoechst 33342 (Yeasen Corporation, Shanghai, China) for 1 h, and subjected to fluorescence microscopy (TE2000; Nikon, Shanghai, China).

Western blot analysis. Western blot analysis was performed according to a standard method (8). In brief, 10 mg protein lysates were loaded onto the SDS-PAGE gel and transferred onto the membrane. The blots were quantified by Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to measure the density of the bands. The following antibodies were used: Anti-p38 (cat. no. 8690; 1:1,000 dilution), anti-phosphorylated (p)-p38 (cat. no. 4511; 1:800 dilution), anti-AKT (cat. no. 9272S; 1:1,000 dilution), anti-p-AKT (cat. no. 4058S; 1:1,000 dilution), anti-extracellular signal-regulated kinases (ERK) (cat. no. 4376S; 1:1,000 dilution), anti-p-ERK (cat. no. 4370S; 1:1,000 dilution) and anti-GAPDH (cat. no. 5174; 1:1,500 dilution) purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA); anti-caspase 3 (cat. no. ab4051; 1:400 dilution; Abcam, Cambridge, MA, USA); anti-Bcl-2 (cat. no. sc-492; 1:100 dilution) and anti-Bax (cat. no. sc-493; 1:150 dilution) purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase (HRP)-anti-rabbit immunoglobulin G (IgG) (cat. no. A0208; 1:1,000 dilution) and HRP-anti-mouse IgG (cat. no. A0216; 1:1,000 dilution) were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Statistical analysis. All experiments were performed in triplicate and the results were presented as the mean ± standard deviation. Data were analyzed using Student's t-test, and IBM SPSS 20 software (IBM SPSS, Armonk, NY, USA) was used to conduct analyses.

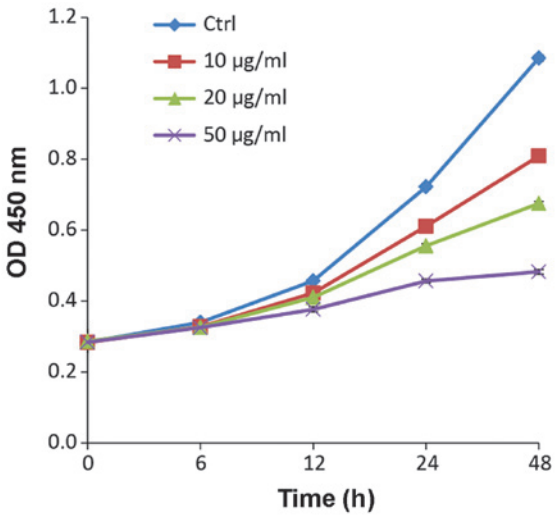

Figure 1. Ponicidin suppressed cell proliferation. HT29 human colorectal cancer cells were treated with various doses of ponicidin (10, 20 and 50 μg/ml) for 6, 12, 24 and 48 h. After treatment, cell proliferation was measured using the Cell Counting kit-8 assay ($P<0.05$, n=3). OD, optical density.

Results

Ponicidin suppresses cell growth. To determine the suppression of cell growth by ponicidin in colorectal cancer cells, HT29 cells were treated with various doses of ponicidin for 0, 6, 12, 24 or 48 h. As shown in Fig. 1, ponicidin exerted cell growth inhibitory effects on HT29 cells in a dose-dependent manner. The growth of the HT29 cells was markedly suppressed following 48 h treatment with 50 μg/ml ponicidin, an ~4 fold reduction as compared with the untreated control cells. The suppressive effects of 10 and 20 μg/ml ponicidin were 1.5- and 2.1- fold, respectively.

Ponicidin induces G1 cell cycle arrest. In order to investigate the underlying mechanism of ponicidin-induced cell growth suppression, cell cycle analysis was performed on the HT29 cells treated with various doses of ponicidin. Treatment with ponicidin suppressed DNA synthesis and cell proliferation, as evidenced by a reduced percentage of S-phase cells (19.02% in the 10 μg/ml group, 11.23% in the 20 μg/ml group, and 7.17% in the 50 μg/ml group, as compared with 14.05% in the control group). G1 cell cycle arrest was markedly increased following treatment with ponicidin (53.89% in the 10 μg/ml group, 60.71% in the 20 μg/ml group, and 66.33% in the 50 μg/ml group, as compared with 51.02% in the control group) (Fig. 2).

Ponicidin induces cell apoptosis. The present study also investigated whether ponicidin-induced cell growth suppression was caused by the induction of apoptosis. The HT29 cells were stained with PI and analyzed by flow cytometry. Following treatment with ponicidin, the apoptotic rate of the HT29 cells was increased to 10.2% in the 10 μg/ml group, 26.6% in the 20 μg/ml group, and 70.9% in the 50 μg/ml group, as compared with 3.9% in the untreated cells

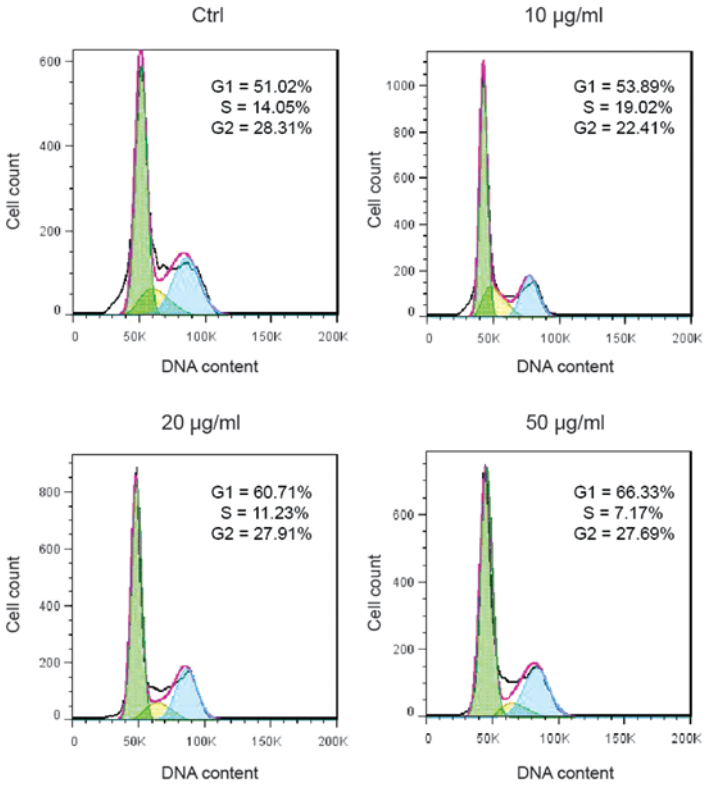

Figure 2. Ponicidin induced cell cycle arrest. HT29 human colorectal cancer cells were treated with various doses of ponicidin (10, 20 or 50 μg/ml) for 24 h. Cell cycle analysis was performed by fluorescence-activated cell sorting (FACS) in HT29 cells. DNA was stained with propidium iodide. Relative phase group was calculated by FACS ($P<0.05$, n=3).

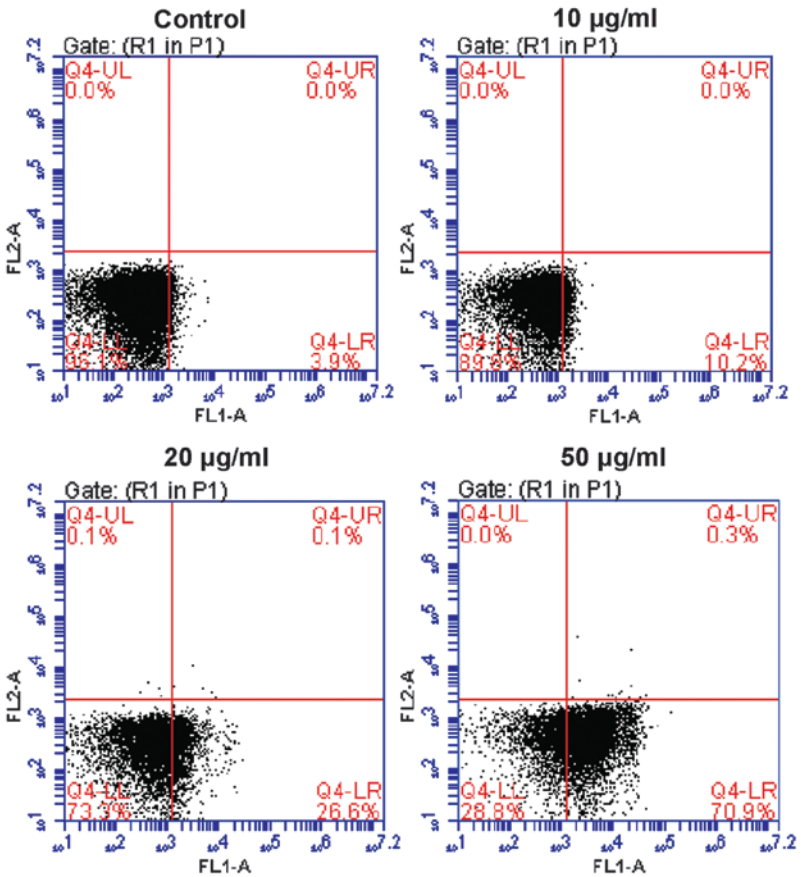

Figure 3. Ponicidin induced cell death. HT29 human colorectal cancer cells were treated with various doses of ponicidin (10, 20 and 50 μg/ml) for 24 h. The early apoptosis assay was performed by fluorescence-activated cell sorting (FACS) analysis in HT29 cells. The percentage of apoptotic cells in relation to total cell counts were plotted in the form of stacked bar diagrams. ($P<0.05$, n=3).

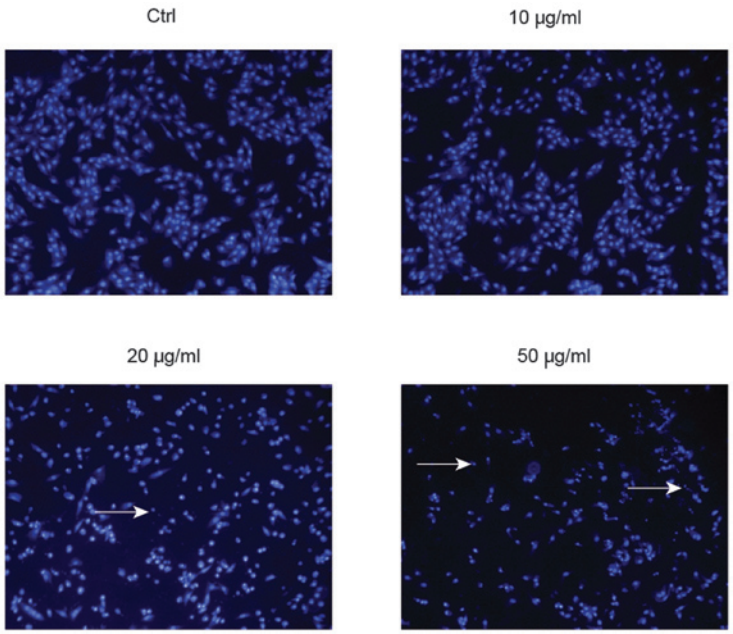

Figure 4. Ponicidin increased the number of apoptotic bodies. HT29 human colorectal cancer cells were treated with various doses of ponicidin (10, 20 and 50 μg/ml) for 24 h. Apoptotic bodies were visualized following Hoechst 33342 staining (magnification, x20). After treatment, apoptosis-associated morphological changes, such as chromatin condensation and apoptotic body formation were observed in HT29 cells.

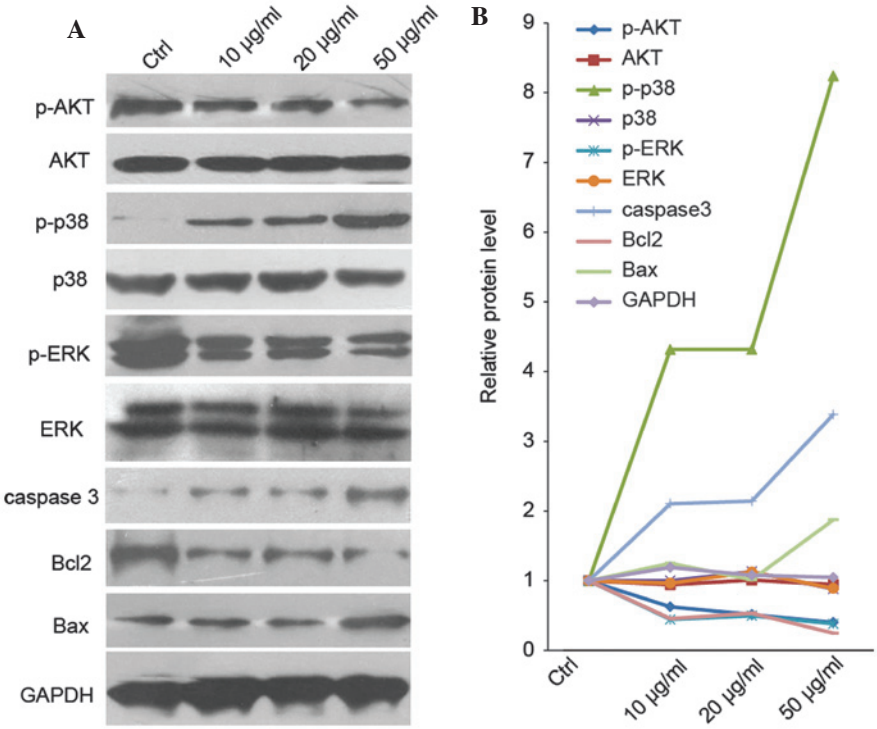

Figure 5. Ponicidin regulated apoptosis-associated protein expression. (A) HT29 human colorectal cancer cells were treated with various doses of ponicidin (10, 20 and 50 μg/ml) for 24 h. The expression levels of cell apoptosis-associated proteins were measured by western blotting. (B) Relative protein expression levels were quantified by Quantity One software.

(Fig. 3). To further validate the apoptotic effects of ponicidin, the number of apoptotic bodies was determined in the treated cells (Fig. 4). Following treatment with ponicidin, a marked increase in the number of apoptotic bodies was detected in the HT29 cells. Treatment with 50 μg/ml ponicidin resulted in the most apoptotic bodies, and the highest apoptotic rate in the HT29 cells. These results were concordant with the findings regarding cell growth suppression.

Ponicidin alters the expression levels of apoptosis-associated proteins. In order to investigate the molecular mechanism underlying ponicidin-induced apoptosis in HT29

cells, apoptosis-associated protein expression was measured by western blot analysis. As shown in Fig. 5, the expression levels of p-p38 were markedly increased following treatment with ponicidin (4.4-fold increase in the 10 μg/ml group, 4.4-fold increase in the 20 μg/ml group and 8.3-fold increase in the 50 μg/ml group), whereas total p38 protein expression levels remained unchanged. Similarly, apoptotic markers, caspase 3 and Bax were markedly upregulated in the treated cells; however, p-AKT and p-ERK were downregulated in the treated cells. These results indicate that proliferation signaling pathways were suppressed following treatment with ponicidin (Fig. 5).

Discussion

Chinese herbal medicine is important in traditional Chinese medical treatment. Recent studies have demonstrated that Chinese herbal extracts may induce cancer cell death (11,12). Mujumdar *et al* (13) reported that the herbal extract triptolide resulted in the downregulation of GRP78 protein expression in the cells, reduced cancer cell survival, and facilitated cell death in human pancreatic cancer cells and tissue in culture. Ponicidin is a natural ent-kaurane diterpenoid compound that is extracted from the traditional Chinese herb *Isodon adenolomus* (14,15). Recent studies have reported the successful and large-scale purification of ponicidin by liquid chromatography-tandem mass spectrometry (16-19), thus indicating the practical usage of ponicidin in therapeutic applications. Xu *et al* (20) identified the DNA binding and cleavage properties of ponicidin; these properties may provide the basis for the rational construction of novel, more efficient drugs targeted to DNA, thus enabbling the development of effective therapeutic agents for the target gene.

Notably, ponicidin has been shown to act as a cytotoxic drug for hepatocellular cancer, lung cancer and monocytic leukemia (1,7,12), and therefore has high potential in translational research in colorectal cancer. The present study examined the biological function of ponicidin in the HT29 colorectal cancer cell line. The results of the present study demonstrated that ponicidin was able to significantly suppress the cell growth of HT29 cells by inducing G1 cell cycle arrest and apoptosis. Treatment of HT29 cells with 50 μg/ml ponicidin resulted in a ~4 fold suppression of cell growth, a ~15% increase in G1 cycle arrest, and a 67% increase in cell death. Further investigation demonstrated that ponicidin significantly upregulated p-p38, and suppressed the activation of the AKT and MEK signaling pathways. The p38 signaling pathway is activated by apoptotic stimuli, and induces apoptosis via its downstream targets (14,21). Subsequent activation of apoptotic marker genes, such as caspase 3 and Bax, was consistent with the activation of the p38 signaling pathway. These results suggested that ponicidin may act as an apoptotic stimulus and trigger activation of the p38 signaling pathway in colorectal cancer cells. Inducers of apoptosis have been applied in cancer therapy and activation of apoptosis is a vital process by which cytotoxic drugs destroy cancer cells (15,22). In conclusion, the results of previous studies and of the present study suggest that ponicidin may be used as a therapeutic cytotoxic drug for the treatment of human cancers, including colorectal cancer.

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