Original Article

Ponicidin induces apoptosis of human cervical cancer HeLa cell line through the PI3K/Akt and MAPK signaling pathways

Yongping Liu1*, Yongling Fan1*, Yonghua Chen1, Yuanyuan Hou2

Departments of 1 Gynecology, 2 Physical Examination, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai 264000, Shandong, China. *Equal contributors.

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Abstract: Ponicidin, an ent-kaurane diterpenoid of Rabdosia rubescens or Isodon japonicas, has been associated with a broad range of biological properties including antitumor activity. The aim of this study is to investigate the antitumor activity of ponicidin on the human cervical cancer HeLa cell line and the mechanism through which ponicidin exerts its antitumor effect. Cell viability was assessed by MTT assay. Cell apoptosis and the apoptosis-related activation in HeLa cells were evaluated by flow cytometry and western blot analysis. The results demonstrated that ponicidin treatment inhibited the proliferation of HeLa cells and induced cell apoptosis in a dose-dependent manner. The apoptosis induced by ponicidin was evidenced by Bcl-2/Bax dysregulation, cleavage of caspase-3, -9 and PARP. In addition, ponicidin treatment decreased the phosphorylation of Akt, while LY294002 pretreatment promoted the dephosphorylation of Akt and cell death induced by ponicidin. Furthermore, ponicidin treatment increased the phosphorylation of ERK1/2, JNK1/2 and p38 MAPK, while cell apoptosis was significantly attenuated when cells were co-treated with ponicidin and pharmacologic inhibitors U0126 (ERK1/2 kinase inhibitor), SP600125 (JNK kinase inhibitor) and SB202190 (p38 kinase inhibitor). In conclusion, ponicidin treatment induced apoptosis in HeLa cells through PI3K/Akt and MAPK signaling pathways and ponicidin may be a promising option in the treatment of cervical cancer.

Keywords: Ponicidin, cervical cancer, HeLa cells, apoptosis, PI3K/Akt, MAPK

Introduction

Cervical cancer, the second most common female cancer worldwide, is the leading malignancy in incidence and mortality among women in some developing countries [1, 2]. Cervical cancer in patients with a poor prognosis is characterized by rapid cellular proliferation and strong expression of antiapoptotic genes. At present, most cervical cancer patients receive radiation as well as chemotherapy as a part of their treatment regimen [3]. Although the combined therapy reduces the risk by 30-50%, the prognosis in advanced cervical cancer is still very poor [4]. Therefore, it is urgent to develop novel therapeutics useful to manage the progression of cervical cancer and improve the quality of life more effectively.

Traditional herb formulations have been re-evaluated by clinicians because these medicines have fewer side-effects and are more suitable for long-term use compared with chemically synthesized medicines [5]. Ponicidin is an ent-kaurane diterpenoid derived from the traditional Chinese herbs Rabdosia rubescens or Isodon japonicas [6, 7]. Ponicidin has been demonstrated to possess a variety of biological effects including antibacterial activity, anti-inflammatory and immunoregulatory functions [8]. Accumulating evidence indicate that ponicidin is a potent antitumor agents with profound effects on human hepatocellular cancer [9], lung cancer [10], monocytic leukemia [11], breast cancer [12], colorectal cancer [13], prostate cancer [14]. Although ponicidin has been proved to be effective in numerous malignancies, there are no available data on the mechanism of ponicidin in human cervical cancer HeLa cells. The aim of the present study was to investigate the potential anticancer effects of ponicidin on human cervical cancer HeLa cells.
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and to elucidate the underlying molecular mechanisms.

Materials and methods

Chemicals and antibodies

Ponicidin (HPLC ≥ 98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and its chemical structure is shown in Figure 1. Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin, streptomycin, DMSO, PBS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was provided by Gibco (BRL Life Technologies, Grand Island, NY, USA). Antibodies to Bcl-2, Bax, caspase-3,-9, poly(ADP ribose) polymerase (PARP), cleaved PARP, p-Akt, c-jun N-terminal kinases 1/2 (JNK1/2), p-JNK1/2, extracellular signal-related kinase 1/2 (ERK1/2), p-ERK1/2, p38 mitogen-activated protein kinase (p38 MAPK), p-p38 MAPK, β-actin and goat anti-rabbit IgG (H&L)-HRP secondary antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). Z-VAD-FMK was obtained from Abcam (Cambridge, MA, USA). Specific inhibitors LY294002, U0126, SP600125 and SB202190 were provided by Calbiochem (San Diego, CA). Annexin V-phycocerythrin (PE) and 7-amino-actinomycin by using the Guava Nexin apoptosis kit were obtained from Cell Signaling Technology (Danvers, MA, USA). Micro BCA kit was obtained from Beyotime Biotechnology (Haimen, China).

Cell line and cell culture

Human cervical cancer HeLa cells and human primary normal cervical squamous cells (NCSC) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin, and were kept at 37°C in a humidified incubator containing 5% CO₂.

Cell viability assay

The viability of the cell was determined by MTT assay. In brief, the cells were cultured in 96-well plates and suspended at a final concentration of 1×10⁴ cell/well. After 12 hour’s incubation, the cells were treated with 0 μM, 10 μM, 20 μM and 40 μM ponicidin for 24 h. Subsequently, MTT was added to the medium and cells were incubated for 4 h at 37°C. The culture media were then replaced with DMSO. The absorbance of the formazan solution was detected at 570 nm with a plate reader (Epoch, BioTek Instruments, USA).

Apoptosis assays

Flow cytometry was used primarily to measure percentage apoptosis of ponicidin-induced HeLa cells. Apoptotic cells or viable cells were stained with Annexin V-phycocerythrin (PE) and 7-amino-actinomycin by using the Guava Nexin apoptosis kit (containing annexin V-PE to detect externalised phosphatidylserine and 7-AAD, a cell impermeant dye, as an indicator of late stage apoptosis) according to the manufacturer’s instructions. After incubation at room temperature for 30 min with mixing in the dark, the cells were examined by flow cytometry.

Western blot analysis

Western blot analysis was performed according to previously published procedure with slight modification [15]. Briefly, after being treated with different concentration of ponicidin, the collected cells were lysed immediately in RIPA buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM Na₂P₂O₇, and 1 mM phenylmethyl-sulphonyl fluoride) supplemented with a protease inhibitor cocktail. Protein concentration was detected using Micro BCA kit. Equal amounts of protein from cell lysate was denatured in a protein loading buffer for 10 min at 100°C and separated on a 12% sodium dodecyl sulfate-poly-acrylamide gel (SDS-PAGE). Pro-
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Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes. After being blocked with 5% non-fat milk, blots were incubated with corresponding primary antibodies overnight at 4°C, and then exerted to incubation with the secondary antibody at room temperature for 2 h. Immunoreactivity was detected by enhanced chemiluminescence (ECL) (Bio-Rad, USA). β-actin was used as a loading control. Quantitative analysis was performed by Image Lab™ software.

Statistical analysis

Data were presented as means ± SD. The SPSS 19.0 software package was used to perform all statistical analysis. Comparisons between two groups were performed using the Student’s-t test and between multiple groups using ANOVA analysis. The level of statistical significance was set at P < 0.05.

Results

Ponicidin suppresses the proliferation of HeLa cells

Cells were treated with different concentrations of ponicidin including 0 μM, 10 μM, 20 μM and 40 μM for 24 h. The cell viability was assessed by MTT assay. As demonstrated in Figure 2A and 2B, ponicidin treatment significantly decreased viability of HeLa cells in a dose-dependent manner, with IC50 values of 23.1%. Interestingly, ponicidin was found to be less cytotoxic on normal cervical squamous cells (NCSC). These results indicated that ponicidin significantly suppressed the viability of HeLa cells with lower toxicity against NCSC, suggesting that ponicidin is a specific and effective inhibition agent against the growth of human cervical cancer cell.

Ponicidin induced apoptosis of HeLa cells

To determine whether ponicidin could induce apoptosis in HeLa cells, cells were treated with different concentration of ponicidin (0 μM, 10 μM, 20 μM and 40 μM) for 24 h and cell apoptosis was determined by flow cytometry. As illustrated in Figure 3A, ponicidin treatment induced apoptosis in HeLa cells in a dose-dependent manner and percentage of apoptotic cell increased from 15.2% to 22.7%, 45.3% and 52.1%, respectively. To further investigate the underlying mechanism of ponicidin-induced apoptosis in HeLa cells, the apoptosis related proteins including Bax, Bcl-2, caspase-3, -9 and PARP were determined by western blot assay. As shown in Figure 3B and 3C, pretreated with ponicidin significantly upregulated the expression of cleaved-caspase-3, -9, cleaved PARP and Bax, while downregulated the expression of Bcl-2 and Bcl-2/ Bax ratio. Furthermore, pretreated HeLa cells with Z-VAD-FMK (a pan-caspase inhibitor) and then incubated with ponicidin (40 μM) for 24 h could significantly alleviate ponicidin-induced cell apoptosis and cleaved caspase-3 and PARP (Figure 3D and 3E). These results indi-
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Figure 3. Ponicidin induce cell apoptosis of HeLa cells. (A) Cells were treated with 0 μM, 10 μM, 20 μM and 40 μM ponicidin for 24 h, cell apoptosis were determined by flow cytometry. The levels of apoptosis-related proteins, including Bax, Bcl-2, cleaved caspase-3, -9 and PARP in HeLa cells were detected by western blot analysis (B) and...
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cated that ponicidin could trigger apoptosis in HeLa cells through the caspase-dependent apoptosis pathway.

**Ponicidin inhibits the PI3K/Akt signaling pathway in HeLa cells**

The PI3K/Akt signaling pathway is one of the major pathways regulating cell proliferation and apoptosis. As demonstrated in Figure 4A, ponicidin treatment downregulated the phosphorylation of Akt in a dose-dependent manner, indicating PI3K/Akt pathway may participate in the inhibition of HeLa cells induced by ponicidin. To further confirm this, we used the PI3K kinase inhibitor LY294002. As shown in Figure 4B and 4C, pretreatment of LY294002 further promoted the dephosphorylation of Akt and cell death induced by ponicidin, demonstrating that ponicidin induced cell apoptosis was partly mediated by PI3K/Akt pathway.

**Ponicidin activates MAPK signaling pathway in HeLa cells**

It has been well documented that MAPK signaling pathway played a crucial role in cell proliferation, autophagy and apoptosis. As illustrated in Figure 5A, ponicidin treatment increased the phosphorylation of ERK1/2, JNK1/2 and p38 MAPK, but no obvious effects were found on total ERK1/2, JNK1/2 and p38 MAPK, indicating that activation of ERK1/2, JNK1/2 and p38 MAPK in HeLa cells. To further investigate the mechanism, we evaluated the effects of a panel of pharmacologic inhibitors, including U0126 (ERK1/2 kinase inhibitor), SP600125 (JNK kinase inhibitor) and SB202190 (p38 MAPK kinase inhibitor) on rescuing of cell death induced by ponicidin. As demonstrated in Figure 5B, compared with cells treated with ponicidin alone, cell apoptosis was significantly attenuated when cells were co-treated with...
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Multistep processes and molecular markers have been confirmed to be involved in the tumorigenesis, invasiveness of cervical cancers [3]. Apoptosis, otherwise known as programmed cell death, plays an essential role in the anticancer properties of many anticancer molecules by preventing or controlling abnormal cell development [16, 17]. Caspases are key factors that control apoptosis and are involved in common pathway of various apoptotic signals [18]. Caspase-3, which is activated by caspase-9, can cleave proteins in damaged DNA recovery or PARP. Caspase activation and PARP cleavage are typical characteristics of apoptosis [19]. In the present study, caspase-3 and -9 were activated, as evidenced by the increased levels of the respective cleaved forms, as well as PARP segmentation. It appears that caspase plays a significant role in the apoptosis induced by ponicidin in HeLa cells, and the activation of caspase-3 and -9 leads to PARP segmentation. In addition, Bcl-2 family proteins, located in the mitochondrial membrane, control membrane permeability and play a crucial role in deciding whether cells will live or die [16]. Bcl-2 acts on apoptotic signals to maintain the stability of mitochondria [20]. Bax can directly induce mitochondria to release cytochrome c for its ability to form ion channels indicating that it can open pores in the outer mitochondrial membrane, allowing the exit of cytochrome c [21]. Specifically, Bcl-2/Bax regulates the release of cytochrome c from mitochondria into the cytosol, which results in caspase activation and eventually apoptosis [22, 23]. In our study, ponicidin treatment increased Bax expression and decreased Bcl-2 expression, which resulted in the reduction of the Bcl-2/Bax ratio, suggesting that ponicidin induced apoptosis in cells. Meanwhile, the result of flow cytometric analyses further confirmed this conclusion.

PI3K/Akt signaling pathway serves as a pivotal signaling axis in cell growth, proliferation, and cell survival [24]. Numerous evidences have demonstrated that PI3K/Akt signaling pathway is aberrantly activated in various types of cancers including breast cancer, lung cancer and cervical cancer [22, 25-27]. PI3K could catalytically produce the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) at cell membrane, which leads to recruiting and activating of downstream targets, including the serine-threonine protein kinase Akt [28]. PI3K/
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Akt pathway may manipulate the apoptotic Bcl-2 family proteins. Overactive PI3K/Akt pathway would mitigate the process of apoptosis and promote tumor cell cycle progression, which acts as an “on” or “off” switch [29]. Therefore, the suppression of PI3K/Akt pathway may be an effective approach to treatment of cancer. In this study, ponicidin treatment significantly inhibited the phosphorylation of Akt. We incubated HeLa cells with LY294002 to confirm that ponicidin induced cell apoptosis through regulating PI3K/Akt signaling pathway.

MAPK signaling pathways which consist of ERK, JNK and p38 MAPK signaling pathways are involved in many cellular processes including cell growth, proliferation, autophagy and apoptosis [30]. The ERK, JNK and p38 MAPK, which are activated by phosphorylation, are key regulatory proteins in these pathways [31, 32]. Each group is controlled by its own pathway and performs distinct functions. ERK1/2 is activated by growth factors, such as those that promote apoptosis and further control cell growth, survival and division. p38 MAPK is activated by chemical and environmental stresses and inflammatory factors that affect cellular levels [19]. JNK, a downstream kinase of the MAPK family, has been proved to modulate the expression of receptors such as Fas and the Fas ligand in apoptosis [33]. We observed that the expression levels of p-p38 MAPK, p-ERK1/2 and p-JNK1/2 were activated by ponicidin. To further investigate whether ponicidin-induced cell apoptosis was related to p-p38 MAPK, p-ERK1/2 and p-JNK1/2 activation, cells were pretreated with the pharmacologic inhibitors SB202190, SP600125 or U0126 respectively. The result indicated that cell apoptosis was significantly attenuated when cells were co-treated with ponicidin and these inhibitors. These findings indicated that activation of MAPK signaling pathway, at least in part, plays an important role in ponicidin-induced apoptosis in HeLa cells.

Conclusion

In conclusion, ponicidin inhibited the proliferation and induced apoptosis of cervical cancer HeLa cells via suppressing the PI3K/Akt and activating MAPK signaling pathways. Therefore, ponicidin may be an alternative therapeutic agent in the treatment of cervical cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Yuanyuan Hou, Department of Physical Examination, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, 20# Yuhuangding East Road, Yantai 264000, China. Tel: +86 13176386366; E-mail: houyy0702@126.com

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