Regular Article

Pterostilbene Inhibits Human Renal Cell Carcinoma Cells Growth and Induces DNA Damage

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Received May 8, 2019; accepted November 16, 2019

Pterostilbene (PTE) has inhibitory effect on a wide array of tumors. However, the therapeutic potential of PTE in renal cancer cells and the underlying mechanisms have not been evaluated. In this study, the aim is to demonstrate the growth inhibitory and the underlying mechanisms of PTE on human renal cell carcinoma (RCC) cells in vitro. By cell viability, cell morphology and colony formation assays, we found that PTE significantly suppressed the proliferation of RCC cells, while had little toxicity to the normal renal cell line HK-2. Flow cytometry assay revealed that PTE potently induced the apoptosis of RCC cells in a concentration-dependent manner, which was also testified by up-regulation of the pro-apoptosis-related protein (Cyto C, Bad, Bak, Bax, Cleaved-caspase 3, Cleaved-caspase 9, Cleaved-poly(ADP-ribose)polymerase (PARP)) and down-regulation of the anti-apoptosis-related protein Bcl-2. Moreover, cell cycle being arrested in S phase and down-regulation of p-Akt and p-extracellular signal-regulated kinase (ERK)1/2 were observed following treatment with PTE in RCC cells, indicating that PTE exerted remarkable anti-tumor activity in RCC cells possibly via cell cycle arrest and inactivation of Akt and ERK1/2 signaling pathways. Immunofluorescence analysis of γH2AX and detecting the expression levels of γH2AX, proliferating cell nuclear antigen (PCNA) and Rad51 by Western blot showed that PTE induced the DNA damages response in RCC cells. Taken together, the results of the present study demonstrated that PTE was a potential preventive and therapeutic agent for human renal cell carcinoma.

Key words  pterostilbene; human renal cell carcinoma; apoptosis; cell cycle arrest; DNA damage

INTRODUCTION

Renal cancer, the most dangerous and common cancer of the urinary system, is the sixth leading death cause of cancer in Western world.1) The incidence of renal cell carcinoma (RCC) is increasing at an annual rate of 2.5%.2) Clear cell renal cell carcinoma accounts for almost 5% of all types of epithelial cancer diagnosed in the United States each year.3) Currently, the treatment methods of RCC include surgery, radiotherapy and chemotherapy. If patients with renal cancer are not suitable for surgery, they will choose radiotherapy or chemotherapy.4) Nevertheless, a lot of studies have shown that renal cell carcinoma is extremely insensitive to radiotherapy and chemotherapy.5) In addition, these therapies are frequently expensive, with low efficiency and serious side effects. Therefore, there is an urgent need to develop novel therapeutic agents for renal cell carcinoma, which has low cost and few harmful effects.

Previous studies have found that natural products and phytochemicals in plants have certain curative effects on various types of cancers, including anticancer activity and low toxicity. Pterostilbene (PTE), a natural polyphenolic compound, primarily found in fruits, such as blueberries, grapes, and tree wood,6) has received a great deal of attention for its potent antioxidant and anti-inflammatory properties7); and anticancer effect on a wide range of cancers, including prostate, bladder, colorectal, and oral cancer and so on.8) But the molecular mechanism is still unclear. Many studies indicated that PTE induced cell cycle arrest or apoptosis in lung cancer, leukemia, breast cancer, and prostate cancer.9) PTE could also trigger apoptosis and further induce cell death in esophageal cancer through activation of endoplasmic reticulum stress (ERS) signaling.10) Ko et al.11) suggested that PTE inhibited the growth and induced autophagy and apoptosis in oral cancer cells via activation of c-Jun N-terminal kinase (JNK)1/2 signaling pathway but inactivation of extracellular signal-regulated kinase (ERK)1/2, Akt, and p38. Dong et al.12) demonstrated powerful cytotoxicity of PTE on ovarian cancer cells by triggering caspase-dependent apoptosis. Pan et al. indicated that PTE selectively inhibited the expression of ER-a36, contributing to growth-inhibiting effect on ERα36-positive breast cancer.13)

To the best of our knowledge, the effects of PTE on renal cancer have not yet been evaluated. The present study aimed to investigate the potential anti-proliferative effect of PTE and the molecular mechanisms.

MATERIALS AND METHODS

Cell Culture  The normal renal cell line HK-2 and RCC cells A498 and ACHN, were obtained from Guangzhou Jennio Biological Technology Co., Ltd. (Guangzhou, China). The authenticity of cell sources was guaranteed. HK-2 and ACHN cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco, U.S.A.) and A498 cells were using RPMI-1640 medium (Gibco, U.S.A.). All medium contained 10% fetal bovine serum and 1% streptomycin/penicillin, in an incubator with 37°C and a humidified atmosphere of 5% CO2.

Reagents and Antibodies  Antibodies against Bad, Bak, Bax, Bel2, Caspase-3, Caspase-9, poly(ADP-ribose)polymerase

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(PARP), Akt, ERK, p-ERK, p-Akt, Cytochrome c, proliferating cell nuclear antigen (PCNA), Rad51, and γH2AX were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.), antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Abcam (Cambridge, U.K.), immunoglobulin G (IgG)-HRP antibodies were obtained from EarthOx (Millbrae, CA, U.S.A.). TEMED, RIPA buffer, ammonium persulfate (APS), phenylmethylsulfonyl fluoride (PMSF), Western Antibody diluent, Crystal violet solution, dimethyl sulfoxide (DMSO) solution, Tween20 solution, streptomyacin–penicillin solution and lactate dehydrogenase (LDH) assay kit were procured from Beyotime Institute of Biotechnology (Shanghai, China). PageRuler Prestained Protein Ladder and fetal bovine serum were procured from Thermo Fisher Scientific, Inc. (Waltham, MA, U.S.A.). PTE was kindly provided by Solarbio (Beijing, China).

**Cell Viability** We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate cell viability according to the manufacturer’s protocol. Briefly, after A498, ACHN and HK-2 cells were reaching 90% confluences, the cells were trypsinised, counted and plated in 96-well plates at a density of 5 × 10^3 cells/well, three parallel replicates were prepared and the cells were cultured overnight. Then the cells were incubated with different concentrations of PTE (0, 5, 10, 20, 50, 100 μM) for 24, 48, and 72 h. After MTT were added into each well, cells were incubated for 4 h at 37°C, then formazan crystals were formed, we removed the MTT solution and added 150 μL DMSO to dissolve formazan crystals. Cell viability was determined as the optical density (OD) value. We used a Multispan Ascent microplate photometer (EnSpire 2300 Multilabel Reader, PE) to evaluate the OD value at 492 nm. Surely, we designed arbitrarily and repeated the experiments three times.

**Cytotoxicity Assay** Evaluation of cytotoxicity was performed with an LDH Cytotoxicity Assay Kit. A498 and ACHN cells were harvested, counted and plated at a density of 4000 cells per well in a 96 well plate and incubated for 24 h. Each experimental well was set up in triplicate, then we treated the cells with different concentrations of PTE (0, 10, 20, 50 μM) for 24 h, untreated cells were considered as the negative control group. After the plates were centrifuged for 5 min at 400 × g, we transferred 120 μL of supernatant from each well to a new 96-well flat-bottomed plate and added LDH detection reagent (60 μL) into each well and put on a shaker for 10 min, and measured at 492 nm using a 96-well plate reader (EnSpire 2300 Multilabel Reader, PE).

**Colony Formation Assay** After A498 and ACHN cells treated with different concentrations of PTE (0, 10, 20, 50 μM) for 24 h, untreated cells were considered as the negative control group. After the plates were centrifuged for 5 min at 400 × g, we transferred 120 μL of supernatant from each well to a new 96-well flat-bottomed plate and added LDH detection reagent (60 μL) into each well and put on a shaker for 10 min, and measured at 492 nm using a 96-well plate reader (EnSpire 2300 Multilabel Reader, PE).

**Western Blot** A498, ACHN and HK-2 cells were treated with PTE (0, 10, 20, and 50 μM) for 24 h. After washing with PBS, we used the mixed solution (PMSF and RIPA buffer) to lyse the cells on the ice. 40 μL of the cell lysate was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then proteins were transferred to poly-vinylidene fluoride membranes (polyvinylidene difluoride (PVDF), EMD Millipore, Billerica, MA, U.S.A.). We blocked the membranes with 5% fat-free powdered milk in TBST, the membranes were subsequently incubated on a shaker with the primary antibodies overnight at 4°C. Antibodies against Bad, Bak, Bax, Becl2, Caspase-3, Caspase-9, PPAR, Akt, ERK, p-ERK, p-Akt, Cytochrome c, PCNA, Rad51, γH2AX and GAPDH were used at a dilution of 1:1000. After incubation, we used TBST to wash the membranes three times, and then incubated the membranes with the secondary antibody (EarthOx, U.S.A.). The blots were measured using an ECL Prime Western blotting Detection Reagent. Fluorescence was detected using a BioRad imaging system.

**Flow Cytometric Analysis of Cell Apoptosis Assay** A498 and ACHN cells were treated with PTE (0, 10, 20, 50 μM) for 24 h, then the cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) by using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer’s directions. The examples were measured by a flow cytometer.

**Cell Cycle Assay** ACHN and A498 cells were treated with PTE (0, 10, 20, 50 μM) for 24 h, then the cells were harvested, washed in cold PBS, fixed with cold ethanol (70%) overnight and stained with propidium iodide (10 μg/mL) for DNA content. The results were measured by fluorescence-activated cell sorting (FACS).

**Immunofluorescence Analysis** ACHN and A498 cells grown on the cover glass were treated with different concentrations (0, 10, 20, 50 μM) of PTE for 24 h, the specific operation process refered to the previous article[39] and then photographed under Fluorescence microscopy.

**Statistical Analysis** Each experiment was performed in triplicate. Data analysis was evaluated using SPSS 19.0 software. One-way ANOVA was used to evaluate statistical significance; *p < 0.05, **p < 0.01, ***p < 0.001 were considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation (S.D.).

**RESULTS**

**Effect of PTE on the Viability of RCC Cells** To extensively investigate the effect of PTE on the growth of RCC cells, ACHN and A498 cells were exposed to concentrations of PTE (0, 5, 10, 20, 50, and 100 μM) for 24, 48, and 72 h. As shown in Fig. 1A, MTT measurements showed that the proliferation of ACHN and A498 cells was inhibited by PTE in a dose- and time-dependent manner. Nevertheless, following administration of HK-2 with relatively low concentrations of PTE (0, 5, 10, 20, and 50 μM) for 24, 48, and 72 h, the viability of HK-2 was hardly reduced markedly (Fig. 1C). In addition, the cytotoxicity of RCC cells was increased in a dose-dependent manner after treatment with various concentrations of PTE (0, 10, 20, and 50 μM) for 24 h (Fig. 1B). These results indicated that PTE potently exhibited anti-proliferative effects on RCC cells but not on normal renal cell.

**PTE Changes Cell Morphology and Inhibits Colony Formation of RCC Cells** Morphologic changes of RCC cells were displayed by phase contrast microscopy. As shown in Figs. 2B and C, administration of various concentrations of PTE (0, 10,
260 Vol. 43, No. 2 (2020)


20, and 50 µM) inhibited the colony forming potential of RCC cells in a dose-dependent manner.

PTE Induces Cell Cycle Arrest via Akt and ERK1/2 Signaling Pathways  In order to detect whether PTE-induced cell proliferation inhibition was mediated by alterations in cell cycle, we conducted cell cycle analysis. RCC cells were treated with different concentrations of PTE (0, 10, 20, and 50 µM) for 24h. Then cells were labeled with PI for DNA content and analyzed by flow cytometry. The results showed that PTE caused an accumulation of cells in S phase compared with the control group (Figs. 3A, B). To investigate the possible mechanism of PTE-mediated cell cycle arrest on RCC cells, the expression of related proteins were measured by Western blot analysis. The results showed that PTE remarkably induced a dose-dependent down-regulation of phosphorylation levels of Akt and ERK1/2 (Fig. 3C), indicating that inhibition of the

Fig. 1. Effect of PTE on the Viability of RCC Cells

(A) The cell viability of ACHN and A498 cells exhibited a significant decrease in a dose- and time-dependent manner which was measured by MTT assay. (B) The cytotoxicity was measured by an LDH assay. (C) There was no significant decrease in the cell viability of HK-2 cell. Data are representative as the mean ± S.D. of three independent experiments, *p < 0.05, **p < 0.01, ***p < 0.001 compared with the cells treated with DMSO (control).

Fig. 2. PTE Changes Cell Morphology and Inhibits Colony Formation of RCC Cells

(A) Morphologicl and quantitative changes of of ACHN and A498 cells were displayed by phase contrast microscopy. (B) The colony forming potential of both A498 and ACHN cells decreased significantly in a dose-dependent manner. (C) The number of colony formation was counted. Data are representative as the mean ± S.D. of three independent experiments, **p < 0.01, ***p < 0.001 compared with the cells treated with DMSO (control). (Color figure can be accessed in the online version.)
Akt and ERK1/2 signaling pathways contributed to the anti-tumor effect of PTE.

**PTE Induces RCC Cells Apoptosis** RCC cells were treated with different concentrations of PTE for 24h. The number of apoptotic cells was then evaluated by annexin V and PI staining. PTE induced cell apoptosis in a concentration-dependent manner in RCC cells (Figs. 4A, B). To further research the related molecular mechanisms, levels of apoptotic-associated proteins were measured, the results showed that PTE increased the expression levels of pro-apoptotic proteins, bad, bak, bax, Cyto C, Cleaved-Caspase 3, Cleaved-Caspase 9, and cleaved-PARP, while decreased the expression levels of anti-apoptotic proteins Bcl2 compared with control group (Figs. 4C, D), indicating that reduced cell growth by PTE in RCC cells could also be attributed to the induction of apoptosis.

**PTE Activates the DNA Damage Response in RCC Cells** Next, in order to investigate whether PTE induced DNA damage in RCC cells or not, Western blot assay and immunofluorescence analysis were used. The results showed that γH2AX foci (Fig. 5A) and the protein expression levels of γH2AX (Figs. 5B, C) were increased, indicating an accumulation of double strand breaks (DSB). We also found that PTE suppressed the level of Rad51, indicating that PTE inhibited homologous recombination (HR). Then we detected the effect of PTE on the expression of PCNA, an important factor in DNA replication and DNA repair. PTE extensively reduced the expression of PCNA, suggesting that PTE regulated the cellular DNA repair pathway to increase DNA breaks. In addition, Western blot assay was used to investigate whether PTE induced DNA damage in HK-2 cells and the results showed that there was no significant change in the protein expression levels of γH2AX (Figs. 5D, E).

**DISCUSSION**

Apoptosis plays an important role in balancing cell division and death and maintaining the proper number of cells *in vivo*. In fact, the deregulation of apoptosis results in pathologic disorders, including cancer. Thus, apoptosis can protect organisms from tumorigenesis and induction of apoptosis is an effective method and the desirable strategy for anti-tumor therapy. A previous study have showed that apoptosis was measured by dozens of methods such as LDH assay. Then, the present study detected that PTE increase the toxicity to renal cancer cells and induced apoptosis with an LDH Cytotoxicity Assay Kit. The mitochondrial pathway induces cell apoptosis through activating caspases and cleavage of specific cellular substrates. The results of the present study indicated that administration of 5–100 µM PTE induced the apoptosis in RCC cells, these results were in coincidence with those of a previous study by Ko et al., which indicated that PTE effectively suppressed the growth of human oral cancer cells by inducing apoptosis. Furthermore, Schneider et al. indicated that PTE stimulated mitochondria-dependent apoptosis in human lung cancer SK-MES-1 and NCI-H460 cells. These studies demonstrated that PTE could trigger caspase-dependent mitochondria-derived apoptosis in various cancer cells.

In order to investigate whether the mitochondria apoptotic
event was involved in PTE-induced apoptosis in RCC cells, we first analyzed the release of cytochrome c. The results showed PTE increased cytochrome c releasing in ACHN and A498 cells. Bcl2 protein family (Bax, Bak, Bad, Bcl2), essential for apoptotic processes in many tumor cells, mediated the release of cytochrome c and apoptosis-inducing factor from the mitochondrial intermembrane space into the cytosol. Releasing cytochrome c subsequently interacted with pro-Caspase-9 to activate Caspase-9 and triggered the activation of Caspase-3, contributing to apoptosis. Our results showed that PTE increased the expression levels of Bax, Bak, and Bad decreased the expression level of Bcl2, activated Caspase-9, Caspase-3 and PARP in RCC cells. Overall, these results demonstrated that induction of the Caspase-dependent mitochondria-derived apoptosis resulted in the anticancer effects of PTE in RCC cells.

Cell cycle progression is a carefully regulated process and a key determinant of cell growth. On the contrary, many cancers are characterized by uncontrolled cell cycles, resulting in deregulated growth. Previous studies mentioned PTE could induce cell cycle arrest in a wide array of cancers, such as human prostate cancer, human gastric cancer, and human acute myeloid leukemia cells. Some studies have shown that relatively low concentration of PTE (20–25 µM) resulted in S-phase cells cycle arrest in human acute myeloid leukemia cell and human gastric carcinoma cell lines. Accordant with these results, our results indicated that PTE (10–50 µM) induced S-phase cell cycle arrest in RCC cells.

We further searched the molecular mechanisms of PTE inhibiting RCC cells growth, finding that inactivation of Akt and ERK1/2 signaling pathways were required. ERK1/2 and Akt play a vital role in numerous signaling pathways. Akt, as a significant cell survival kinase, regulates other cell functions (migration and integrin activation). Previous study suggested that PTE inhibited activation of Akt markedly, resulting in anti-proliferation and autophagy-inducing effects of PTE in oral cancer cells. ERK1/2 pathway, being thoroughly considered as a potential pharmacological
target in the process of studying targeted therapy for cancer, plays a significant role in tumorigenesis and development by facilitating cell survival and growth. With regard to the effect of ERK activity on apoptosis, previous research have declared clearly that activation of ERK pathway accelerated cell growth, whereas, inactivation of ERK pathway makes cancer cells prone to apoptosis.29) So inactivation of Akt and ERK exerted an apoptosis-inducing effect on tumor cells. The present study tested the Akt and ERK1/2 activity in RCC cells following treatment with PTE, and indicated that phosphorylation of Akt and ERK1/2 reduced in a dose-dependent manner. Similar result could be discovered in oral cancer cells.11) Accumulation of DNA damage has been thought to be highly correlated with occurrence and development of cancer.30) Thus, evaluation of DNA damage, such as DSB, may be an effective indicator of cancer risk. Previous study had suggested that γH2AX assay was an accurate and powerful tool to evaluate DNA DSBs in cancer research.31) The result of our study suggested that PTE increased the expression level of γH2AX, indicating that PTE induced DNA damage in RCC

Fig. 5. PTE Activates the DNA Damage Response in RCC Cells

(A) Immunofluorescence analysis was conducted to measure the levels of phosphorylated H2AX (γH2AX) in ACHN and A498 cells following treatment with different concentrations (0, 10, 20, 50µM) of PTE. (B, C) Western blotting analysis was conducted to measure the expression levels of γH2AX, Rad51, and PCNA in ACHN and A498 cells. (D, E) Western blotting analysis was conducted to measure the expression levels of γH2AX, Rad51, and PCNA in HK-2 cell. Data are representative as the mean ± S.D. of three independent experiments, *p < 0.05, **p < 0.01, ***p < 0.001 compared with the cells treated with DMSO (control). (Color figure can be accessed in the online version.)
cells. PCNA, functioning as an auxiliary protein of eukaryotic DNA polymerase, increases the binding of DNA polymerase on particular DNA templates. In addition, research has shown that PCNA participate in DNA replication. Therefore, the increase of PCNA synthesis may initiate the reproduction of DNA, resulting in cell growth. The result of our study suggested that PTE decreased the expression level of PCNA, indicating that PTE inhibited the reproduction of DNA, ultimately leading to the inhibition of proliferation in RCC cells. DNA homologous recombination has always been an important metabolic pathway in organisms. Rad51 plays a pivotal role in DNA homologous recombination for DNA double strand breaks. A series of studies have showed the remarkable role of Rad51 in regulating cellular chemo responsiveness to chemotherapeutic drugs. In soft tissue sarcomas, the absence of Rad51 increases the sensitivity of cells to chemotherapeutic drugs. Similar results were observed in colon cancer cells treated with inhibitors of thymidylate synthase and in imatinib-resistant chronic myeloid leukemia cells. Rad51 has become a potential anti-tumor target for chemotherapy. Our study showed that PTE decreased Rad51 in RCC cells, demonstrating that PTE-induced inhibition on Rad51 may result in failure of repaired DNA double strand breaks, contributing to the growth retardation in RCC cells.

Taken together, these results demonstrated that PTE inhibited cell viability through suppressing cell cycle progression, inducing cell apoptosis, and regulating cellular DNA repair and inducing the DNA breaks, further promoting the RCC cells death via Akt and ERK1/2 signaling pathways.

Acknowledgments This work was supported by Scientific Research Fund of Guangdong Medical University (No. M2017038); the National Natural Science Funds (No. 81272833) of China.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


