Paris Polyphylla Monomer PP-26 Induces Mitochondrial Pathway-Mediated Cell Apoptosis by Inhibiting the PI3K/Akt Pathway in Human Gastric Cancer Cells

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Research Article

Keywords: Paris polyphylla, gastric cancer, apoptosis, mitochondrial apoptotic pathway, Akt

Posted Date: December 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1185625/v1
Abstract

Background: *Paris polyphylla* is a traditional Chinese medicinal herb that has been used as a haemostatic, antimicrobial and anticancer agent. Gastric cancer (GC) is a global health problem, with more than 1 million people newly diagnosed with gastric cancer worldwide each year.

Methods: The MTT and colony formation assay were used to test the anti-proliferative effects of PP-26 on MGC-803 and BGC-823 cells. Flow cytometry assays, Hoechst 33258 staining assay and Caspase inhibitor Z-VAD-FMK were used to test apoptosis. JC-1 staining used to measure changes in mitochondrial membrane potential and western blot analysis were used to test apoptotic and PI3K/Akt pathway related proteins.

Results: PP-26 had a dose-dependent inhibitory effect on the proliferation of MGC-803 and BGC-823 cells, but had no obvious anti-proliferative effect on normal liver LO2 cells and normal embryonic kidney HEK-293 cells. Additionally, PP-26 induced typical apoptotic morphological changes, such as nuclear pyknosis, nuclear cracking and apoptotic bodies. Moreover, PP-26 induced a decrease in mitochondrial membrane potential. And PP-26 modulated the expression of Bcl-xL, Mcl-1, Bax, caspase-9/-3 and PARP proteins and induced cell apoptosis through the mitochondrial apoptotic pathway. Next, using an irreversible general caspase inhibitor (Z-VAD-FMK), we confirmed the activation of the mitochondrial apoptotic pathway induced by PP-26. Furthermore, PP-26 inhibited the phosphorylation of Akt and GSK-3β. The inhibition of Akt protein activated the mitochondrial apoptotic pathway.

Conclusion: Collectively, these results indicated that PP-26 inhibited the proliferation of MGC-803 and BGC-823 cells by inhibiting the Akt signalling pathway and activating the mitochondrial apoptotic pathway.

Introduction

Gastric cancer (GC) is a global health problem, with more than 1 million people newly diagnosed with gastric cancer worldwide each year. Despite its worldwide decline in incidence and mortality over the past 5 decades, gastric cancer remains the third leading cause of cancer-related death (1). Recently, the incidence rate and death rate of GC has decreased in Western countries, but a high level of GC occurrence remains in Eastern countries (especially in Korea, Mongolia, Japan, and China). Moreover, the 5-year survival rate and early diagnosis rate of GC remain low. Currently, a combination of surgery and chemotherapy can improve outcomes of gastric cancer patients, but the side effects of chemotherapy cannot be underestimated (2). Therefore, more effective therapies are urgently needed for the treatment of gastric cancer.

*Paris polyphylla*, named Chong Lou, a perennial medicinal plant, originates from Yunnan, Sichuan, Guangxi, and Guizhou Provinces of China. Studies on its phytochemical properties have shown that steroidal saponins are the major active components in this commonly used traditional Chinese medicine (3, 4). Previous studies have shown that *Paris polyphylla* possesses anti-inflammatory, haemostatic,
antifebrile and antimicrobial effects, thereby contributing to curing injuries, fractures, parotitis, tumours, analgesia, and haemorrhages (5–9). In recent years, Paris polyphylla has attracted considerable interest owing to its antitumour activities.

Using silica gel column chromatography and preparative HPLC, we isolated and identified 51 monomer compounds, known as PP-1~PP-51, from Paris polyphylla. We found that PP-26 and 13 other monomers had strong anti-proliferation effects in CNE-2 cells, and the IC50 of these 14 monomers was less than that of cisplatin on CNE-2 cells. However, PP-26 was abundant in Paris polyphylla and had strong antitumour effect on cancer cells. In our previous study, we have reported that PP-26 triggers G2/M-phase arrest and induces apoptosis in HepG2 cells (10). Its molecular mechanism has not previously been reported in gastric cancer cell line. Hence, we investigated the effect of PP-26 on the proliferation of human gastric cancer cells and characterized the potential mechanism involved.

**Materials And Methods**

**Isolation and purification of PP-26 from Paris polyphylla**

The dried roots of Paris polyphylla var. yunnanensis were collected from Yunnan Province, China. The species was identified by Prof. Guang-Xiong Zhou (College of Pharmacy, Jinan University). The air-dried roots of P. polyphylla var. yunnanensis (10 kg) were extracted with 70% ethanol at room temperature. The EtOH solution was evaporated in vacuum to produce a residue (1.5 kg). The residue was later suspended in water and subjected to a Diaion HP-20 column using the EtOH/H2O (30: 70, 60: 40, 95:5, v/v) solvent system. The 95% EtOH fraction (550.0 g) was separated by a silica gel column chromatography (CHCl3/MeOH, 100:0 to 0:100, v/v) to afford fourteen subfractions (1–14). Fr. 10 was separated by preparative HPLC (MeOH/H2O, 75:25, v/v) to yield compound PP-26 (45.5 mg). The purity of the isolate was more than 95%, as determined by HPLC analysis. The spectral data of PP-26 revealed that it was the same as (3β,17α,25R)-spirost-5-ene-3,17-diol3-O-α-l-rhamnopyranosyl-(1→4)-α-l-rhamnopyranosyl-(1→4)-β-d-glucopyranoside. Data on PP-26: White amorphous powder; [d]D25 -118.6° (c 1.64, C5H5N); IR (KBr) νmax: 3421, 2931, 2879, 1642, 1456, 1380, 1130, 1052, 979, 892 cm⁻¹; ESI-MS m/z: 1053.8 [M+Na]⁺; ¹H NMR (400 MHz, C5D5N) δH: 6.38 (1H, s, H′′rha-1), 6.26 (1H, s, H′′′′rha-1), 5.82 (1H, s, H′′′rha-1), 5.29 (1H, d, J = 4.3 Hz, H-6), 4.91 (1H, overlapped, H′′′′′′rha), 1.75 (3H, d, J = 6.0 Hz, H′′′′rha-6), 1.58 (3H, m, H′′′′rha-6), 1.58 (3H, m, H′′′rha-6), 1.22 (3H, d, J = 7.1 Hz, H-21), 1.08 (3H, s, H-19), 0.95 (3H, s, H-18), 0.68 (3H, d, J = 5.1 Hz, H-27); ¹³C NMR (100 MHz, C5D5N) δC: 140.8 (C-5), 121.9 (C-6), 109.8 (C-22), 103.3 (C′′′′rha-1), 102.2 (C′′′rha-1), 102.2 (C′′rha-1), 100.3 (C′′′glc-1), 90.2 (C-17), 90.1 (C-16), 80.4 (C′′′rha-4), 78.1 (C-3), 78.0 (C′′glc-2), 77.8 (C′′glc-3), 77.7 (C′′′glc-4), 77.0 (C′′glc-5), 74.1 (C′′′rha-4), 74.0 (C′′′′rha-4), 73.3 (C′′rha-2), 72.8 (C′′′′rha-3), 72.8 (C′′rha-3), 72.8 (C′′′′rha-3), 72.6 (C′′′rha-2), 72.5 (C′′rha-2), 70.4 (C′′′′rha-5), 69.5 (C′′rha-5), 68.3 (C′′′rha-5), 66.7 (C-26), 61.2 (C′′glc-6), 53.1 (C-14), 50.3 (C-9), 45.1 (C-13), 44.8 (C-20), 39.0 (C-4), 37.6 (C-1), 37.2 (C-10), 32.4 (C-7), 32.4 (C-8), 32.1 (C-12), 32.1 (C-23), 31.8 (C-15), 30.4 (C-25), 30.2 (C-2), 28.8 (C-24), 21.0 (C-11), 19.4 (C-19), 18.9 (C′′′′rha-6), 18.6 (C′′rha-6), 18.4 (C′′′rha-6), 17.3 (C-27), 17.1 (C-18), 9.7 (C-21).
Cell culture and reagents

The human gastric carcinoma cell lines MGC-803 and BGC-823, the normal liver cell line LO2 and the normal embryonic kidney cell line HEK-293 were obtained from China Center for Type Culture Collection (CCTCC). PP-26((3β,17α,25R)-spirost-5-ene-3,17-diol3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-{α-L-rhamnopyranosyl-(1→2)}-β-D-glucopyranoside, C51H82O21) (Fig. 1A), isolated from *Paris polyphylla* and provided by the Institute of Traditional Chinese Medicine and Natural Products of Jinan University, was dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) at 16 mmol/l and diluted using Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, USA) to a 16 µmol/l working solution (DMSO final concentration <0.01%, v/v). Foetal bovine serum (FBS), trypsin, and EDTA were purchased from GIBCO (Grand Island, USA). Annexin V-fluorescein isothiocyanate (FITC), propidium iodide (PI), mitochondrial membrane potential assay kit with JC-1, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China). The Hoechst 33258 Staining Kit (no. C0003) was purchased from Beyotime (Shanghai, China). Antibodies against Akt (#4691 Rabbit mAb), p-Akt (Ser473) (#4060 Rabbit mAb), p-Akt (Thr308) (#9275 Rabbit mAb), GSK-3β (#9315P Rabbit mAb), p-GSK-3β (#5558P Rabbit mAb), Bcl-2 (#2872 Rabbit mAb), Bcl-xL (#2764 Rabbit mAb), Mcl-1 (#5453 Rabbit mAb), Bax (#5023 Rabbit mAb), Bak (#12105 Rabbit mAb), Caspase-3 (#9668 Mouse mAb), cleaved Caspase-3 (#9661 Rabbit mAb), Caspase-9 (#9502 Rabbit mAb), cleaved Caspase-9 (#7237 Rabbit mAb), PARP (#9532 Rabbit mAb), cleaved PARP (#9541 Rabbit mAb), and Z-VAD-FMK (Pan-Caspase inhibitor VI) were purchased from Cell Signalling Technology (Boston, USA).

Cell culture

MGC-803, BGC-823, LO2 and HEK-293 cells were cultured in RPMI 1640 supplemented with 10% FBS in a 5% CO₂-humidified atmosphere at 37°C. The cells were regenerated every 3 days as they grew to 70%-90% confluence. Once in the logarithmic growth phase, the cells were ready for the following experiments.

MTT assay

MGC-803, BGC-823, LO2 and HEK-293 cells were seeded onto a 96-well plate at a density of 5.0 × 10³ cells per well and incubated at 37°C overnight. Next, the cells were treated with different concentrations (0, 0.5, 1, 1.5, 2, and 2.5 µmol/l) of PP-26 for 24, 48, and 72 h under standard culture conditions (i.e., 37°C with 5% CO₂). At the end of the incubation period, 20 µl of MTT solution (0.5 mg/ml) was added to each well. After 4 h of incubation, the supernatant was discarded, and 150 µl of DMSO was added to each well. Subsequently, the cells were shaken vigorously to dissolve the purple precipitate. The absorbance of each well was measured with a microplate reader (Bio-Rad Laboratories, USA) at a reference wavelength of 570 nm. The inhibition rate of cell proliferation was calculated using the following formula: inhibition rate (%) = (1 – A_treatment / A_blank)×100%.

Colony formation assay

MGC-803 and BGC-823 cells were seeded onto a 12-well plate at a density of 500 cells per well and incubated in RPMI 1640 medium supplemented with 15% FBS in a 5% CO₂-humidified atmosphere at
37°C overnight. Next, the cells were treated with different concentrations (0, 0.5, 1.0, 1.5, 2.0, and 2.5 µmol/l) of PP-26 for another 7 days (two repeated wells were set in each group). At the end of the incubation period, the cells were washed three times with phosphate-buffered saline (PBS), and fixed with a fixative comprising methanol-glacial acetic acid (3:1) for 10 min. Next, the cells were stained in crystal violet for 15 min and subsequently washed with flowing water. Images of the colonies were captured with a digital camera.

**Annexin V-FITC/PI double-staining assay**

MGC-803 and BGC-823 cells were seeded onto a six-well plate at a density of 2×10^5 per well and incubated at 37°C. After cell attachment, MGC-803 cells were treated with different concentrations (0, 0.5, 1.0, 1.5, 2.0, and 2.5 µmol/l) of PP-26 for 24 h at 37°C, and BGC-823 cells were treated for 48 h. The cells were harvested and washed twice with PBS. Next, the cells were re-suspended in 200 µl of staining buffer and incubated at room temperature in the presence of 10 µl of Annexin V-FITC and 5 µl of PI for 15 min at 4°C in the dark. The cells were analysed by a flow cytometer (Becton, USA). A total of 10,000 cells were analysed in each sample.

**Hoechst 33258 staining**

MGC-803 and BGC-823 cells were seeded onto a six-well plate at a density of 2×10^5 per well and incubated at 37°C overnight. MGC-803 cells were treated with the indicated concentrations (0 and 2.5 µmol/l) of PP-26 for 24 h at 37°C, and BGC-823 cells were treated for 48 h. At the end of the incubation period, the cells were harvested and fixed with 500 µl of fixative solution at 4°C overnight. After cell immobilization, the cells were washed twice with PBS and stained with Hoechst 33258 for 5 min in the dark. Images of the apoptotic cells were captured with a fluorescence microscope (Leica, Germany).

**Measurement of mitochondrial membrane potential**

MGC-803 and BGC-823 cells were seeded onto a six-well plate at a density of 2×10^5 per well and incubated at 37°C overnight. Next, MGC-803 cells were treated with indicated concentrations (0 and 2.5 µmol/l) of PP-26 for 24 h at 37°C, and BGC-823 cells were treated for 48 h. At the end of the incubation period, the cells were incubated with 500 µl of JC-1 solution for 20 min at 37°C in the dark and washed with buffer solution. Changes in the mitochondrial membrane potential were detected under a fluorescence microscope (Leica, Germany). Red fluorescence indicates potential-dependent aggregation in the mitochondria. Green fluorescence, reflecting the monomeric form of JC-1, appeared in the cytosol after mitochondrial membrane depolarization.

**Western blot analysis**

MGC-803 and BGC-823 cells were seeded onto a six-well plate at a density of 2×10^5 per well and incubated at 37°C overnight. In the caspase inhibition experiment, the cells were pretreated with Z-VAD-FMK (20 nmol/l) for 1 h followed by treatment with PP-26 (2.5 µmol/l) for 24 and 48 h. After PP-26 treatment, the cells were collected, lysed for 30 min
at 4°C in ice-cold lysis buffer, and centrifuged at 12,000 rpm for 15 min at 4°C. The protein concentrations were determined using the Bradford Protein Assay kit. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to lysates and incubated at 95°C for 5 min. Equal amounts of protein were loaded in each well of a 10%-15% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Subsequently, the membranes were blocked with 5% dried skimmed milk for 1 h and incubated with the appropriate primary antibody at 4°C overnight. Next, the membranes were washed three times with Tris-buffered saline and Tween 20 (TBST) and incubated with a secondary antibody for 1 h at room temperature. After washing with TBST, the bound antibody complex was detected using an ECL chemiluminescence reagent on an UVItec Gel Documentation System (Cambridge, UK).

**Statistical analysis**

The SPSS18.0 software package was employed to perform all statistical analysis. All results are expressed as the means ± standard deviation (SD). Data were obtained from at least three independent experiments. Student’s t-tests and one-way analysis of variance (ANOVA) were used to evaluate the statistical significance of the results at a 95% confidence level, and $p<0.05$ was considered to be statistically significant.

**Results**

**Anti-proliferative effects of PP-26 on MGC-803 and BGC-823 cells.**

PP-26 is a steroidal saponin compound extracted and separated from *Paris polyphylla* (Fig. 1a). To investigate the cytotoxic effect of PP-26 on human gastric cancer cells, we assessed the viability of MGC-803, BGC-823, LO2 and HEK-293 cells by MTT assay. The cells were treated with different concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3.0 µmol/l) of PP-26 for 24, 48 and 72 h, and the results showed that the anti-proliferative effects of PP-26 in MGC-803 and BGC-823 cells were more significant than those of LO2 and HEK-293 cells (Fig. 1c, d, e, f). The growth of MGC-803 and BGC-823 cells was inhibited in a dose-and time-dependent manner. The IC_{50} of PP-26 on MGC-803 cells at 48 and 72 h was 2.45 ± 0.11 and 1.57 ± 0.07 µmol/l, respectively, and the IC_{50} of PP-26 on BGC-823 cells at 72 h was 2.41 ± 0.08 µmol/l. However, the IC_{50} of PP-26 on LO2 and HEK-293 cells was not detected at the experimental doses. In addition, MGC cells were treated with carboplatin and 5-fluorouracil at different concentrations for 48h as positive control group, and the results showed that the anti-proliferative effects of PP-26 in MGC-803 were more significant than carboplatin and 5-fluorouracil with the IC_{50} were >160 µmol/l and 132.4 µmol/l, respectively (Fig. 1b). These results indicated that PP-26 inhibited the proliferation of MGC-803 and BGC-823 cells but had weaker cytotoxicity to LO2 and HEK-293 cells.

Moreover, a cell colony formation assay was used to detect the cell colony forming ability of MGC-803 and BGC-823 cells treated with different concentrations of PP-26. The results showed that clones of
MGC-803 and BGC-823 cells were decreased under doses of PP-26 at 1~1.25 µmol/l, indicating that the cell colony forming ability of MGC-803 and BGC-823 cells was inhibited by treatment with PP-26 (Fig. 1g, h, i, j).

**PP-26-induced apoptosis in MGC-803 and BGC-823 cells.**

To determine whether the anti-proliferative effect of PP-26 is associated with the induction of cell apoptosis, MGC-803 cells were treated with PP-26 at the indicated concentrations and stained with Annexin V-FITC and PI staining solution for 24 h, and BGC-823 cells were treated for 48 h. The control group (0 µmol/l group), did not show any significant apoptosis, however, after PP-26 stimulation, the apoptosis rate increased to 18.1% and 9.9% in MGA-803 and BGC-823 cells, respectively. (Fig. 2a, b, c). Furthermore, Hoechst 33258 staining was used to observe the cell morphology changes of tumour cells. Cell morphological changes in MGC-803 or BGC-823 cells were observed in the 2.5 µmol/l treatment group for 24 or 48 h (Fig. 2d). Additionally, cell typical apoptotic morphological changes, such as nuclear pyknosis, nuclear cracking and apoptotic bodies, were observed under a fluorescence microscope.

**Activation of the mitochondrial apoptotic pathway induced by PP-26 in MGC-803 and BGC-823 cells.**

The mitochondrial apoptotic pathway plays an important role in cell apoptosis (11). To determine whether the anti-proliferation of PP-26 was executed by involving the mitochondrial apoptotic pathway, MGC-803 cells were detected by the fluorescent probe JC-1 after PP-26 stimulation at 2.5 µmol/l for 24 h, and BGC-823 cells were detected after stimulation for 48 h. The red fluorescence of J-aggregates was present in areas with high mitochondrial membrane potential (MMP), while the green fluorescence of JC-monomers was prevalent in areas with low MMP. The decrease of MMP is one of the markers of early apoptosis (12). We observed a remarkable decrease in the red fluorescence of J-aggregates while the green fluorescence of JC-monomers remained illuminated in PP-26 treated cells, indicating a reduction in MMP and activation of mitochondrial apoptotic pathway (Fig. 2e, f, g).

To further investigate the regulation of the mitochondrial apoptotic pathway induced by PP-26, Bcl-2 family proteins and apoptosis-associated proteins were analysed by Western blotting after PP-26 stimulation at the indicated concentrations. The results showed that the anti-apoptotic factors, Bcl-xL and Mcl-1 were downregulated and the proapoptotic factor Bax was upregulated in both gastric carcinoma cell types (Fig. 3a, b). In addition, Bcl-2 was downregulated in BGC-823 cells and Bak was upregulated in MGC-803 cells (Fig. 3a, b). For the proteins downstream of Bcl-2 family proteins, the expression of caspase-3 and -9 and PARP were decreased, while the cleaved fragment of caspase-3 and PARP were increased in both gastric carcinoma cells (Fig. 4a, b).

Next, to further prove whether PP-26 induced caspase-dependent apoptosis, Z-VAD-FMK, a inhibitor of caspase was used. After pretreatment with Z-VAD-FMK for 20 min, MGC-803 cells and BGC-823 cells were
treated with PP-26 at 2.5 µmol/l for 24 h and 48 h, respectively. The results showed that total PARP and caspase-3 was decreased which indicated that PP-26 induced caspase dependent apoptosis (Fig. 5a, b). Furthermore, MGC-803 and BGC-823 cells were treated with 2.5 µmol/l PP-26 with or without 20 nmol/l Z-VAD-FMK for 24 and 48 h. Annexin V-FITC and PI staining assay were performed. In PP-26 group, the apoptosis rates were 20.08% and 26.98% in MGC-803 and BGC-823 cells, but in PP-26 + Z-VAD-FMK groups the apoptosis rate were reversed, which further demonstrated that caspase-3 was involved in PP-26-induced apoptosis of MGC-803 and BGC-823 cells (Fig. 5c, d).

**PP-26-induced apoptosis is dependent on the inhibition of the Akt signalling pathway.**

To further explore the relationship between the Akt signalling pathway and PP-26 induced apoptosis, proteins of PI3K/Akt pathway was analysed by Western blotting after PP-26 stimulation at the indicated concentrations in MGC-803 cells for 24 h and in BGC-823 cells for 48 h and at 2.5 µmol/l for various times. The results showed that the phosphorylation of Akt proteins (Ser-473 and Thr-308) was decreased in both cell types treated with PP-26 in dose- and time-dependent manners (Fig. 6a, b, c, d). Additionally, the phosphorylation of GSK-3β, downstream of Akt, was also decreased. These results showed that the Akt signalling pathway, upstream of the mitochondrial apoptotic pathway was down regulated.

**Discussion**

*Paris polyphylla*, as a traditional Chinese medicinal herb, has been used as an anticancer agent for hundreds of years. In a previous study, 51 monomer compounds, named PP-1~PP-51, were isolated from *Paris polyphylla*. Recent studies confirmed that the aqueous, ethanolic extracts or monomeric compounds of *Paris polyphylla* anticancer activity on several types of cancer cell lines (13–16). For example, PP-7 exhibited cytotoxicity against HepG2 and HEK-293 cell lines with IC<sub>50</sub> values of 2.9 ± 0.5 µmol/l and 5.0 ± 0.6 µmol/l, respectively (17). Polyphyllin D displayed anti-proliferative effects against all OVCA cell lines tested, with IC<sub>50</sub> values ranging from 0.2 to 1.4 µmol/l (18). We found PP-22, a natural pennogenyl saponin, could inhibit the growth of human tongue squamous cell carcinoma SCC-15 cells in dose- and time-dependent manners (19).

In the present study, we investigated the antitumour activity of PP-26 in gastric cancer cells. We examined the antitumour potential of PP-26 in MGC-803, BGC-823, LO2 and HEK-293 cells and found that PP-26 inhibited the proliferation of MGC-803 and BGC-823 cells but exhibited weaker cytotoxicity to LO2 and HEK-293 cells. Furthermore, PP-26 has better anticancer effect than carboplatin and 5-fluorouracil. The cytotoxicity of PP-26 in gastric carcinoma cells was also confirmed by cell colony formation assay. In this study, we found that compare with BGC-803 cells, MGC-803 cells were more sensitive to PP-26. This phenomenon has also been reported in other literature, for example, extracts of Swertia Mussotii can induce apoptosis in MGC-803 and BGC-823 cells, and the IC<sub>50</sub> for MGC-803 cells was 395 µg/mL, while the IC<sub>50</sub> for BGC-803 cells was 1284µg/mL (20). Besides, the essential oil extracted from Cyrtomium
fortumei (J.) Smith leaves can also inhibited the proliferation of gastric cancer MGC-803 and BGC-823 cells, and MGC-803 cells were more sensitive to it (21).

Apoptosis is a spontaneous and programmed death process of cells under normal physiological or pathological conditions (22). Apoptosis occurs via three pathways: the death-receptor-mediated pathway (extrinsic pathway), the mitochondrial-mediated pathway (intrinsic pathway), and the endoplasmic reticulum-associated pathway (23). In the present study, the apoptosis rates of both MGC-803 and BGC-823 cells gradually increased with PP-26 incubation in a dose-dependent manner, as detected by Annexin V-FITC/PI double staining. The results also showed that the stimulation of PP-26 caused the loss of MMP and induced cell apoptosis, with typical apoptotic morphology changes, such as nuclear pyknosis, nuclear cracking and apoptotic bodies, in both MGC-803 and BGC-823 cells.

The mitochondrial death pathway is controlled by members of the Bcl-2 family (24, 25), which play a central regulatory role in deciding the fate of cells via the interaction between pro- and anti-apoptotic members (26). In the present study, antiapoptotic proteins, such as Bcl-xL and Mcl-1, were downregulated, and the proapoptotic protein Bax was upregulated in both gastric carcinoma cell lines in the process of cell apoptosis. Additionally, Bcl-2 was downregulated in BGC-823 cells, and Bak was upregulated in MGC-803 cells. Moreover, Western blotting also showed that full-length caspase-9, caspase-3 and PARP were decreased, and cleaved caspase-3 and cleaved PARP were upregulated, in both MGC-803 and BGC-823 cells treated with PP-26, demonstrating the occurrence of cell apoptosis. Furthermore, the downregulation of full-length caspase-3 and PARP was blocked by the caspase inhibitor Z-VAD-FMK, and Z-VAD-FMK reversed the apoptosis induced by PP-26, indicating that caspase-dependent apoptosis occurred in both MGC-803 and BGC-823 cells in the PP-26-treated groups.

The PI3K-Akt signalling pathway plays important roles in cell proliferation, cell cycle, cell growth, cell metabolism, cell survival and angiogenesis by affecting the activity of downstream molecules and is closely associated with the development and progression of human tumour cells (27–29). Therefore, we measured the activation of Akt and its downstream protein GSK-3β by Western blotting, and the results showed that the expression of both phospho-Akt and its downstream protein phospho-GSK-3β was downregulated after stimulation by PP-26 in time- and concentration-dependent manners; these results demonstrated that the PI3K/Akt signal pathway was inhibited by PP-26 in both cell types.

In conclusion, PP-26 inhibited proliferation and caused apoptosis in human gastric cancer MGC-803 and BGC-823 cells. However, this treatment showed weaker cytotoxicity to normal human liver LO2 cells and human embryonic kidney HEK293 cells. The proliferative inhibition effect of PP-26 is achieved by inhibiting the Akt signalling pathway and further activating the mitochondrial apoptosis pathway. (Fig. 7)

**Declarations**

**Ethics approval and consent to participate**

Not applicable.
Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Jiajin Chen and Junzhen Zhou contributed to the conception and design of the study and drafted the manuscript; Jiajin Chen, Junzhen Zhou, Jun Xu, Zhao Na and Qiang Li contributed to data analysis and interpretation; Xiaoying Zhang, Zhilong Liu, Guocai Wang and Qing Zhang participated in data collection and literature research. Chunyue Wang and Jianwei Jiang contributed to review and edit. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by Guangzhou Health Science and Technology Project (2021A011118), Science and Technology Planning Project of Guangzhou (202102080543, 202002030087), the First Affiliated Hospital of Jinan University Flagship specialty construction project-General surgery (711003), The National Natural Science Foundation of China (82074064), The Administration of Traditional Chinese Medicine of Guangdong Province (20201078), the Natural Science Foundation of Guangdong Province in China (2114050002009).

Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

Research involving Human Participants and/or Animals

Not applicable.

Informed consent

Not applicable.
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Figures

Figure 1
Anti-proliferative effects of PP-26 in MGC-803 and BGC-823 cells. (a) Chemical structure of PP-26. Anti-proliferative effects of carboplatin and 5-fluorouracil in (b) MGC-803 cells. Anti-proliferative effects of PP-26 in (c) MGC-803 cells, (d) BGC-823 cells, (e) LO2 cells, and (f) HEK-293 cells. Cells were seeded at 5000 cells per well onto a 96-well plate, exposed to different concentrations of PP-26 and incubated for 24, 48, and 72 h. Inhibitory rates of MGC-803 and BGC-823 cells were presented as the means ± SD of triplicate determinations (vs. group (time=24 h), *P < 0.05, **P < 0.01). Cell clones of (g) MGC-803 and (h) BGC-823 cells were observed after treatment with different concentrations of PP-26 and incubation for 7 days. The growth-inhibitory effects of PP-26 were observed in MGC-803 (i) and BGC-823 (j) cells. Clones of MGC-803 and BGC-823 cells were presented as the means ± SD of triplicate determinations (vs. control group (PP-26=0), *P < 0.05, **P < 0.01).
Figure 2

PP-26 induced apoptosis in MGC-803 and BGC-823 cells. Annexin V-FITC/PI analysis of (a) MGC-803 (24 h) and (b) BGC-823 (48 h) cells treated with different concentrations of PP-26. (c) The apoptosis rates of MGC-803 and BGC-823 cells were presented as the means ± SD of triplicate determinations (vs cell control group, *P < 0.05, **P < 0.01). (d) The morphological changes in MGC-803 and BGC-823 cells treated with PP-26 detected by Hoechst 33258 staining. (e) Effect of mitochondrial transmembrane...
potential on MGC-803 and BGC823 cells treated with PP-26 by JC-1 staining. The photometric ratio of MGC-803 (f) and BGC-823 (g) cells were presented as the means ± SD of triplicate determinations (vs. control group (PP-26=0), *P < 0.05).

**Figure 3**

Effect of the expression of Bcl-2 family proteins on MGC-803 and BGC-823 cells induced by PP-26. The expression of Bcl-2 family proteins was examined by Western blotting after (a) MGC-803 (24 h) and (b) BGC-823 (48 h) cells were incubated with different concentrations of PP-26. The experiment was repeated three times with similar results (vs. control group (PP-26=0), *P < 0.05).
Figure 4

Effect of apoptosis-associated proteins on MGC-803 and BGC-823 cells induced by PP-26. The expression of apoptosis-associated proteins caspase9, caspase 3, PARA was examined by Western blotting after (a) MGC-803 and (b) BGC-823 cells were incubated with different concentrations of PP-26 for 24 and 48 h. The experiment was repeated three times with similar results (vs. control group (PP-26=0), *P < 0.05).
Effect of PP-26 on inducing of caspase dependent apoptosis in MGC-803 and BGC-823 cell lines also treated with caspase inhibitor. The expression of apoptosis-associated protein caspase3, PARP in (a) MGC-803 and (b) BGC-823 cells was examined by Western blotting following 2.5 μmol/l PP-26 treatment with or without 20 nmol/l Z-VAD-FMK for 24 and 48 h. The experiment was repeated three times with similar results (*P < 0.05). Annexin V-FITC/PI analysis of (c) MGC-803 (24 h) and (d) BGC-823 (48 h) cells.
treated with 2.5 μmol/l PP-26 with or without 20 nmol/l Z-VAD-FMK. The apoptosis rates of MGC-803 and BGC-823 cells were presented as the means ± SD of triplicate determinations (vs cell control group, (PP-26=0), *P < 0.05, **P < 0.01).

Figure 6
Effect of PP-26 on the regulation of the Akt signalling pathway in MGC-803 and BGC-823 cell lines. The protein expression was examined by Western blotting after (a) MGC-803 (24 h) and (b) BGC-823 (48 h) cells were incubated with different concentrations of PP-26. Protein expression was examined by Western blotting after (c) MGC-803 and (d) BGC-823 cells were incubated with 2.5 μmol/l PP-26 for different time points. The experiment was repeated three times with similar results (vs. the control group (0 μmol/l of PP-26), *P < 0.05).
Figure 7

Schematic graph demonstrating mechanism of PP-26 on apoptosis in MGC-803 and BGC-823 cell lines. PP-26 inhibited the Akt signalling pathway and further activating the mitochondrial apoptosis pathway.