



Promotion of Ros-mediated Bax/Cyt-c apoptosis by polyphyllin II leads to suppress growth and aggression of glioma cells

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Background: Gliomas remain among the most difficult cancers to treat, with a 5-year overall survival no greater than 5%. Many saponins showed a wide spectrum of anti-cancer activities at low concentration. Polyphyllin II is one of the common saponins from Paris polyphylla. However, the effect of Polyphyllin II on glioma cells has not been evaluated. Objective of the present study was to investigate whether Polyphyllin II have inhibition on glioma cells, and the possible mechanisms.

Methods: The viability of U87 and U251 cells was detected by cell counting kit-8, cell counting real time cellular analysis and cell clone formation methods. Transwell was used to estimate the aggression of U87 and U251. The cell apoptosis rate was tested by flow cytometry. The morphological change was determined by transmission electron microscopy. The levels of AKT, phosphorylation of AKT, Bax, Bcl-2, cytochrome c, and cleaved caspase 3 proteins were assessed by Western blot. N-acetyl-L-cysteine was used to check the role of ROS in polyphyllin II inhibition to glioma cells.

Results: Polyphyllin II showed significant suppress to proliferation and aggression of U87 and U251 in a dose- and time- dependent manner. Result of flow cytometry confirmed that Polyphyllin II induced apoptosis to U87 and U251 cells. Transmission electron microscopy observation revealed majority of the glioma cells treated with Polyphyllin II had turgidity of mitochondrion, disarrangement, diminution and vacuolization, those refer to mitochondrial apoptosis. Western blot indicated that Polyphyllin II promoted cyt-c, Bax, caspase 3 and cleaved-caspase 3, and decreased Bcl-2, AKT and p-AKT. Rescue experiments using N-acetyl-L-cysteine, a reactive oxygen species scavenger, reversed the levels of Bax and cyt-c, and the inhibition in Polyphyllin II-treated U87 and U251 cells.

Conclusions: The present findings revealed that polyphyllin II may be a potential drug against glioma.

Keywords: Cancer; saponin; mitochondrial apoptosis; reactive oxygen species; Paris polyphylla

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Introduction

In the central nervous system glioma is one of the most common primary tumors, and almost half of glioma is glioblastoma, that is associated with 5-year relative survival not more than 5% (1-3). Therefore, discovery of new drugs for anti-glioma is continued to be meaningful. Natural products have significantly contributed to the discovery of new compounds for new anti-cancer drugs. Saponins as a kind of important natural products have the characteristics of outstanding pharmacological effects, such as anti-inflammatory, antiviral, immune regulatory, cardiovascular protection (4-7) and anti-tumor activities (8,9). Many saponins showed a wide spectrum of anti-tumor activities at low concentration. Including our previous work, anti-cancer saponins were isolated from *Anemone tomentosa* (10), *Clematis argentilucida* (11), *Anemone taipaiensis* (12-15), *Ardisia pusilla* (16,17), starfish *Culcita novaeguineae* (18,19), and sea cucumbers (20-22). But the yield of many anti-cancer saponins isolation is low.

Paris polyphylla is extensively used in traditional system of Chinese medicine and widely distributed in China (23). Clinically and traditionally, Paris polyphylla is used as an analgesic, anti-inflammatory (9) and hemostatic medical herb (24) and shows anti-cancer effects in various cancer types (25-28). Secondary metabolites such as daucosterol, polyphyllin D, β -ecdysterone, polyphyllin I, II, V, VI, VII, H, dioscin, oligosaccharides, heptasaccharide, octasaccharide, trigofenoside A, protogracillin, Paris yunnanosides G-J, padelaoside B, pinnatasterone, formosanin C and 20-hydroxyecdysone saponins have been isolated from Paris polyphylla (23). A series of saponins from Paris polyphylla have better drug accessibility and have been reported anti-cancer effects. Such as polyphyllin I ($C_{44}H_{70}O_{16}$) (29,30), II ($C_{51}H_{82}O_{20}$) (31,32) and VII ($C_{51}H_{82}O_{21}$) (33-37): according to Chinese Pharmacopoeia 2020 edition the total content of polyphyllin I ($C_{44}H_{70}O_{16}$), II ($C_{51}H_{82}O_{20}$) and VII ($C_{51}H_{82}O_{21}$) should not be less than 0.60% in dried Paris polyphylla rhizome (38). Nevertheless, on glioma we still know little about the effect and apoptosis mechanism of Polyphyllin II (PPII). In this study, the effect and underlying mechanism of PPII on U87 and U251 cells were evaluated by RTCA, CCK-8, clone formation, transwell, transmission electron microscopy (TEM), western blot and flow cytometry. U87 cells are wild-type p53 glioma cells and U251 cells contain mutant p53 (39).

The present study aimed to evaluate the role of PPII in the regulation of apoptosis in glioma cells, and to identify

the potential underlying mechanisms. The results revealed PPII significantly suppressed the vitality and aggression of U87 and U251 cells in a dose- and time-dependent manner via AKT/p-AKT/Bax/Bcl-2/cyt-c/caspase 3/cleaved-caspase 3 pathway. Rescue experiments confirmed PPII inhibited the proliferation via ROS-mediated Bax/cyt-c in glioma. The present findings revealed that PPII may be a potential drug against glioma.

We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/tcr-21-966>).

Methods

Cell culture

Human glioma cell lines U87 (catalog number: TCHu138) and U251 (catalog number: TCHu 58) were bought from the Cell Bank of Chinese Academy of Science (Shanghai, China). The cell lines were cultured in DMEM (Gibco, China) supplemented with 10% FBS (Ausbian, Harbin, China) and maintained at 37 °C with 5% CO₂.

Cell proliferation assay

The logarithmic phase cells (5×10^3 cells/well) were seeded in 96-well-plate, and 24 h later the cells were incubated with different doses of PPII for 24 h, four replicated wells were used for each experimental condition. 10 μ L CCK-8 was added to each well (incubated at 37 °C for 2 h) for the measurement of the cell proliferation using a microplate reader (450 nm).

Clone formation

Cells were seeded in a fresh six-well plate and maintained in media containing 10% FBS, replacing the medium every 4 days. After 14 days, methanol and stained with 0.1% crystal violet (ACROS organics) fixed cells.

Transwell

Inserts of transwell flat bottom plate were coated with/without polylysine for invasion/migration (BD Bioscience, San Jose, CA, USA). 2×10^5 cells with 300 μ L Serum-free medium were seeded to the upper chamber. In the lower chamber, 500 μ L of medium (10% FBS) was added. After 24 h, the cells that did not migrate through the pores (upper

surface) were removed by scraping the membrane with a cotton swab. The cells were fixed in 4% paraformaldehyde for 15 to 30 min in the membrane. After fixed, the cells continued to be stained for 10 to 20 min with 0.1% crystal violet.

Flow cytometric analysis

2×10^5 cells were treated with different doses of PPII. After 24 h, the cells were washed with cold PBS (4 °C). The cells apoptosis rates were tested by Annexin V Apoptosis Detection Kit I. The result was evaluated by a flow cytometer (BD Bioscience) after the cells were labeled with Annexin-V and PI.

Transmission electron microscopy analysis

Cells were After 2 h fixed in 2% glutaraldehyde, the cells were washed with PBS for 10 min twice, and then were fixed in 1% OsO₄. Samples were cut and analyzed with a JEM-1400 (JEM-1400, JEOL, Japan).

Real time cellular analysis (RTCA)

1×10^4 cells with 200 μ L were seeded in E-plates at 37 °C and 5% CO₂ for 120 h recorded by iCELLigence system (ACEA Biosciences, Inc., San Diego, CA, USA).

Western blot analysis

Cells were treated with different doses of PPII (1.85 μ g/mL for U87, 5 μ g/mL for U251) for 24 h, and washed twice in cold PBS. Then the treated cells were collected and lysed in RIPA lysis buffer, bicinchoninic acid (BCA) kit was used to determine protein concentration, all the protein samples were quantified to be the same concentration. Cell lysates (50 μ g) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. Then incubated with the primary antibody at 4 °C overnight after blocked with 5% non-fat dry milk, and then incubated again with the secondary antibody in a dark place for 1 h. The protein level was corrected using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). P-AKT (Affinity Biosciences, Inc., USA, product code: AF0908), AKT (Servicebio Inc., China, product code: GB13427), BAX (Servicebio Inc., China, product code: GB11690), caspase 3 (Servicebio Inc., China, product code: GB11767C),

cleaved-caspase 3 (Servicebio Inc., China, product code: GB11009), cytochrome C (Servicebio Inc., China, product code: GB11080), BCL2 (Wanleibio Co, China, product code: WL0234), GAPDH (Santa Cruz Biotechnology, Inc., USA, product code: sc-32233).

Statistical analysis

Experiments were performed in biological replicates and randomly divided to treatments or untreatments, most of which included at minimum technical triplicates (n=3), as indicated in the figure legends. Sample size depended on the assay type. The investigators were blinded to the group allocation during the experiment when assessing the outcome of CCK-8, cell clone formation, RTCA, transwell, western blot, TEM, and flow cytometer detection. Means were compared with one-way analysis of variance or two-way analysis of variance when applicable. Multiple comparisons were performed by Tukey's multiple comparisons test. All data are analyzed using GraphPad Prism. A value of P<0.05 was considered as statistically significant difference.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Results

PPII inhibited glioma cell proliferation, migration and invasion in a dose- and time-dependent manner

To test the effect of PPII (*Figure 1A*) on glioma cell growth, proliferation assay was done in U87 and U251 cells treated with different doses of PPII (20, 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 μ g/mL) for 24 h. We used CCK-8 assay to detect the cell viability and the results showed PPII decreased the OD values of U87 and U251 in a dose-dependent manner compared with the control group (*Figure 1B*). The IC₅₀ values of the saponin against the proliferation of U87 and U251 cells were 3.695 μ g/mL and 10.04 μ g/mL (*Figure 1C*). According to the IC₅₀ values, 3.7 μ g/mL PPII was used as high dose, and 1.85 μ g/mL PPII was used as low dose in U87 cells; 10 μ g/mL PPII was used as high dose, and 5 μ g/mL PPII was used as low dose in U251 cells. To verify the inhibition of PPII on glioma cell growth, proliferation was measured by iCELLigence and cell clone formation. iCELLigence is a real time monitoring system for an overall detection of the cells proliferation. The result showed that both of high and low doses could inhibit the

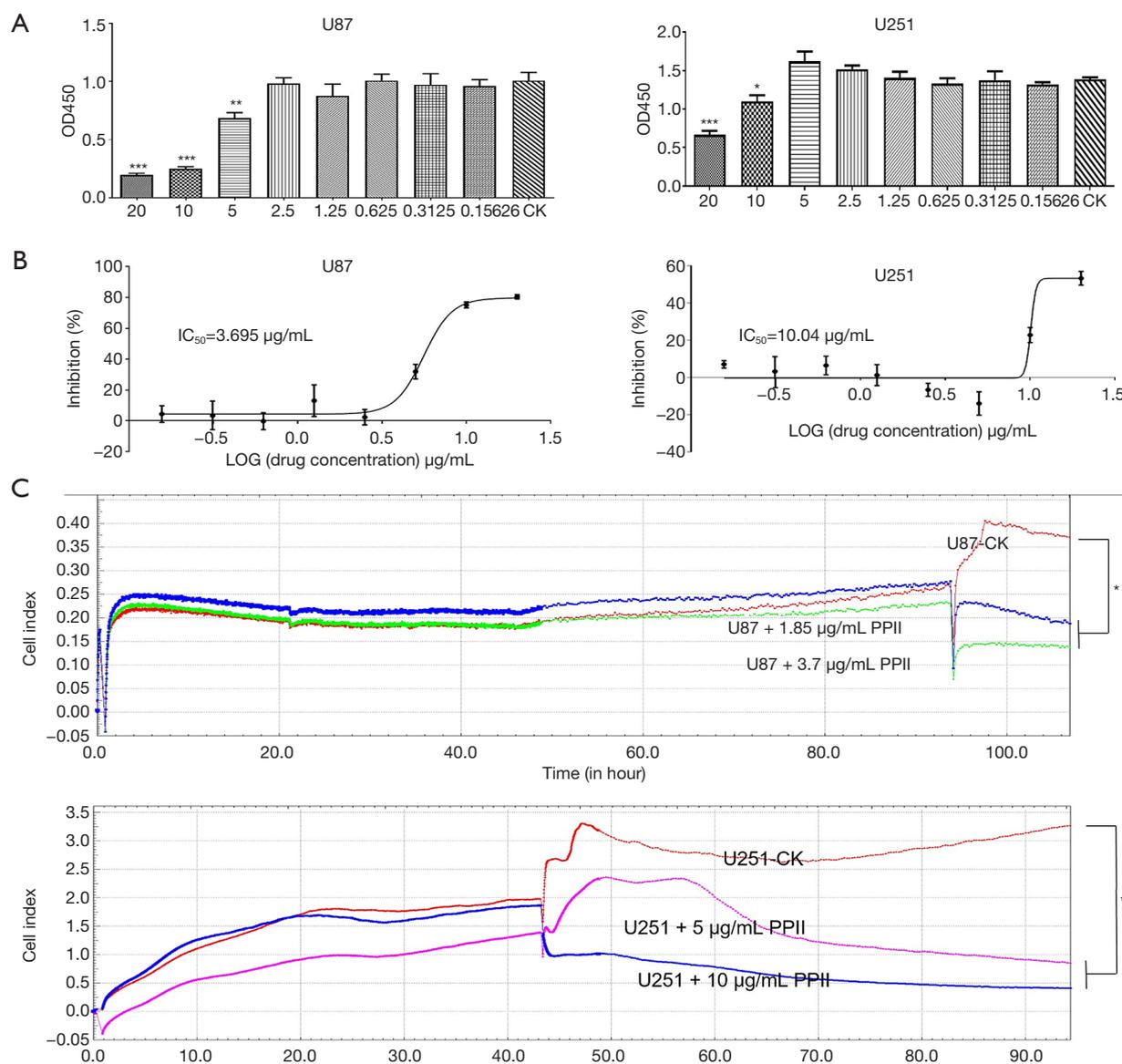


Figure 1 Low concentration of PPII inhibited glioma cell proliferation. (A) Structure of PPII. (B) PPII inhibited proliferation of U87 and U251 cells in a dose-dependent manner ($n=4$, 24 h, *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared with normal glioma cells). (C) IC₅₀ value of 3.695 µg/mL PPII for U87 cells and IC₅₀ value of 10.04 µg/mL PPII for U251 cells ($n=4$, 24 h); (D) iCELLigence showed PPII inhibited proliferation of U87 and U251 cells in a dose- and time-dependent manner ($n=3$, *, $P<0.05$ compared with normal glioma cells). PPII, polyphyllin II.

glioma cells proliferation in a time-dependent manner with PPII treatment (Figure 1D). Cell clone formation provided intuitive pictures that PPII inhibited glioma cell proliferation in a dose-dependent manner (Figure 2A). So did transwell test and the results revealed both of high and low doses of PPII inhibited migration and invasion of the glioma cells (Figure 2B).

PPII induced apoptosis to glioma cells

The above results confirmed that low concentration of PPII could inhibit glioma cell proliferation. The next question is what the mechanism is. Often apoptosis contributes to proliferation inhibition in drug anti-cancer. In order to determine the relationship between PPII and glioma cell

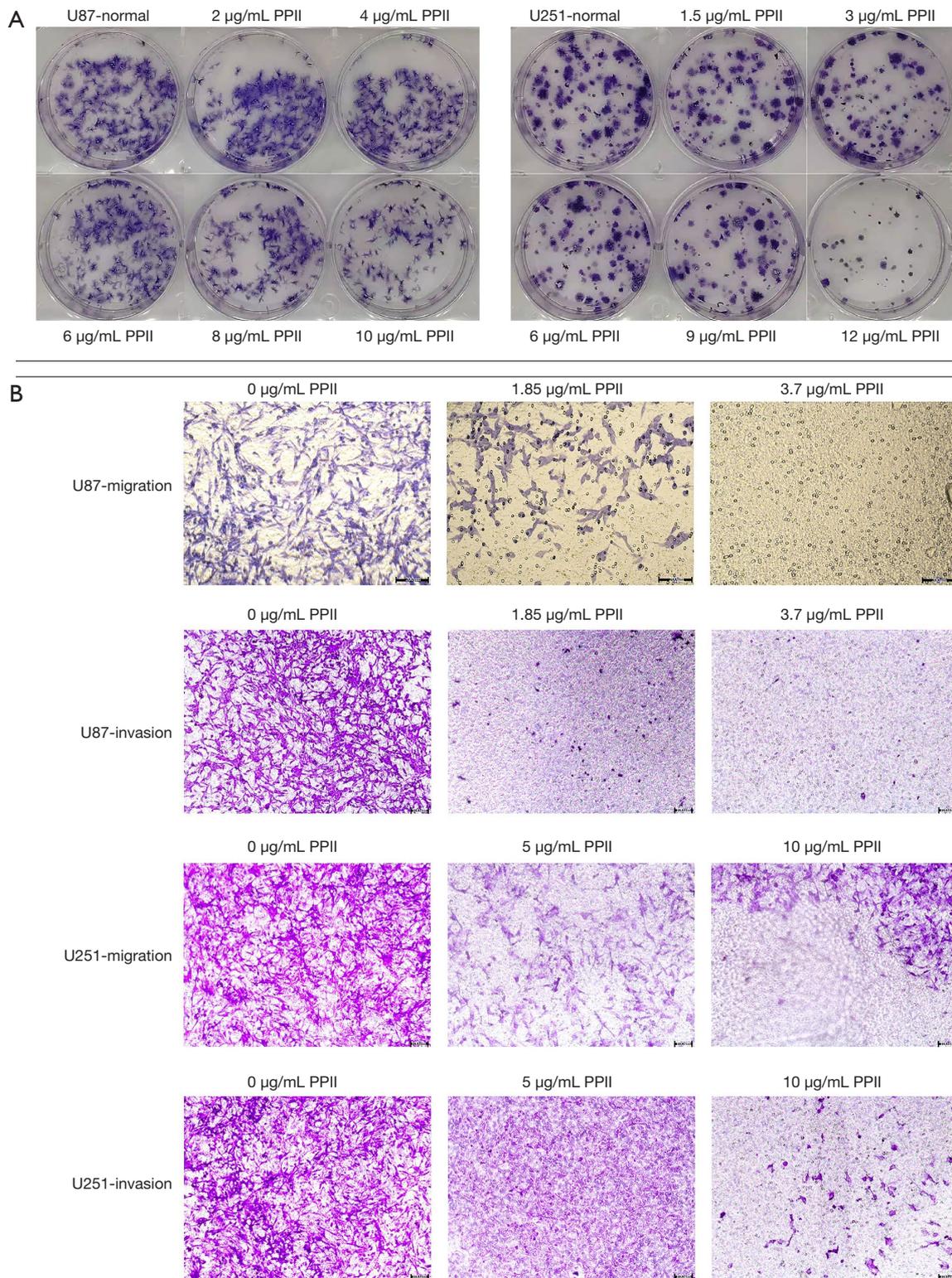


Figure 2 PPII attenuated cell clone and aggression of glioma cells. (A) PPII (Polyphyllin II) reduced the cell clone formation of U87 and U251 cells in a dose-dependent manner ($n=3$, 14 days, stained with 0.1% crystal violet); (B) effects of PPII attenuating migration and invasion of U87 and U251 cells ($n=3$, 24 h, stained with 0.1% crystal violet). PPII, polyphyllin II.

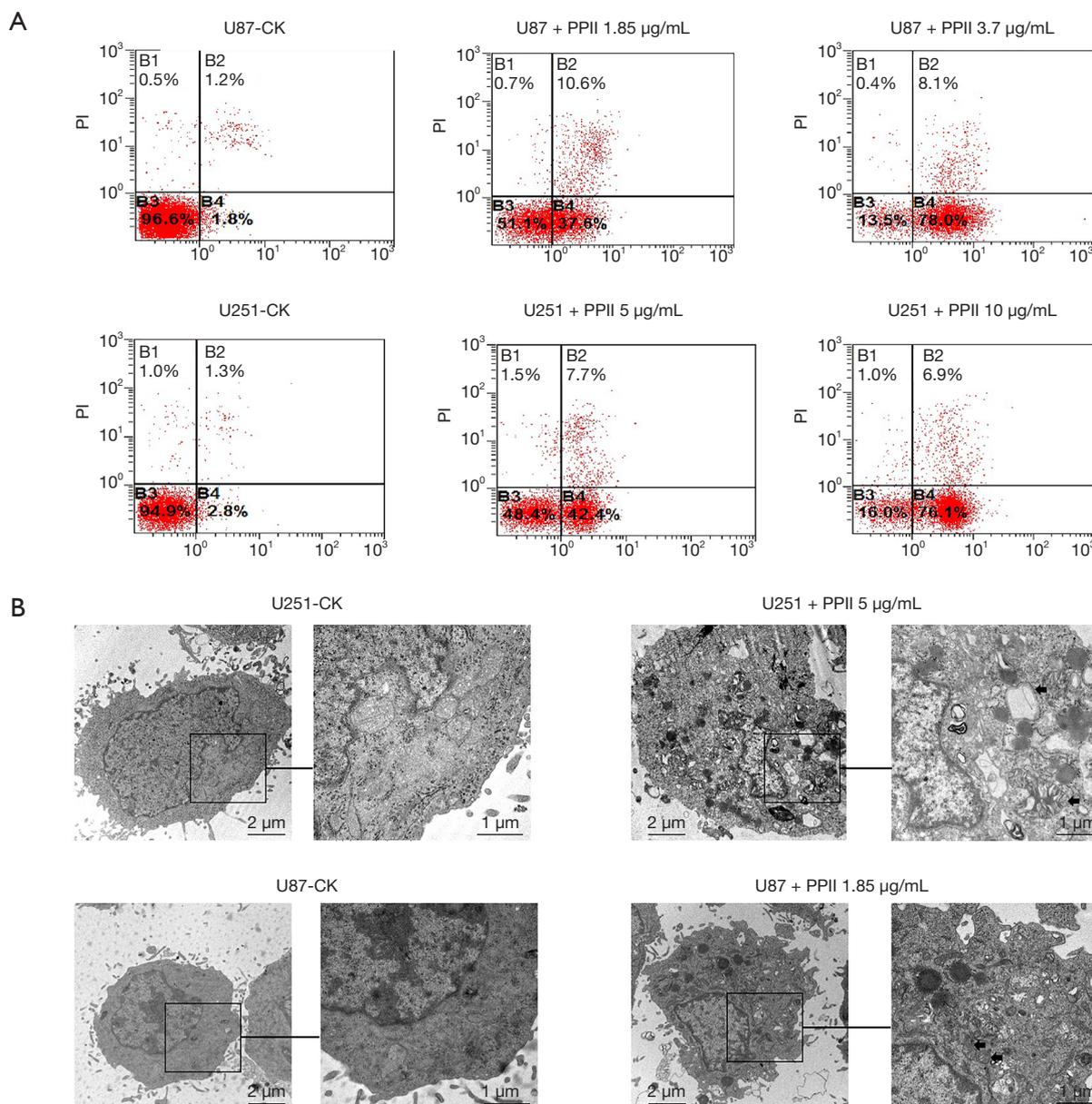


Figure 3 PPII induced apoptosis to U87 and U251 cells. (A) Both of high (3.7 $\mu\text{g/mL}$) and low doses (1.85 $\mu\text{g/mL}$) of PPII promoted the apoptosis to U87 cells, and both of high (10 $\mu\text{g/mL}$) and low doses (5 $\mu\text{g/mL}$) of PPII promoted the apoptosis to U251 cells examined by flow cytometry (n=3, 24 h); (B) TEM revealed that, normal glioma cells exhibited intact cell membranes and normal nuclei, while treated with PPII majority of cells had apoptosis features that mainly include the cytoplasmic shrinkage, the dilation of the endoplasmic reticulum, turgidity of the mitochondrion, the disarrangement, diminution and vacuolization (pointed by arrows, 24 h). PPII, polyphyllin II; TEM, transmission electron microscope.

apoptosis, three methods were performed: flow cytometry assay for apoptosis rate detection, transmission electron microscope (TEM) for observation of organelle structure change and western blot for molecular signaling pathway

test. Results of flow cytometry indicated that both of high and low doses of PPII could increase the percentage of apoptotic cells. In addition, apoptosis rates were positively associated with the drug concentration (Figure 3A). Because

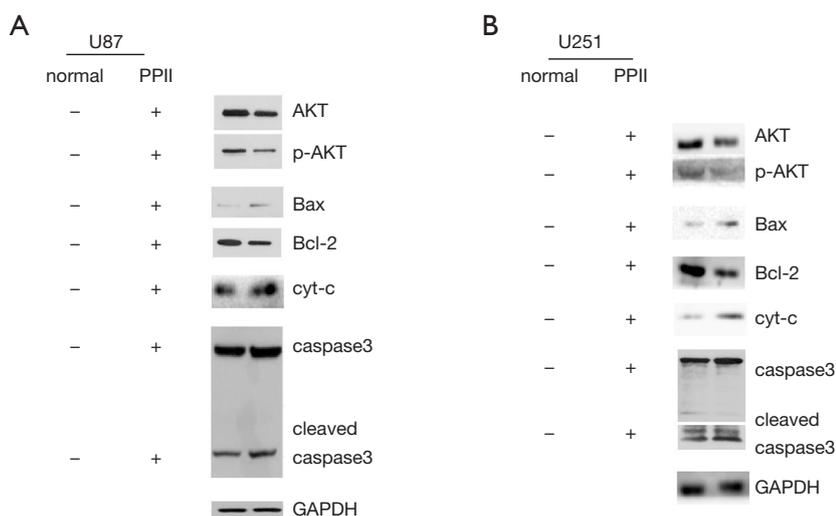


Figure 4 PPII induced mitochondrial apoptosis in U87 and U251 cells. (A) Western blot results showed 1.85 $\mu\text{g/mL}$ PPII increased Bax, cyt-c, caspase 3 and cleaved caspase 3, and decreased AKT, p-AKT, and Bcl-2 in U87 cells, the protein level was corrected using GAPDH ($n=3$, “+” represents positive, and “-” represents negative); (B) Western blot results showed 5 $\mu\text{g/mL}$ PPII increased Bax, cyt-c, caspase 3 and cleaved caspase 3, and decreased AKT, p-AKT, and Bcl-2 in U251 cells, the protein level was corrected using GAPDH ($n=3$, “+” represents positive, and “-” represents negative). PPII, polyphyllin II; Bax, BCL2-associated X protein; cyt-c, cytochrome c; AKT, protein kinase B; p-AKT, phosphorylation protein kinase B; Bcl-2, B-cell lymphoma 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

all the treatments led to significant apoptosis (at least over 37%), we selected the low doses to carry on the subsequent experiments. Under TEM, majority of cells had apoptosis features that mainly include the cytoplasmic shrinkage, the dilation of the endoplasmic reticulum, turgidity of the mitochondrion, the disarrangement, diminution and vacuolization. At the same time, intact cell membrane and normal nucleus were observed in normal glioma cells (Figure 3B). That indicated PPII may induce mitochondrial apoptosis in U87 and U251 cells.

PPII induced mitochondrial apoptosis in U87 and U251 cells through AKT/Bax/Bcl-2/cyt-c/caspase 3 pathway

To determine the further molecular mechanism under PPII inducing apoptosis to glioma cells, western blot assay was used to evaluate the expression of the AKT/Bax/Bcl-2/cyt-c/caspase 3 signaling pathway related proteins. After 24 treatments of PPII, the expressions of caspase 3, cleaved-caspase 3, Bax and cytochrome c (cyt-c) were significantly increased, and AKT, phosphorylated AKT (p-AKT) and Bcl-2 were significantly decreased in U87 and U251 (Figure 4). These results demonstrated that PPII induced mitochondrial apoptosis in U87 and U251 cells via AKT/Bax/Bcl-2/cyt-c/caspase 3 pathway.

ROS mediated PPII inhibition in U87 and U251 cells via Bax/cyt-c

It was reported that natural products generated ROS to promote cancer cell apoptosis (40-44), and in the current study a rescue experiment was performed to reveal the role of ROS in PPII inhibition. With a test of concentration gradient of N-acetyl-L-cysteine (NAC, a ROS scavenger), in U87 cells the PPII inhibition was reversed from 4 mg/mL NAC (Figure 5A), and in U251 cells the PPII inhibition was reversed from 8 mg/mL NAC by CCK-8 assays (Figure 5B). Moreover, on the molecular level, western blot showed NAC also reversed the levels of Bax and cyt-c in PPII treated U87 and U251 cells (Figure 5A, 5B).

Discussion

The present study aimed to evaluate the role of PPII in the regulation of apoptosis in glioma cells, and to identify the potential underlying mechanisms. The cell proliferation was evaluated with CCK-8, cell clone formation and iCELLigence assays to verify the inhibition effect of PPII to U87 and U251 cells. The results revealed PPII significantly inhibited the cell proliferation of glioma cells in a dose- and time-dependent manner and suggested that IC_{50} values of

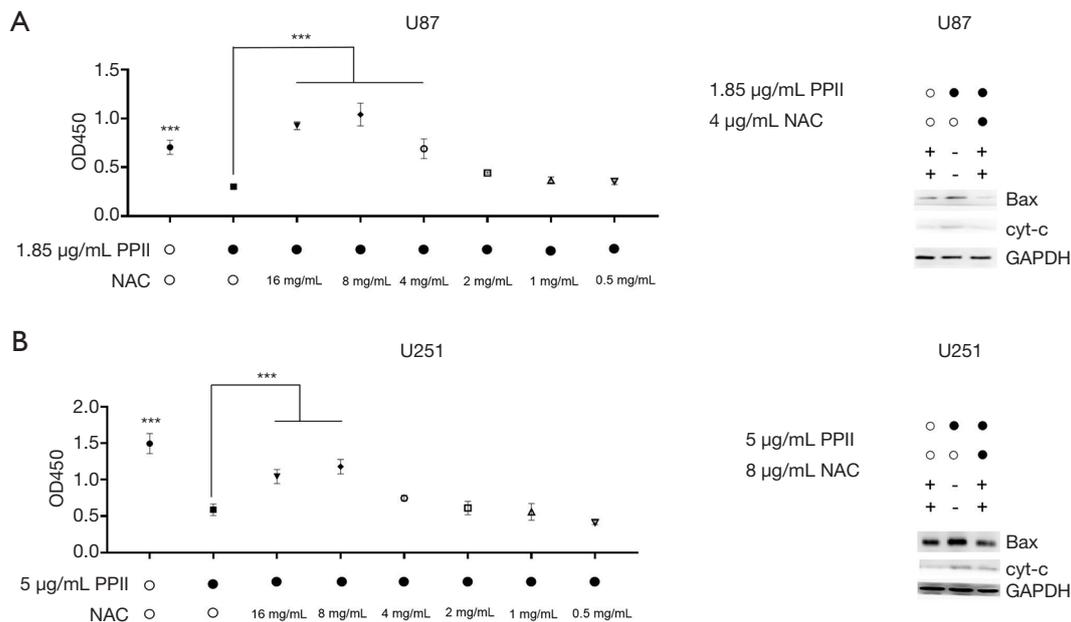


Figure 5 ROS-mediated activation of Bax and cyt-c led to inhibition in PPII-treated U87 and U251 cells. (A) CCK-8 and western blot determined that 4 mg/mL NAC was the optimal dose that could reverse the PPII inhibition via Bax and cyt-c in U87 cells, the protein level was corrected using GAPDH (n=3, ***, P<0.001 compared with PPII treated U87 cell, ○ means not added, ● means added, “+” represents positive, and “-” represents negative); (B) CCK-8 and western blot assay determined that 8 mg/mL NAC was the optimal dose that could reverse the PPII inhibition via Bax and cyt-c in U251 cells, the protein level was corrected using GAPDH (n=3, ***, P<0.001 compared with PPII treated U251 cell, ○ means not added, ● means added, “+” represents positive, and “-” represents negative). PPII, polyphyllin II; CCK-8, Cell Counting Kit-8; NAC, N-acetyl-L-cysteine; Bax, BCL2-associated X protein; cyt-c, cytochrome c; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the saponin against the proliferation of U87 (3.695 µg/mL) and U251 (10.04 µg/mL) (Figures 1,2A). PPII was reported to suppress the migration of T98G and LN18 (although the paper title used “invasion”, in that study transwell inserts were coated with nothing, thus we thought that was migration experiment) (45). In the current study, transwell results showed PPII inhibited both of migration (transwell inserts coated with nothing) and invasion (transwell inserts coated with polylysine) (Figure 2B).

From microscope observation, the cellular morphology changes and the cells become rounder after PPII treatment. After 24 h exposure to PPII the cells were seriously damaged: the cells became round and swollen, and the boundary was not clear. This observation suggested that PPII may induce apoptosis to the glioma cells. Therefore, flow cytometry assay and TEM were employed to confirm the suggestion. Flow cytometry assay showed PPII could induce serious apoptosis to the glioma cells at low doses (Figure 3A). TEM revealed treated with

PPII majority of cells had apoptosis features, mainly including the cytoplasmic shrinkage, the dilation of the endoplasmic reticulum, turgidity of the mitochondrion, the disarrangement, diminution, and vacuolization (Figure 3B).

The induction of apoptosis is one of the most effective approaches for treating cancer (46). Swollen mitochondrion is one of the hallmarks of early apoptosis stages in cells, since it facilitates cyt-c release in cell cytoplasm triggering intrinsic mitochondria-mediated apoptosis (47). Phosphorylation of AKT regulates Bax and Bcl-2 against apoptosis (48). Translocation of Bax to mitochondria membrane results in cyt-c release (49). Bcl-2 antagonizes Bax during the process. Caspase 3 and Bcl-2 are well-known pro- and antiapoptotic regulatory genes in eukaryotes. The Bcl-2 family controls caspase activity by inhibiting cyt-c release and subsequent caspase 3 activation (50-52). In the current study, PPII decreased levels of AKT, p-AKT and Bcl-2, and increased cyt-c, Bax, cleaved caspase 3 and caspase 3 in U87 (Figure 4A) and U251 (Figure 4B). That revealed

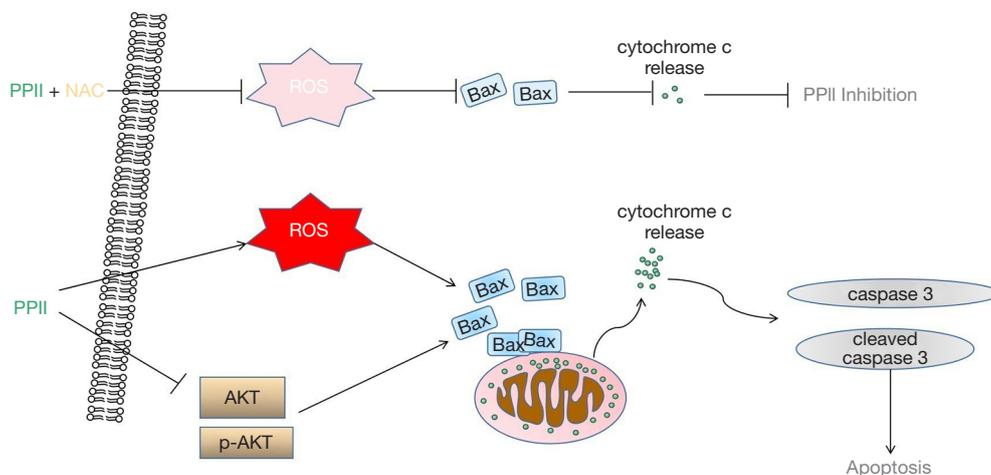


Figure 6 PPII induced apoptosis in U87 and U251 via ROS-mediated activation of Bax and cyt-c. PPII, polyphyllin II; Bax, BCL2-associated X protein; cyt-c, cytochrome c.

PPII inhibited proliferation of the glioma cells may be due to inactivation of the AKT signaling pathway and activation of Bax-mediated mitochondria apoptosis.

Mitochondria is major producer of ROS, and excessively high level of ROS promotes apoptosis (40,41). Previous studies reported several natural products generated ROS to activate apoptosis signaling in cancer cells. Antioxidant glutathione attenuated the piperlongumine-induced apoptosis and ROS of SKBR3, 786-O, Panc1, L3.6pL and A549 (42). A conjugate AlBA-DCA promotes ROS and killing cancer cells selectively (43). Erianin induced apoptosis to osteosarcoma *in vitro* and *in vivo* via ROS (44). In this paper NAC (a ROS scavenger) attenuated the PPII inhibition due to reversing the levels of Bax and cyt-c in PPII treated U87 (Figure 5A) and U251 (Figure 5B) cells.

In conclusion, PPII induced glioma cells mitochondrial apoptosis via ROS-mediated activation of Bax and cyt-c (Figure 6).

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Footnote

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Data Sharing Statement: Available at <https://dx.doi.org/10.21037/tcr-21-966>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/tcr-21-966>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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