Paeoniflorin inhibits human glioma cells via STAT3 degradation by the ubiquitin–proteasome pathway

Abstract: We investigated the underlying mechanism for the potent proapoptotic effect of paeoniflorin (PF) on human glioma cells in vitro, focusing on signal transducer and activator of transcription 3 (STAT3) signaling. Significant time- and dose-dependent apoptosis and inhibition of proliferation were observed in PF-treated U87 and U251 glioma cells. Expression of STAT3, its active form phosphorylated STAT3 (p-STAT3), and several downstream molecules, including HIAP, Bcl-2, cyclin D1, and Survivin, were significantly downregulated upon PF treatment. Overexpression of STAT3 induced resistance to PF, suggesting that STAT3 was a critical target of PF. Interestingly, rapid downregulation of STAT3 was consistent with its accelerated degradation, but not with its dephosphorylation or transcriptional modulation. Using specific inhibitors, we demonstrated that the prodegradation effect of PF on STAT3 was mainly through the ubiquitin–proteasome pathway rather than via lysosomal degradation. These findings indicated that PF-induced growth suppression and apoptosis in human glioma cells through the proteasome-dependent degradation of STAT3.

Keywords: paeoniflorin, glioma, apoptosis, proliferation, signal transducer and activator of transcription 3 (STAT3), ubiquitin–proteasome pathway (UPP)

Introduction

Gliomas are the most common primary tumors of the human central nervous system.1 Despite tremendous efforts to improve the prognosis of glioma patients over the past four decades, very little progress has been made, especially in patients with high-grade gliomas (grades III and IV).2 As in other cancers, glioma progression is accompanied by abnormal molecular changes.3,4 Several molecules are vital in the pro- or anti-survival pathways.5-6 Therefore, the identification and/or development of chemotherapy agents that selectively target molecular events linked to cancer progression may be an effective approach for the treatment of glioma.7

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that integrates signals from extracellular stimuli and regulates genes involved in many important cellular processes, such as cell cycle progression, apoptosis, and angiogenesis.8,9 The role of STAT3 as a crucial oncprotein in tumorigenesis has been extensively studied.10-12 After activation, phosphorylated-STAT3 could induce expression of genes that participate in oncogenesis, such as apoptosis inhibitors (HIAP-1, Bcl-2, and Survivin), and cell-cycle regulators (cyclin D1).13,14 Its expression and activation have also been correlated with reduced survival and poor prognosis in patients with glioma.15,16 Therefore, STAT3 is considered a promising target for glioma therapy, and its inhibition using multiple approaches has been shown to induce growth arrest and apoptosis in glioma cells both in vitro and in vivo.17-19

Interest in the identification of novel anticancer agents derived from natural sources has been growing exponentially. These agents provide an alternative approach to...
improve the existing standard of care for cancer patients.\textsuperscript{20,21} Paoniflorin (PF), an active component of \emph{Paeonia lactiflora} Pall. (also called \emph{P. alba}), has been widely used in traditional Chinese medicine. Previous studies have demonstrated that PF has many biological activities, such as immunoregulation,\textsuperscript{22,23} neuroprotection,\textsuperscript{24,25} and hepatoprotection.\textsuperscript{26} Recent research has indicated that PF might be a potent antitumor agent. It induced cell cycle arrest of HT29 colorectal cancer cells through the activation of p53/14-3-3 zeta.\textsuperscript{27} It also suppressed NF-κB activation and enhanced 5-fluorouracil-induced apoptosis in human gastric carcinoma cells.\textsuperscript{28} In this study, we demonstrated that PF inhibited proliferation and induced apoptosis of glioma cells. Additionally, we explored its antitumor activity via ubiquitylation and downregulation of STAT3. These attributes make PF a promising candidate compound for the treatment and prevention of glioma.

Materials and methods

Chemicals, reagents, antibodies, and cell culture

All human cell experiments were conducted according to the protocols approved by the Institutional Ethics Committee of Southern Medical University and the Military General Hospital of Beijing PLA. The study was approved by the Institutional Ethics Committee of Southern Medical University and Military General Hospital of Beijing PLA. DMSO (#D4540), Annexin V-FITC/PI apoptosis detection kit (#APOAF), proteasome inhibitor MG132 (#M7449), chloroquine (CQ) (#C6628), and thiazoyl blue tetrazolium bromide (MTT) (#M2128) were purchased from Sigma-Aldrich (St Louis, MO, USA). Protein A/G plus-agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA, #sc-2003). PF was purchased from Tianjin Shilian Technology (Tianjin, People’s Republic of China, #PCM-PL-002). PF diluted in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Valencia, CA, USA, #11965) was prepared as a stock solution of 400 mM and stored at −20°C until use. Antibodies specific for p-STAT3 (Tyr705) (#9145), STAT3 (#9139), cyclin D1 (#2978), Survivin (#2808), ubiquitin (#3936), and β-actin (#3700) were obtained from Cell Signaling Technology (Danvers, MA, USA). Human U87 and U251 cell lines were obtained from the Chinese Academy of Medical Sciences (Beijing, People’s Republic of China). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, #12664-025) in a humidified incubator with 5% CO\textsubscript{2} at 37°C. The cells were passaged twice weekly and used for experiments when in the exponential growth phase.

Cell viability assay

Cells (4×10\textsuperscript{4} cells per well) were seeded into 96-well plates in triplicate and incubated overnight before treatment with PF (10–20 mM) for 12–72 hours. After incubation, MTT dye was added, and the plates were incubated for 4 hours. Absorbance was measured using an ELISA reader (Multiskan EX; Labsystems; Dynex Technologies, Denkendorf, Germany) at a test wavelength of 490 nm and a reference wavelength of 690 nm.

Annexin V-FITC/PI staining

Approximately 1.5–2×10\textsuperscript{5} cells per well were plated in six-well plates, treated with 10–20 mM PF for 24 hours or 20 mM for 6, 12, and 24 hours, then collected and stained with Annexin V-FITC/PI according to the manufacturer’s instructions (Sigma-Aldrich). Briefly, the cells were washed twice with cold phosphate-buffered saline (PBS), resuspended in binding buffer at 5×10\textsuperscript{4} cells/500 μL, stained with 5 μL Annexin V and 10 μL Propidium iodide (PI), and incubated for 15 minutes at room temperature in the dark prior to flow cytometric analysis.

Western blot

Treated cells were washed in ice-cold PBS and extracted with ProteoJET\textsuperscript{TM} Mammalian Cell Lysis Reagent (Fermentas, Burlington, ON, Canada) supplemented with protease and phosphatase inhibitors (Fermentas) according to the manufacturer’s instructions. Protein (20–40 μg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 8%–10% gels and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Blots were blocked for 1 hour at room temperature with 5% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline/0.1% Tween-20. The blots were then incubated with specific primary antibodies at 4°C overnight. The blots were then incubated with horseradish peroxidase-linked secondary antibodies for 1 hour at room temperature. After additional washes, signals were detected using SuperSignal ECL (Pierce, Rockford, IL, USA).

Overexpression of wild-type STAT3

Semiconfluent cultures of U87 cells in 60 mm\textsuperscript{2} dishes or 96-well plates were transfected with indicated empty or STAT3 expression vector (pCMV6-STAT3) (Bioworld, Beijing, People’s Republic of China) using Lipofectamine\textsuperscript{TM} 2000 reagent following the manufacturer’s protocol. Approximately 24 hours after transfection, cells were incubated with PF or vehicle for 24 hours. Overexpression of STAT3 in transfected cells was confirmed by immunoblotting.
RNA preparation and real-time PCR
Total RNA was extracted from U87 and U251 cells after treatment with PF for 24 hours using an E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA). RNA was then reverse-transcribed using a Prime-Script II 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan). For validation, real-time polymerase chain reaction (PCR) was performed using a SYBR Premix Ex Taq Kit (Takara) and an ABI Vii7 detection system (Applied Bio-systems, Foster City, CA, USA). The reaction conditions were: 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 34 seconds (40 cycles). The nucleotide sequences of the primers used for amplification were: STAT3 (forward, 5′-GCTTCCTGCAAGAGTCGAAT-3′; reverse, 5′-ATTTGCTTCTCAAGATACCTG-3′) and β-actin (forward, 5′-AGCGCGGCTACAGCTTCA-3′; reverse, 5′-GGCCATCTCTTGCTCGAAGT-3′).

Immunoprecipitation of STAT3 for ubiquitination assay
To assess the ubiquitin regulatory ability of PF in glioma, cells were treated with PF or vehicle for 24 hours, extracted with lysis buffer, and then centrifuged at 13,000× g for 20 minutes at 4°C. The cleared supernatants were removed, mixed with STAT3 antibody, and incubated for 1 hour at 4°C. Next, 20 μL of resuspended volume of Protein A/G PLUS-Agarose was added to the mixture, followed by incubation at 4°C on a rotating device overnight. The beads were collected by centrifugation at 10,000× g for 2 minutes and washed three times in cold PBS containing protease inhibitors and phosphatase inhibitors. The immunoprecipitation complex was subjected to SDS–PAGE, followed by immunoblotting with antiubiquitin antibody to detect polyubiquitinated STAT3 protein. Immune complexes were visualized using an enhanced chemiluminescence kit.

Statistical analysis
SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were presented as the means ± SD. Statistical analysis of in vitro drug assays was performed by using the one-way ANOVA test and post hoc Bonferroni-corrected t-test. P<0.05 was considered statistically significant.

Results
Paeoniflorin inhibits human glioma cell proliferation
Cells were treated with different concentrations of PF for the indicated time periods, and MTT assay was used to measure cell viability. As shown in Figure 1A and B, cell viability was significantly decreased in a dose- and time-dependent manner in PF-treated cells (P<0.05). To assess the effect of PF on apoptosis, PF-treated cells stained with Annexin V-FITC/PI were analyzed by flow cytometry. Data shown in Figure 1C–F indicate that PF treatment increased apoptosis of glioma cells.

STAT3 mediates the role of paeoniflorin in glioma cell proliferation and apoptosis
The ability of PF to modulate STAT3 in glioma cell lines was investigated. PF was found to downregulate both total STAT3 and pSTAT3 protein levels in a time- and dose-dependent manner (Figure 2A–H, P<0.05). Several signaling molecules downstream of STAT3, including HIAP, Bcl-2, cyclin D1, and Survivin, were also decreased by PF treatment in a time- and dose-dependent manner (Figure 3A and B). These findings confirm that PF modulates STAT3 signaling in U87 and U251 cells.

If STAT3 is a key target of PF, then forced overexpression of STAT3 should attenuate its antitumor effects. To test this

Figure 1 (Continued)
Figure 1 Effects of PF on proliferation and apoptosis in U87 and U251 cells.

Notes: (A) Cells were treated with various concentrations of PF (0–20 mM) for 24 hours. (B) Cells were treated with 20 mM of PF for various time periods (12, 24, 36, and 48 hours). (C-F) Cells were treated with various concentrations of PF (0–20 mM) for 24 hours (C and D) or incubated with 20 mM PF for different times (0, 6, 12, and 24 hours) (E and F). After staining with Annexin-V-FITC/PI, cell apoptosis was analyzed using flow cytometry, and at least 10,000 cells were analyzed per sample. The data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 versus respective controls.

Abbreviation: PF, paenoflorin.
Paeoniflorin induces STAT3 ubiquitination and degradation

Figure 2 PF downregulated STAT3 and p-STAT3 protein expression in U87 and U251 cells. 

Notes: (A–D) The concentration course study for the expression of total and phosphorylated STAT3 by immunoblot assay. (E–H) Cells were treated with 20 mM PF for indicated time points (0, 6, 12, and 24 hours). Cell lysates were subjected to Western blot with the indicated antibodies. (B, D, F, H) Quantification of protein levels normalized to β-actin. The data are expressed as mean ± SD of three independent experiments, and significant differences from the control are indicated by *P<0.05. 

Abbreviations: PF, paeoniflorin; STAT3, signal transducer and activator of transcription 3.
Figure 3 Effects of PF on downstream gene products of STAT3.

Notes: The immunoblot analysis was done using glioma cells exposed to different concentrations (0, 10, 15, and 20 mM) of PF for 24 hours (A), or to 20 mM PF for indicated time points (0, 6, 12, and 24 hours) (B). β-actin served as loading control.

Abbreviations: PF, paeoniflorin; STAT3, signal transducer and activator of transcription 3.

Figure 4 (Continued)
Figure 4 Resistance to PF-induced apoptosis in STAT3-overexpressing glioma cells.

Notes: Group definition: empty vector transfected cells with vehicle alone (a) or with PF treatment (b); STAT3-expressing plasmid transfected cells with vehicle exposure (c) or with the PF exposure (d). (A) U87 cells were transfected with STAT3-expressing plasmid or empty vector as described and confirmed by Western blot analysis. (B) Quantification of protein levels normalized to β-actin. (C) Cell extracts from each group were immunoblotted for STAT3 and p-STAT3. (D) Quantification of protein levels normalized to β-actin. (E) Cell viability was determined by MTT assay. (F-G) Cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry. *P<0.05 compared with group a. #P<0.05 compared with group b. The data are representative of three separate experiments and presented as the mean ± SD; error bars indicate SD.

Abbreviations: PF, paeoniflorin; STAT3, signal transducer and activator of transcription 3; MTT, thiazolyl blue tetrazolium bromide.
hypothesis, we conducted a STAT3-overexpression experiment using cDNA of wild-type STAT3. The wild-type STAT3-overexpressing U87 cells showed increased expression of total and phosphorylated STAT3 (Figure 4A–D). STAT3 overexpression in U87 cells significantly attenuated the PF-mediated decrease in p-STAT3 (Figure 4C and D), indicating that the decrease of p-STAT3 might be due to STAT3 modulation. As expected, STAT3 overexpression attenuated the anti-proliferative and proapoptotic effects of PF, in comparison with their respective control groups (Figure 4E–G). These findings suggest that PF might function, at least in part, via downregulation of STAT3 in glioma cells.

**Paeoniflorin promotes ubiquitin-mediated STAT3 degradation**

To investigate the mechanism by which PF decreases STAT3 expression, we first evaluated mRNA expression using real-time PCR after incubation with 20 mM PF or vehicle for 24 hours in both cell lines. There was no significant change in mRNA levels following PF treatment compared to the control group (Figure 5A). Next, using cycloheximide (CHX), an inhibitor of de novo protein synthesis, we showed that PF treatment influences STAT3 protein stability. As shown in Figure 5B–E, the half-life of STAT3 protein in cells treated with PF and CHX was much shorter (~12 hours in U87 and ~13 hours in U251, respectively) than in cells treated with CHX alone (~24 hours for both). Thus, the degradation of STAT3 was significantly accelerated when treated with PF and CHX. These results indicate that PF influences STAT3 protein stability without affecting its transcription in glioma cells.

Both proteasomes and lysosomes are important mediators of degradation of cellular proteins. To determine which process was involved in PF-induced turnover of STAT3, cells were pretreated with the proteasomal inhibitor, MG132, or

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**Figure 5** PF modulates STAT3 via protein degradation and not via its transcriptional.

**Notes:** (A) Cells were treated with 20 mM PF or vehicle for 24 hours. STAT3 mRNA levels detected by qRT-PCR and normalized to the levels of β-actin are expressed as fold-change relative to the untreated cells. *P<0.05 compared with control group. (B and D) Time course study for STAT3 degradation. Cycloheximide (CHX, 100 μg/ml) was added to glioma cells in the presence or absence of PF (20 mM) for 24 hours, followed by Western blot analysis. (C–E) Quantification of STAT3 band intensities in the experiment are representative of three separate determinations by Image J (National Institute of Mental Health, Bethesda, MD, USA); the relative protein levels in each band from the cell samples were quantified by densitometry as a function of time, with the dashed line (-----) indicating the half-life ($t_{1/2}$) of the STAT3 protein in U87 and U251 cells. Similar results were obtained in three independent experiments.

**Abbreviations:** PF, paeoniflorin; STAT3, signal transducer and activator of transcription 3.
Figure 6 PF enhances the proteasome-mediated degradation/ubiquitination of STAT3 in glioma cells.

Notes: (A) Cells were treated with 25 μM CQ (4 hours), followed by incubation with 20 mM PF (20 hours) and then harvested for immunoblotting with STAT3 antibody. (B) Quantification of protein levels normalized to β-actin. (C) Cells were treated with 20 μM MG132 (4 hours), followed by incubation with 20 mM PF (20 hours) and then harvested for immunoblotting. (D) Quantification of protein levels normalized to β-actin. All aforementioned experiments were repeated at least three times with similar results and presented as the mean ± SD; error bars indicate SD. (E) Cells were incubated with 20 mM PF for 24 hours. The detection of ubiquitin (Ub) levels was assessed by the immunoprecipitation of STAT3. *P<0.05 compared with control. †P<0.05 compared with group (PF+MG132−).

Abbreviations: PF, paeoniflorin; STAT3, signal transducer and activator of transcription 3.

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the lysosomal inhibitor, chloroquine (CQ), for 4 hours, followed by PF treatment or vehicle for an additional 20 hours. As shown in Figure 6A–D, lysosomal inhibition did not rescue PF-induced STAT3 downregulation. In contrast, MG132 pretreatment completely blocked PF-mediated degradation of STAT3 in both cell lines. We next performed an in vitro ubiquitination activity assay to examine the role of ubiquitination in PF-mediated proteasomal degradation of STAT3 in both glioma cell lines. Interestingly, degraded STAT3 could be detected using an antiubiquitin antibody, indicating that STAT3 was ubiquitinated during PF-induced degradation. Specifically, we demonstrated that the intensity of the smeared bands of STAT3 in PF-treated cells was stronger than that in control cells (Figure 6E). These results indicate that the ubiquitin–proteasome pathway (UPP) plays an important role in PF-mediated STAT3 degradation in glioma cells.

Discussion
In this study, we have shown that PF inhibits proliferation and induces apoptosis of human glioma cells. Importantly, we have provided evidence that PF regulates STAT3 turnover via the ubiquitin–proteasome system. Therefore, PF is the first natural product targeting STAT3 for ubiquitin-mediated proteasomal degradation.

STAT3 protein is present in the cytoplasm in an inactive form until it is phosphorylated by receptor-associated kinases. Activated JAK kinases phosphorylate STAT3 at the tyrosine residue in its SH2 domain. This phosphorylation event releases p-STAT3 from the receptor, allowing it to then homo- or heterodimerize with other STAT proteins. Dimerized STAT3 translocates to the nucleus and binds to promoters containing its cognate DNA-binding sequence. The targeting and disruption of oncogenic STAT3 signaling may be achieved via multiple approaches. Newly discovered chemotherapeutic reagents targeting STAT3 mainly act by inhibiting its tyrosine 705 phosphorylation. In this study, we found that PF strongly inhibits the STAT3 pathway. Specifically, PF decreases levels of total and phosphorylated STAT3 as well as several of its downstream targets. Unlike other chemotherapeutic agents that target the STAT3 pathway, PF did not directly inhibit phosphorylation at tyrosine 705. As shown in Figure 2, the expression levels of p-STAT3 protein correlated with those of STAT3. When STAT3 was overexpressed in PF-treated U87 cells, it rescued p-STAT3 protein level as well as cellular proliferation and antiapoptosis (Figure 4). These data clearly demonstrate that PF inhibits cell proliferation and induces apoptosis by suppressing STAT3 signaling, which was not mediated by dephosphorylation of STAT3, but by the levels of STAT3 itself.

In all tissues, proteins are mainly degraded via the UPP or by lysosomes. Lysosomes mainly degrade extracellular and some cell-surface proteins, while proteasomes degrade intracellular proteins that are aberrantly folded or normally short lived. These proteins are recognized by a specific E3 ubiquitin ligase and conjugated to polyubiquitin, which is then degraded by the proteasome in an ATP-dependent manner. These changes in the fates of individual proteins can, in turn, induce global changes in cell signaling, thus regulating cell proliferation and apoptosis. The clinical success of bortezomib, which targets the UPP-mediated degradation of proteins in cancer cells, illustrated the importance of ubiquitin-mediated signaling in cancer and stimulated further research in this field. Natural products can also trigger the ubiquitin-mediated turnover of some proteins, leading to antiproliferative and proapoptotic effects in cancer cells. A previous study showed that fucoidan, a polysaccharide extracted from brown seaweed, enhanced the UPP-mediated degradation of transforming growth factor receptor (TGFR) and reversed TGFR-induced epithelial-to-mesenchymal transition in breast cancer cell lines. In this study, STAT3 mRNA expression was not altered by PF treatment (Figure 5A). In addition, CHX and PF increased the rate of STAT3 degradation (Figure 5B–E). Therefore, the reduction of STAT3 by PF was not caused by synthesis inhibition, but by its accelerated degradation. MG132, a proteasome inhibitor, reversed STAT3 degradation (Figure 6C and D), whereas CQ, a known lysosome inhibitor, had no effect on STAT3 regulation (Figure 6A and B). We also detected increased ubiquitin–STAT3 complexes following PF treatment in both cell lines (Figure 6E).

Recently, Wang et al observed that PF inhibited the growth of HT29 colon carcinoma xenografts in nude mice. A dose of 1 g/kg PF/d caused no loss in bodyweight or diet consumption by oral gavage for 6 weeks compared with the control group. However, its inhibitory effects have not been evaluated yet. On the other hand, PF is an orally administered bioactive reagent that may act in the central nervous system, its ability to penetrate the blood–brain barrier should also be evaluated in future studies. Although we have demonstrated that PF promotes the UPP-mediated degradation of STAT3, the exact molecular mechanism is still unclear. As mentioned earlier, E3 ubiquitin ligase is a key player during UPPs, and many small molecules including some natural compounds specifically target E3 ubiquitin ligase to initiate
the ubiquitin-mediated proteasomal degradation. As a result, we attempted to determine if any molecules (especially E3 ubiquitin ligase) were involved in PF-induced degradation of STAT3, but research in this field is limited. TMF/ARA160 and the E3 ligase TRAF6, which were reported to be involved in the UPP-mediated degradation of STAT3, were also investigated in this study (data not shown). Unfortunately, no significant differences were observed in their expression after PF treatment in two glioma cell lines.

Conclusion
In conclusion, we present the anticancer properties of PF in glioma cell lines. PF may achieve its antiproliferative and proapoptotic effects by reducing protein expression of STAT3 and its downstream targets. Furthermore, PF caused STAT3 degradation via the UPP. This is the first evidence that STAT3 is targeted for UPP-mediated degradation by a natural product. These data not only suggest that PF is a potential therapeutic candidate for glioma, but also demonstrate that the inhibition of STAT3 by its UPP-mediated degradation is an efficient strategy for cancer prevention and the discovery of new chemotherapeutic agents.

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Disclosure
The authors report no conflicts of interest in this work.

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