

Paeoniflorin inhibits glioblastoma growth in vivo and in vitro: a role for the Triad3A-dependent ubiquitin proteasome pathway in TLR4 degradation

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Background: Paeoniflorin, a polyphenolic compound derived from *Radix Paeoniae Alba* (*Paeonia lactiflora*), has exhibited anticancer activity in various human cancers, including glioblastoma. However, the mechanisms underlying the effects of this compound have not been fully elucidated. Toll-like receptor 4 (TLR4) plays an important role in the regulation of cancer cell proliferation and progression, and high TLR4 expression in glioblastoma specimens is associated with a poor prognosis. The present study aimed to investigate whether paeoniflorin suppresses glioblastoma via inhibition of TLR4 expression.

Methods: CCK-8 experiments and clone formation assay were performed to detect the cell proliferation. Western blotting was used to analyze protein expression levels. Detection of Triad3A binding with TLR4 was assessed by the immunoprecipitation. Orthotopic xenograft mouse model was used to evaluate the effect of paeoniflorin in vivo. MST was used to analyze the interaction between paeoniflorin and TLR4 protein.

Results: In our study, we found that paeoniflorin effectively inhibited glioblastoma growth and suppressed TLR4 protein levels, as well its downstream effectors both in vivo and in vitro. Moreover, when overexpressed TLR4 in glioblastoma abolished the effects of paeoniflorin on cell proliferation, migration, and invasion. Furthermore, we found that paeoniflorin decreased TLR4 protein through ubiquitination proteasome pathway (UPP)-mediated degradation in glioblastoma cells. Mechanistically, paeoniflorin promoted Triad3A to conjugate with TLR4, resulting in degradation. In addition, *Triad3A*-shRNA abolished paeoniflorin-enhanced UPP-mediated TLR4 degradation. Finally, we found that paeoniflorin could directly bind with TLR4 protein as assessed by MST assay.

Conclusion: Our study is the first to identify a novel mechanism for the antitumor activity of paeoniflorin, specifically: it decreases tumor growth by directly targeting TLR4 and modulating the TLR4/Triad3A-dependent axis, leading to TLR4 protein degradation and inhibition of glioblastoma cell progression in vitro and in vivo. Our current findings indicate that paeoniflorin is a potential glioblastoma therapeutic agent due to its Triad3A-dependent ubiquitin degradation of TLR4.

Keywords: paeoniflorin, glioblastoma, TLR4, ubiquitin, Triad3A

Introduction

Glioblastoma is the most common malignant tumor in the central nervous system.^{1,2} Over the past few decades, various approaches of treating this disease, including surgery, chemotherapy, radiotherapy, and combination treatments, have been developed; however, the survival rate among patients diagnosed with glioblastoma is seldom >18 months.^{3,4} Therefore, the identification of new therapeutic agents and the exploration

of novel intervention targets may be clinically beneficial for patients undergoing glioblastoma therapy.

Paeoniflorin, one of the polyphenols isolated from *Paeonia lactiflora* Pall, exhibits anticancer activation by inducing apoptosis, reducing cell proliferation, and suppressing migration and invasion via inhibition of diverse pro-onco molecular and signaling pathways in tumor cells.⁵⁻⁷ It has been reported that paeoniflorin influenced breast cancer cell proliferation and invasion via inhibition of the Notch1 signaling pathway.⁸ Additionally, Li et al reported that paeoniflorin suppressed pancreatic cancer cell growth by upregulating HTRA3.⁹ Hao et al recently reported that paeoniflorin enhanced the inhibitory effects of Erlotinib in pancreatic cancer by reducing ErbB3 phosphorylation; while Zheng reported that paeoniflorin inhibited human gastric carcinoma cell proliferation through a combination of upregulation of microRNA-124 and suppression of PI3K/Akt and signal transducer and activator of transcription 3 (STAT3) signaling.^{10,11} However, the anticancer mechanism of paeoniflorin has not been fully elucidated. Though, Li et al reported that paeoniflorin inhibited proliferation and induced apoptosis in human glioma cells via microRNA-16 upregulation and matrix metalloproteinase-9 downregulation and in our previous study, we found paeoniflorin inhibited human glioma cells via inhibiting STAT3,^{12,13} no specific molecular or signaling pathway was identified as the target for paeoniflorin-mediated glioblastoma suppression. Therefore, the definitive targets of paeoniflorin and clarification of the underlying mechanisms involved in glioblastoma suppression are needed.

Toll-like receptor 4 (TLR4) is a member of TLR family of proteins, which plays an important role in multiple cancers.¹⁴⁻¹⁷ Accumulating evidence demonstrates that TLR4 is highly expressed in glioblastoma and is associated with poor patient prognosis.¹⁸⁻²¹ Moreover, TLR4 is involved in the regulation of a variety of molecular and signaling pathways, of which the most important is the nuclear factor (NF)- κ B signaling pathway, a pathway involved in the upregulation of inflammation-related molecules, including nucleotide-binding domain and leucine-rich repeat containing protein 3 (NLRP3), caspase-1, and interleukin-1 β (IL-1 β), all of which are associated with glioblastoma progression.²²⁻²⁴ Furthermore, paeoniflorin was shown to regulate TLR4 in various pathological processes. For example, Li et al reported that paeoniflorin ameliorated atherosclerosis by suppressing TLR4-mediated NF- κ B activation,²⁵ while Zhang et al reported that paeoniflorin abrogated dextran sulfate sodium-induced colitis via a TLR4-dependent pathway.²⁶ Moreover, in Zhang et al study, paeoniflorin inhibited B-cell activation, proliferation, and differentiation by selectively blocking the lipopolysaccharide/TLR4 signaling pathway.²⁷ In addition,

Zhu et al reported that paeoniflorin suppressed the inflammatory response via inhibition of the TLR4/NF- κ B pathway in diabetic retinopathy.²⁸ Finally, paeoniflorin prevented TLR2/4-mediated inflammation in type 2 diabetic nephropathy.²⁹ However, whether paeoniflorin can regulate TLR4 to suppress glioblastoma is unclear; as is whether TLR4 is a direct target of paeoniflorin.

The TLR4 degradation signaling has been reported to be regulated by ubiquitin-dependent proteasomal pathways (UPPs).^{30,31} In general, ubiquitination controls the turnover of short-lived proteins in a cell. The ubiquitination process involves the activation of 3 specific enzymes, including ubiquitin-activation enzyme, ubiquitin-conjugation enzyme, and ubiquitin ligase enzyme (E3),³² which regulate ubiquitin molecules to attach to specific target proteins. Subsequently, these ubiquitinated target proteins are disrupted and degraded by the 26S proteasome complex. Triad3A is an E3 ubiquitin-protein ligase that interacts with the Toll/IL-1 receptor domain of TLRs and that this interaction enhances ubiquitination and proteolytic degradation of TLR4.³⁰ Therefore, we explored whether paeoniflorin could regulate TLR4-Triad3A remodeling.

In the present study, we demonstrate that paeoniflorin suppressed TLR4 in glioblastoma in vitro and in vivo. Furthermore, we show that paeoniflorin enhanced TLR4 degradation via a Triad3A-mediated ubiquitin-proteasome pathway. Finally, we identify TLR4 as a direct target of paeoniflorin. Taken together, our findings show that paeoniflorin has potential as an anticancer agent through regulating Triad3A-dependent TLR4 degradation.

Materials and methods

Chemicals, reagents, and antibodies

Paeoniflorin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in normal saline (0.9% NaCl) and stored at 4°C. DMEM and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Antibodies against NLRP3, ubiquitin (P4D1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA), and antibodies against TLR4, pro-IL-1 β (IL-1 β inactivated form), caspase-1, Triad3A, and NF- κ B were purchased from Abcam (Cambridge, MA, USA). Chloroquine (CQ), MG-132, and actidione cycloheximide (CHX) were purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

The human glioblastoma cell lines U87, U251, and U87-luciferase were purchased from the Chinese Academy of Medical Sciences (Beijing, China) and were cultured in high-glucose

DMEM supplemented with 10% FBS. All cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cell viability assay

Cells were seeded in a 96-well plate at a density of 4×10³ cells/well for 24 h and then treated with paeoniflorin. A total of 10 μL of Cell Counting Kit-8 (CCK-8) solution was then added to each well, and the cells were incubated for 1 h at 37°C. The absorbance of the reaction mixture was subsequently measured by a microplate reader.

Wound-healing assay

A wound-healing assay was used to compare the migratory ability of cells in the control and experimental groups. Cells (5×10⁵ cells) were seeded and cultured in 6-well plates and upon reaching 80%–90% confluency, scratches of a predetermined length were introduced into the monolayers by a sterile pipette tip. The monolayers were rinsed with PBS to remove detached cells, and the medium was then replaced with medium containing paeoniflorin or normal saline (0.9% NaCl). To distinguish the contributions of cell proliferation to wound closure from those of migration, we treated the cells with the cell cycle blocker, hydroxyurea (5 mM, Sigma-Aldrich), at the time of the experiment. To analyze cell migration, we photographed the wounds at the indicated time points with a Leica microscope (Melville, NY, USA). The images were processed using Image Pro-Plus Software (NIH). The wound-healing percentage was determined as follows: [1– (empty area × h/empty area 0 h)]×100.

Cell invasion assay

The transwell system used for the cell invasion assay and was obtained from Corning (Corning, NY, USA). Cells (1×10⁵ in 200 μL of DMEM or extracellular matrix (ECM) supplemented with 1% FBS) were seeded in the upper chamber (8 μm), which was coated with 100 μL of Matrigel (BD Biosciences, San Diego, CA, USA), while 600 μL of DMEM or ECM supplemented with 20% FBS was added to the lower chamber. After 24 h, the cells in the lower chamber were fixed with methanol and stained with 0.1% crystal violet in methanol. Three independent fields in each well were photographed at 100× magnification, after which the cells in each field were counted.

Real-time polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using Trizol reagent (Sigma-Aldrich). cDNA was synthesized with a FastQuant RT Kit

(Tiangen Biotech, Beijing, China) and used as template in a 2-step quantitative real-time polymerase chain reaction (RT-PCR). SYBR Green I (Takara, Dalian, China) labeling was conducted on a real-time thermocycler (Applied Biosystems Inc., Carlsbad, CA, USA). PCR was performed with Taq DNA polymerase (Takara) using the following primers: human *TLR4*: 5'-TCCCTCCAGGTTCTTGATTA-3' (forward) and 5'-GTAGTGAAGGCAGAGCTGAAA-3' (reverse); and human *GAPDH*: 5'-TTGGTATCGTGGAAGGACTCA-3' (forward) and 5'-TGTCATCATATTTGGCAGGTT-3' (reverse) (Takara).

Immunohistochemistry

Tissue slides were incubated for 1 h at 37°C and then deparaffinized. Antigen retrieval was performed by treating the tissues in citrate buffer in a microwave for 10 min. After peroxidase activity was blocked with 3% H₂O₂/methanol for 10 min, the sections were incubated with normal goat serum for an additional 10 min to block nonspecific antibody binding. The sections were incubated with the appropriate primary antibodies for 1 h at 25°C before being incubated with biotinylated anti-rabbit/mouse IgG and peroxidase-labeled streptavidin for 10 min each. The images were acquired by a microscope and were analyzed by Image Pro-Plus software.

Transfection

To overexpress TLR4, we transfected the previous cell lines with plasmids carrying *TLR4* or vector plasmid (GeneCopoeia, Rockville, MD, USA) using Lipofectamine 3000, according to the manufacturer's protocol.

Western blotting

Western blotting was performed using cell lysates or xenograft glioblastoma tissue homogenates. Protein was extracted using the Pro-prep TM protein Extraction Solution (iNtRON Biotechnology, Daejeon, Korea), according to manufacturer's instructions. Equal amounts of total protein were separated by 10%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (Merck, KGaA, Darmstadt, Germany), which were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature before being incubated with specific primary antibodies overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The resulting signals were obtained using Super Signal ECL (Pierce, Rockford, IL, USA).

Co-immunoprecipitation

Cells or tumor tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The lysates were precleared with protein A/G plus agarose beads (Santa Cruz, Dallas, TX, USA) for 2 h and then incubated with antibody-conjugated beads overnight at 4°C. The beads were washed 3 times with RIPA buffer, resuspended in SDS electrophoresis sample buffer, and then boiled for 5 min at 95°C, after which the samples were subjected to SDS-PAGE and Western blot analysis.

Treatment of the U87 xenograft mouse model with paeoniflorin

Female BALB/c nude mice were obtained from Vital River Laboratories (Beijing, China). The mice were aged 8 weeks and were maintained in accordance with a standard protocol approved by the Institutional Animal Care Committee of Army General Hospital. All procedures performed in the study involving the animals were compliant with the ethical standards of the institution or practice at which the studies were conducted. The mice were anesthetized with 3.6% chloral hydrate in 0.9% sterile saline. Each mouse was then intracranially injected with 3 μ L of cultured U87-luciferase cells (5×10^5 cells per mouse) at a rate of 0.5 μ L/min using a Micro 4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, FL, USA) attached to a Hamilton syringe with a 33-gauge needle (Hamilton, Reno, NV, USA). The injection was performed in the mid-right striatum at the following coordinates (in mm from the bregma): +0.5 anterior-posterior, +2.0 medio-lateral, and -2.8 dorso-ventral. Seven days after cell transplantation, tumor-bearing mice were distributed into 2 groups ($n=6$ each) and intraperitoneally injected with paeoniflorin (400 mg/kg/day) or vehicle (equivalent amount of PBS). Tumor sizes and body weights were measured once every 7 days. At the end of the experiments, the mice were sacrificed, and the tumors were resected for subsequent experiments.

Bioluminescence imaging

D-luciferin was purchased from Abcam (Cambridge, MA, USA) and was resuspended in PBS at a concentration of 100 mg/mL. The mice were intraperitoneally injected with 150 mg/kg per total body weight D-luciferin and imaged 10 min thereafter using an IVIS[®] Spectrum optical imaging system fitted with an XGI-8 Gas Anesthesia System (Caliper Life Sciences, Hopkinton, MA, USA). Bioluminescent images were acquired using the exposure

function and all the parameters were the same in every measurement. We measured the total flux (photons/s) of the tumor bioluminescence signal by placing a region of interest at the tumor using Living Image[®] Software (Caliper Life Sciences).

Microscale thermophoresis (MST) assay

MST was used to analyze the interaction between paeoniflorin and TLR4 protein. Purified TLR4 protein was labeled with the Monolith NT[™] Protein Labeling Kit RED (Cat # L001) according to the supplied labeling protocol. Labeled TLR4 protein was kept constant at 10 μ M, and different concentrations of paeoniflorin solutions were prepared and mixed with the TLR4 protein solution at a volume ratio of 1:1 in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) and 0.05% (v/v) Tween-20. After 10 min incubation at room temperature, samples were loaded into Monolith[™] standard-treated capillaries and after 30 min of thermophoresis was measured at 25°C using a Monolith NT.115 instrument (Germany) (laser power set to 20% using a 30 second on-time, while the LED power was set to 20%). The dissociation constant K_d values were fitted using NT Analysis software.

Statistical analysis

The data are presented as the mean \pm SD from at least 3 independent experiments. Simple comparisons between 2 groups were analyzed using independent *t*-tests, and multiple comparisons between the groups were assessed with one-way analysis of variance, followed by post hoc analyses, which were performed with the LSD test or Dunnett's T3 test. All analyses were performed using SPSS 20.0 software. $P < 0.05$ was considered statistically significant.

Results

Paeoniflorin inhibits cell proliferation and downregulates TLR4 signaling in glioblastoma cells

In our previous study, we reported that paeoniflorin inhibited U87 and U251 cell proliferation. To confirm the effects of paeoniflorin on cell proliferation, we performed CCK-8 experiments in U87, U251, U118, and T98G cells. As shown in Figure 1A, a 24-h paeoniflorin treatment inhibited cell growth in 4 glioblastoma cell lines in a dose-dependent manner. Cell viability declined from 90% to 20% in all 3 cell lines when the concentration of paeoniflorin was increased from 5 to 40 μ M. Next, we performed cell colony formation assays to validate the effects of paeoniflorin on cell growth in U87

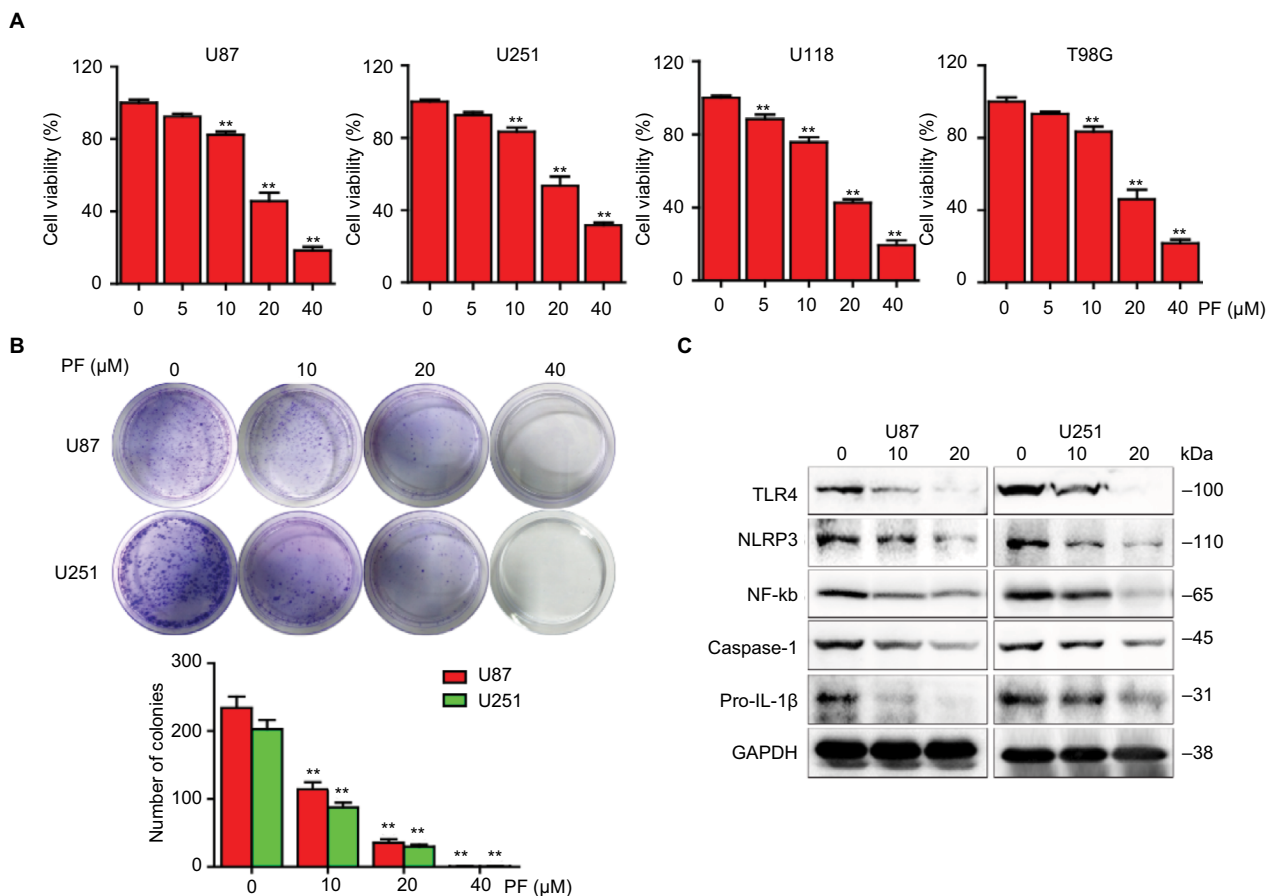


Figure 1 The effects of paeoniflorin on cell proliferation and TLR4 signaling in glioblastoma cells.

Notes: (A) U87, U251, U118, and T98G cells were treated with different concentrations of paeoniflorin for 24 h and after which cell viability was examined using the CCK-8 assay. (B) U87 and U251 cells were cultured for 7 days after treatment with varying concentration of paeoniflorin and stained with 0.1% crystal violet. Colonies containing >50 cells were counted (C) in U87 and U251 cells that were incubated with the indicated concentrations of paeoniflorin for 24 h; Western blotting was then performed to analyze TLR4 protein expression levels as well as downstream effectors: n=3 and n=4 for cell counts and Western blotting, respectively. All tests were performed in triplicate. **P<0.01 compared with control (0 μM).

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; NF-κB, nuclear factor κB; NLRP, nucleotide-binding domain and leucine-rich repeat containing protein 3; CCK-8, Cell Counting Kit-8; TLR4, Toll-like receptor 4; PF, paeoniflorin.

and U251 cells. As shown in Figure 1B, paeoniflorin decreased colony formation in a dose-dependent manner. These results confirm that paeoniflorin can effectively inhibit glioblastoma cells growth in vitro.

TLR4 played an important role in glioblastoma and is, therefore, highly expressed in glioblastoma. To validate the effect of paeoniflorin on TLR4 expression in glioblastoma cells, we treated U87 and U251 cells with different concentrations of paeoniflorin for 24 h and then performed Western blotting to detect TLR4 expression, as well as related downstream effector expression. As shown in Figure 1C, paeoniflorin downregulated TLR4 expression as well as related downstream effectors, such as NLRP3, NF-κB, caspase-1, and pro-IL-1β, in a dose-dependent manner in U87 and U251 cells. These results suggest that TLR4 may be a target of paeoniflorin in glioblastoma cells.

TLR4 overexpression decreases the effects of paeoniflorin in glioblastoma cells

To investigate whether paeoniflorin suppresses the effects of TLR4 overexpression, we overexpressed TLR4 in U87 and U251 cells and incubated these cells with paeoniflorin or vehicle. Results showed that TLR4 overexpression decreased the inhibitory effects of paeoniflorin on proliferation in U87 and U251 cells (Figure 2A). We also found that TLR4-induced glioblastoma cell migration and invasion were abolished by treatment with paeoniflorin (Figure 2B). U87 and U251 cells were treated with different doses of paeoniflorin for 24 h, which resulted in dose-dependent downregulation of TLR4 and related downstream effectors. However, TLR4 overexpression in U87 and U251 cells attenuated the effects

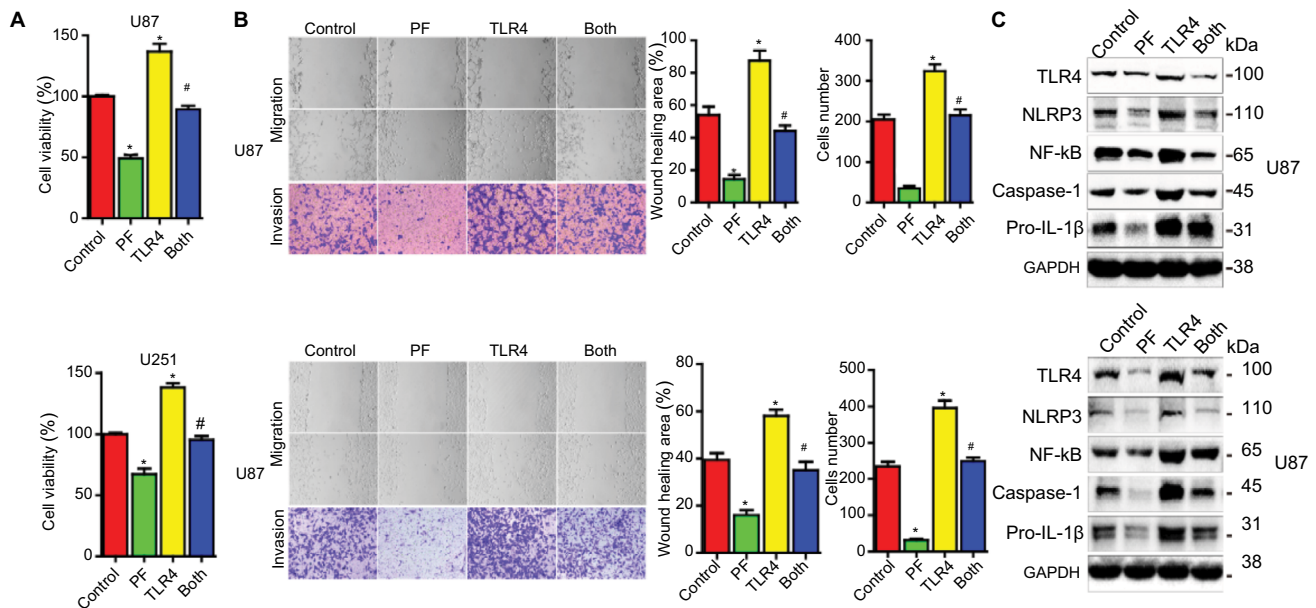


Figure 2 TLR4 overexpression reduces the effects of paeoniflorin on cell proliferation, migration, invasion, and TLR4 signaling.

Notes: U87 cells and U251 cells transfected with TLR4 or vector plasmids for 12 h were harvested; **(A)** 4×10^3 cells were seeded in a 96-well plate and then incubated with 20 μ M paeoniflorin or PBS for 24 h. The Cell Counting Kit-8 assay was used to detect cell proliferation. **(B)** 1×10^5 cells were seeded in 6-well plates and then incubated with 20 μ M paeoniflorin or PBS for 24 h. Wound-healing assays and Transwell assays were performed to detect U87 and U251 cell migration and invasion, respectively (scale bar = 100 μ m). **(C)** U87 and U251 cells were transfected with TLR4 or vector plasmids for 12 h then treated with 20 μ M paeoniflorin for 24 h. Western blotting was performed to examine protein expression. Control: transfected with vector; paeoniflorin: transfected with vector + 20 μ M paeoniflorin; TLR4: transfected with TLR4; both: transfected with TLR4 + 20 μ M paeoniflorin: n=3 or n=4. All tests were performed in triplicate. * $P < 0.05$ vs. control; # $P < 0.05$ compared with either paeoniflorin treatment or TLR4 transfection alone.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; NF- κ B, nuclear factor κ B; NLRP, nucleotide-binding domain and leucine-rich repeat containing protein 3; TLR4, Toll-like receptor 4.

of paeoniflorin-mediated downregulation of TLR4 and most related downstream effectors, except NF- κ B in U87 and NLRP3 in U251 cells, which suggests paeoniflorin may have cell-specific effects on these molecules (Figure 2C). In general, these results suggest that paeoniflorin exerts anticancer effects by suppressing TLR4-induced epithelial–mesenchymal transition in glioblastoma cells.

Paeoniflorin promotes TLR4 degradation via ubiquitin–proteasome-dependent pathway in glioblastoma cells

We first examined *TLR4* mRNA expression by RT-PCR after cells were treated with paeoniflorin for 24 h and found that there was no significant difference in *TLR4* mRNA expression in either U87 or U251 cells (Figure 3A, B). Using CHX, a de novo protein synthesis inhibitor, we detected the effects of paeoniflorin on TLR4 protein stability. As shown in Figure 3C, D, the half-life of TLR4 protein in cells treated with paeoniflorin and CHX was shorter (~3 h in U87 and U251 cells) than that in cells treated with CHX alone (~6 h in U87 and U251 cells). Thus, TLR4 degradation was accelerated (i.e., TLR4 stability was dramatically reduced) by treatment

with paeoniflorin and CHX. These results indicate that paeoniflorin promotes TLR4 degradation in U87 and U251 cells without interfering with its transcription.

The 2 most important posttranscriptional protein degradation pathways are the autophagy–lysosome and ubiquitin–proteasome pathways. Therefore, we investigated whether TLR4 was degraded through the autophagy–lysosome pathway or the ubiquitin–proteasome pathway by analyzing TLR4 protein expression levels in cultured cells treated with paeoniflorin in combination with autophagy–lysosome or ubiquitin–proteasome blockers. The results showed that MG-132, a proteasome blocker, successfully prevented the degradation of TLR4 caused by paeoniflorin; however, CQ, a lysosome inhibitor, did not inhibit the effects of paeoniflorin on TLR4 (Figure 3E). An in vitro ubiquitination activity assay was then conducted to examine the role and involvement of ubiquitin (or ubiquitination) in paeoniflorin-mediated proteasome degradation of TLR4 in U87 and U251 cells. The cells were preincubated with MG-132 and treated with paeoniflorin, after which whole cell lysates were incubated with anti-TLR4 antibody. Immunoprecipitated proteins were then assessed using an anti-ubiquitin antibody. It was noteworthy that degraded TLR4 could be detected using an anti-ubiquitin antibody,

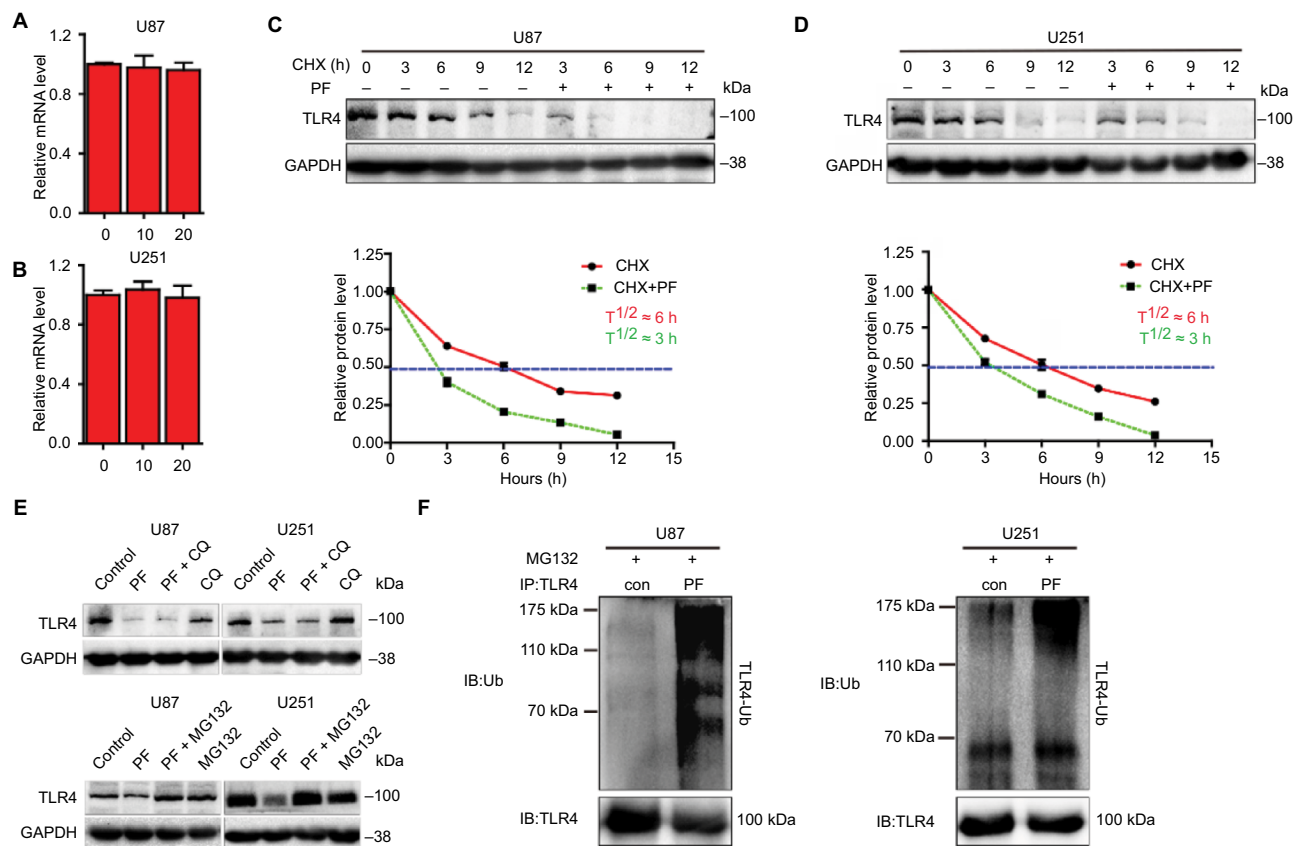


Figure 3 Paeoniflorin promotes TLR4 degradation via the ubiquitin–proteasome pathway in glioblastoma cells.

Notes: (A, B) TLR4 mRNA expression was detected by quantitative real-time polymerase chain reaction in cells treated with different concentrations of paeoniflorin and was normalized to GAPDH expression. Expression was expressed as a fold change relative to 0 μ M paeoniflorin-treated U87 and U251 cells. (C, D) Time course of TLR4 degradation. Top panel, CHX (100 μ g/mL) was added to U87 and U251 cells treated with or without 20 μ M paeoniflorin for 24 h, after which Western blot analysis was performed. Bottom panel, quantified TLR4 band intensities, which are representative of 3 separate analyses by Image J. The relative intensities of each band from the cell samples were quantified by densitometry as a function of time, with the dotted line (---) indicating the half-life ($T_{1/2}$) of TLR4 protein in U87 and U251 cells. (E) U87 and U251 cells were incubated with 20 μ M CQ or 5 μ M MG-132 for 6 h before being treated with 20 μ M paeoniflorin or PBS for 18 h. TLR4 protein expression was estimated by Western blotting. (F) U87 cells and U251 cells were pretreated with MG-132 (10 μ M) for 6 h, followed by additional incubation with paeoniflorin (20 μ M) for 18 h. The lysates were subjected to immunoprecipitation, which was performed with antibodies against TLR4 and immunoblot analysis, which was performed with antibodies against ubiquitin; $n=3$ or $n=4$. All tests were performed in triplicate.

Abbreviations: CHX, cycloheximide; CQ, chloroquine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TLR4, Toll-like receptor 4; IB, immunoblotting; PF, paeoniflorin; Ub, ubiquitin.

indicating that TLR4 was ubiquitinated during paeoniflorin-induced degradation. Specifically, we demonstrated that the intensity of the smeared bands of TLR4 in paeoniflorin-treated cells was stronger than that in control cells (Figure 3F). Taken together, these results indicated that paeoniflorin promoted TLR4 degradation via the ubiquitin–proteasome-dependent pathway in glioblastoma cells.

Paeoniflorin enhances the conjugation of Triad3A and TLR4, while Triad3A-shRNA (shTriad3A) disturbs paeoniflorin-induced TLR4 degradation

Triad3A is one of the most important E3 ligases regulating TLR4 expression and has a critical function in promoting

TLR4 degradation through binding with TLR4. Here, we examined whether Triad3A is involved in the processing step of the paeoniflorin-mediated ubiquitin–proteasome degradation of TLR4 in glioblastoma cells. As shown in Figure 4A, during paeoniflorin treatment of U87 and U251 cells, there were increases in the amounts of Triad3A protein bound to TLR4 in the paeoniflorin-treated cells compared with those in control cells. These results suggest that Triad3A may be involved in paeoniflorin enhanced, ubiquitin-dependent TLR4 degradation. Next, we performed *Triad3A*-knockdown experiments in U87 cells. First, using *Triad3A* siRNAs, such as sh-T1, sh-T2, and sh-T3, we found that Triad3A knockdown led to reductions in Triad3A expression, as assessed by Western blotting analysis: specifically, 53%, 62%, and 34% reduction for sh-T1, sh-T2, and sh-T3, respectively

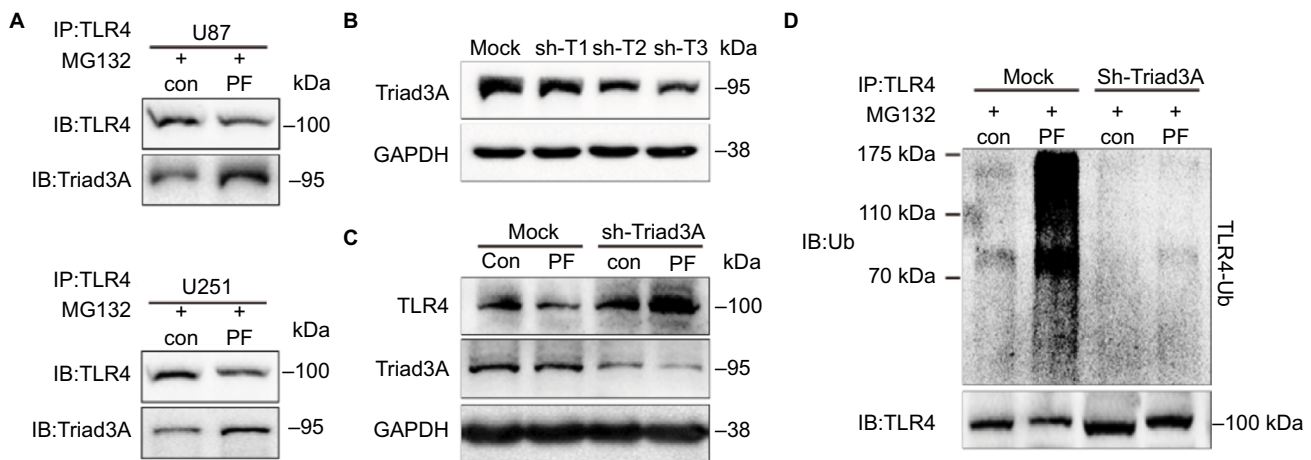


Figure 4 Paeoniflorin enhances the interaction of Triad3A and TLR4, while sh-Triad3A disturbs paeoniflorin-induced degradation of TLR4 in glioblastoma cells.

Notes: (A) Detection of Triad3A binding with TLR4 was assessed by the immunoprecipitation of TLR4 in U87 and U251 cells treated with MG-132 (10 μ M) for 6 h followed by paeoniflorin (20 μ M) for 18 h. After incubation, whole cell lysates were immunoprecipitated using anti-TLR4 antibodies. Protein levels of Triad3A and TLR4 were measured by Western blotting. (B) U87 cells were transfected with 3 types of sh-Triad3A plasmids (sh-T1, sh-T2, and sh-T3) for 24 h and the expression of Triad3A was detected by Western blotting of whole cell lysates. (C) U87 cells transfected with mock or sh-Triad3A were incubated with paeoniflorin (20 μ M) for 24 h followed by Western blotting of whole cell lysates to detect the expression of TLR4 and Triad3A. GAPDH was used as an internal control. (D) U87 cells transfected with mock or sh-Triad3A were treated with MG-132 (10 μ M) for 6 h followed by with or without paeoniflorin (20 μ M) for 18 h. Ubiquitination level of TLR4 were detected by the immunoprecipitation assay. All tests were performed in triplicate.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TLR4, Toll-like receptor 4; Triad3A-shRNA, sh-Triad3A; IP, immunoprecipitation; PF, paeoniflorin.

(Figure 4B). Second, we found that *Triad3A* knockdown (sh-T2) substantially blocked the paeoniflorin-induced reduction of TLR4 expression in U87 cells. Specifically, as expected in the mock-transfected U87 cells, paeoniflorin decreased TLR4 expression compared with that in control cells. In contrast, in sh-Triad3A-transfected U87 cells, no difference was noted in TLR4 expression with or without paeoniflorin treatment (Figure 4C).

Effect of Triad3A knockdown on the paeoniflorin-mediated UPP in TLR4 protein degradation in U87 cells

To further explore the role of Triad3A in paeoniflorin-mediated TLR4 protein degradation, an *in vitro* ubiquitination activity assay was applied against ubiquitin proteins to evaluate the effect of Triad3A in paeoniflorin enhancement of UPP-induced TLR4 protein degradation in si-Triad3A-transfected U87 cells. Cells were preincubated with MG-132, treated with paeoniflorin, after which whole cell lysates were incubated with anti-TLR4 antibodies. Immunoprecipitated proteins were then assessed using an anti-ubiquitin antibody. In mock si-RNA transfected cells, a higher level of TLR4 ubiquitination was found in cells treated with MG-132 and paeoniflorin than in cells treated with MG-132 alone (Figure 4D). However, in si-Triad3A-transfected cells pretreated with MG-132 or MG132, which were subsequently treated with paeoniflorin, no differences in the levels of

TLR4 ubiquitination were found. These results suggest that the paeoniflorin-mediated ubiquitination of TLR4 is Triad3A dependent.

Effects of paeoniflorin on the orthotopic xenograft mouse model

To validate the effects of paeoniflorin on glioblastoma *in vivo*, we used a U87-luciferase orthotopic xenograft mouse model. The results showed that tumor volumes were decreased (Figure 5A, B) and mouse survival rates were enhanced (Figure 5C) in the 28-day paeoniflorin-treated group compared with the PBS-treated group. The body weights of mice treated with paeoniflorin were also higher than those in the PBS-treated group (Figure 5D).

Then, we detected TLR4 protein expression by immunohistochemical staining and found that TLR4 expression was reduced in the paeoniflorin-treated group compared with the PBS-treated group (Figure 5E). Furthermore, we found the endogenous expression of TLR4, NLRP3, NF- κ B, caspase-1, and pro-IL-1 β was suppressed in tumor tissues from U87 xenograft mice treated with paeoniflorin compared with tumor tissues from mice treated with PBS (Figure 5F). We also performed co-immunoprecipitation in tumor tissues and found that paeoniflorin treatment promoted TLR4 ubiquitination and enhanced conjunction of TLR4 and Triad3A in the intracranial xenograft mouse model (Figure 5G). Our *in vivo* study results were consistent with *in vitro* results

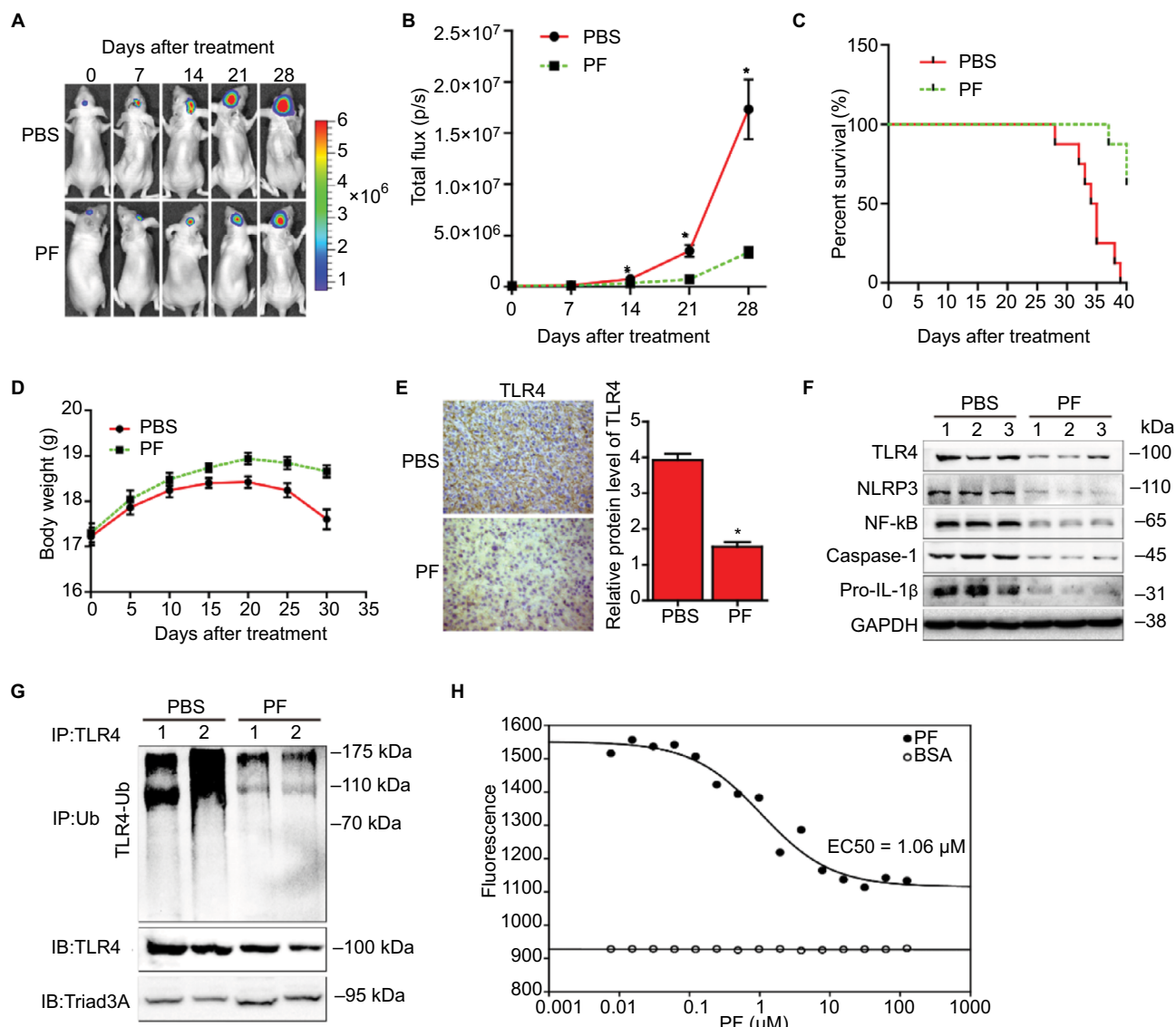


Figure 5 Effects of paeniflorin on the orthotopic xenograft mouse model and specific binding of paeniflorin with TLR4 protein.

Notes: U87-luciferase cells (5×10^5) were intracranially injected into the mid-right striatum of 8-week-old female BALB/c nude mice. Ten days postinjection, tumor formation was detected by bioluminescence imaging, and the mice were separated into the following 2 groups: a group comprising mice that were intraperitoneally injected with PBS (control) and a group comprising mice that were intraperitoneally injected with paeniflorin (400 mg/kg/day). Tumor sizes were measured once every 7 days. Bioluminescence imaging was used to measure tumor volumes. **(A)** A representative tumor volume in each group is shown at each time point, $n=6$. **(B)** Tumor volumes were examined at each time point in each group, $n=6$. **(C)** The survival rate of each group, $n=6$. **(D)** Body weight changes in each group, $n=6$. At the end of the experiment or after the mice had died, the brains were removed **(E)** and were stained for TLR4 (scale bar=20 μm), $n=3$. The images were analyzed by Image-Pro-Plus. At the end of the experiment or after the mice had died, the tumor tissues were excised, and the protein lysates, $n=3$ **(F)** were used to estimate TLR4 signaling, $n=3$. **(G)** The lysates were immunoprecipitated with anti-TLR4 antibody and immunoblotted with antibodies against TLR4 and Triad3A, $n=2$. **(H)** MST assay was used to measure the binding between paeniflorin and TLR4 protein, $n=3$. EC50 values were automatically calculated by the curve fitting. All tests were performed in triplicate. * $P < 0.05$ compared with control.

Abbreviations: MST, microscale thermophoresis; TLR4, Toll-like receptor 4; IB, immunoblotting; PF, paeniflorin; Ub, ubiquitin.

demonstrating that paeniflorin plays a critical role in the suppression of glioblastoma growth in an intracranial tumor model by inhibiting TLR4.

Specific binding of paeniflorin with TLR4 protein

The MST assay has been previously used to assess protein–protein, small organic molecule–protein, nucleic acids–protein, and antibody–protein interactions. Hence, to verify the

interaction of paeniflorin with TLR4, we used this method. Compared with the control group (BSA), the paeniflorin group displayed interactions with TLR4 protein with an EC50 of 1.06 μM (Figure 5H), which suggests that TLR4 may be a direct target of paeniflorin.

Discussion

In our present study, we noted that paeniflorin suppressed glioblastoma by degrading TLR4 both in vitro and in vivo.

Mechanically, we demonstrated that paeoniflorin reduced TLR4 in a Triad3A-dependent ubiquitin–proteasome pathway manner. Furthermore, we identified TLR4 as a direct target of paeoniflorin.

According to data from the US Food and Drug Administration, ~25%–48% of currently approved anticancer agents come from plants.^{33,34} Paeoniflorin, a monomeric natural compound extracted from *Radix Paeonia Alba*, has shown anticancer activity in some types of cancer, including glioblastoma.^{6,9,12} Several lines of evidence have showed that paeoniflorin regulates multiple molecular and signaling pathways,^{8,35,36} but until now, there are no reports elucidating a direct target of paeoniflorin. Here, we not only report that paeoniflorin downregulates TLR4 but also demonstrate that TLR4 may be a direct target of paeoniflorin.

A major limitation of agents that inhibit/suppress glioblastoma is in their ability to penetrate the blood–brain barrier (BBB). Previously, He et al found that paeoniflorin entered into hippocampus soon after administration and that the concentration remained high after a considerable period of time following administration.³⁷ Similarly, Cao et al reported that paeoniflorin penetrated the BBB and entered cortex at a concentration enabling the effective treatment of ischemia–reperfusion in rats.³⁸ In our study, we found that paeoniflorin exerted potent anti-glioblastoma effects in an intracranial glioblastoma model by suppressing TLR4.

TLR4, a member of the TLR family of proteins, has been reported to contribute to progression in various tumors. It has been reported that activated TLR4 signaling promoted proliferation of glioma cell lines and induced chemotactic migration in glioma cells.^{18,20} Once activated, TLR4 induces downstream signaling pathways, including the NF- κ B signaling pathway, which results in an inflammation-related cascade activation. In turn, these activated downstream inflammatory factors contribute to tumor progression.^{39,40} Moreover, it has been demonstrated that inflammation is closely associated with tumorigenesis and progression. In our study, paeoniflorin not only downregulated TLR4 but also suppressed the downstream inflammation-related effectors NF- κ B, NLRP3, caspase-1, and pro-IL-1 β . Therefore, suppression of TLR4-induced inflammation may be a mechanism responsible for inhibiting glioblastoma.

TLR4 signal transduction pathways are regulated at multiple levels by ubiquitin-mediated targeting and proteasomal degradation. Triad3A, an E3 ubiquitin–protein ligase that interacts with the Toll/IL-1 β receptor domain of TLRs, has been reported to be one of the most important E3 ligases regulating TLR4 degradation.^{30,31} Here, for the first time, we demonstrate that paeoniflorin enhances the levels of Triad3A

interacting with TLR4 to regulate TLR4 degradation in glioblastoma. A possible mechanism by which paeoniflorin binds with TLR4 is as follows: the interaction between paeoniflorin and TLR4 causes increased binding of Triad3A with TLR4, which initiates TLR4 degradation. Finally, paeoniflorin induction of the ubiquitin proteasome-mediated pathway in TLR4 degradation is correlated with Triad3A activity.

Conclusion

We found that paeoniflorin suppresses TLR4 in glioblastoma both in vitro and in vivo. Additionally, we demonstrated that paeoniflorin-induced TLR4 ubiquitin–proteasome-dependent degradation is Triad3A dependent. Moreover, we determined that TLR4 may be a direct target of paeoniflorin in glioblastoma.

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Disclosure

The authors report no conflicts of interest in this work.

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