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Paeonol combined with radiotherapy inhibits the growth of human glioblastoma by inhibiting the two angiogenesis pathways of VEGF/VEGFR and ANG/Tie-2 in vitro

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► ***Original article***

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ABSTRACT

Background: The blockade of both VEGF / VEGFR and angiopoietin/TEK receptor tyrosine kinase (Tie-2) pathways can be considered an attractive approach to inhibition of angiogenesis in glioblastomas. Paeonol (Pae), a natural compound, has been identified to possess evidence of antitumor activity. ***Materials and Methods:*** In this study, the impact of Pae combined with radiotherapy on the glioblastoma angiogenesis pathways was investigated in vitro. For this purpose, effects of various doses of Pae alone or in combination of 2 Gy radiation was assessed using various endpoints such as gene expression, $_{\gamma}$H2AX and apoptosis in glioblastoma cells. ***Results:*** Results indicated that Pae could induce the down-regulation of VEGF at the protein level and inhibit the mRNA and protein level of VEGFR1/2 in EA.hy926 cells. Pae and radiation co-treatment inhibited the protein levels of ANG1 and ANG2 in U251 cells and of Tie-2 in EA.hy926 cells. Notably, the combination therapy disrupted endothelial tube formation more effectively than Pae or radiation alone, reducing tubule length by 65% ($p<0.001$). However, we found that Pae had reduced the phosphorylation of Erk pathway in these cells. Pae enhanced radiation-induced DNA damage, as evidenced by increased γ-H2AX foci formation ($p<0.01$). The results further elucidate the multifaceted effects of Pae on tumor cell proliferation and angiogenesis in glioblastoma. ***Conclusion:*** Obtained results might imply that Pae might inhibit glioblastoma angiogenesis by the VEGF/VEGFR and ANG/Tie-2 pathways, with the Erk pathway potentially serving as a key mechanism. Our findings propose that Pae acts as a radiosensitizer while concurrently suppressing angiogenic signaling.

INTRODUCTION

Glioblastoma (GBM), the most prevalent primary intracranial tumor, arises from glial cells in the adult brain's central nervous system, representing approximately 70–80% of all brain tumors. GBM treatment primarily involves surgical tumor removal combined with radiotherapy, chemotherapy, and other integrative therapeutic approaches. For patients with GBM post-surgery, temozolomide (TMZ) is often administered [1]. For postoperative GBM, temozolomide (TMZ) concurrent chemoradiotherapy is strongly recommended as the standard treatment, but the side effects of the treatment are obvious. At the same time, the average survival time of patients is usually about 1 year, and less than 1% survive for more than 5 years [2]. Angiogenesis inhibitors can exert anti-tumor effects by inhibiting angiogenesis regulators, or inhibiting the growth of vascular endothelial cells and other mechanisms [3-5]. GBM are highly vascularized tumors whose growth is dependent on angiogenesis, which is the key factor affecting the progression and prognosis [6, 7]. Angiogenesis mechanisms are intricate and multifaceted, with diverse angiogenesis regulators such as VEGF and VEGFR, ANG, Tie-2, and PDGF, acting on vascular cells and interacting to collectively promote angiogenesis via different pathways [8-11]. Targeting these pathways holds promise as a potential strategy for inhibiting tumor angiogenesis. Some angiogenesis inhibitors have entered clinical use, such as bevacizuamb, apatinib, thalidomide, and trebananib, among others. However, these drugs often exhibit limited therapeutic efficacy when used alone, accompanied by adverse reactions such as hypertension, proteinuria, cytopenia, and intestinal perforation [9]. Trebananib, a recently identified inhibitor of the ANG-related angiogenesis pathway. Conversely, a phase 2 clinical trial evaluating trebananib monotherapy in patients with recurrent glioblastoma reported no significant differences in patients as compared to the control

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group [3, 4]. Thus, it is imperative to explore anti-GBM strategies capable of effectively inhibiting tumor angiogenesis with minimal side effects.

Asia has an extensive past of using traditional Chinese herbal medicine for anti-tumor treatment. Mounting evidence suggests that Chinese herbs possess the ability to inhibit tumor angiogenesis [12, 13]. Pae, a natural plant extract, exhibits a multitude of therapeutic effects, including antibacterial, anti-inflammatory, antipyretic, analgesic, immunity enhancement, blood circulation-promoting, blood stasis removal, and anti-tumor properties [13]. Notably, Paeonol also demonstrates anti-angiogenic properties [14, 15]. Studies have confirmed Pae inhibited the progression of ovarian tumor and fibrosarcoma cell lines [16]. Furthermore, our prior research validated that Pae significantly inhibited the proliferation of U251 cells and reduced the expression of VEGF and enhanced the cytotoxicity of etoposide in U251 cells [17, 18]. Paeonol enhances the radiosensitivity of lung adenocarcinoma by increasing radiation-induced apoptosis and suppressing the PI3K/Akt pathway, as demonstrated in both in vitro and in vivo studies [19]. Paeonol increases ovarian cancer cell radiosensitivity by promoting apoptosis through suppression of the PI3K/Akt/PTEN signaling axis while concurrently reducing VEGF and HIF-1α expression [20].

Glioblastoma development incorporates angiogenesis as one of the essential satisfactions that directly correlates with the communication between endothelial cells and glioma cells. The angiogenic switch in human gliomas is defined by the activation of tumor cell genes involving angiogenesis regulatory factors VEGF, ANG and PDGF, alongside by the coordinated activation of genes in endothelial cells encoding the respective growth factor receptors such as VEGFR, Tie and so on [8]. To delve into the effect and mechanism of Pae on the anti-angiogenesis pathways of VEGF/VEGFR and ANG/Tie-2 in glioblastoma *in vitro*, this study employs U251 cells and EA.hy926 cells as research subjects. We aim to study the impact of Pae on VEGF, VEGFR1/2, and Tie-2 in EA.hy926 cells, as well as ANG1/2 in U251 cells, and its downstream potential mechanisms.

MATERIALS & METHODS

Reagents and antibodies

Pae (product number: H35803, molecular weight: 166.17) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to prepare a 500 mg/mL stock solution for storage. For radiotherapy experiments, a clinical-grade linear accelerator (LINAC) model X-2000 (Varian Medical Systems) was used to deliver precise doses of ionizing radiation (2-8 Gy) to treated cells.

The RNAiso Plus total extraction reagent and the reverse transcription kit were PrimeScript RT Master Mix, while the fluorescent dye for real-time quantitative PCR was SYBR Premix Ex Taq™, all procured from Takara Bio, Inc. (Japan). To assess DNA damage, a γ-H2AX antibody (Abcam, ab26350) was added to the experimental protocol.

Primers for the RT-PCR procedure was designed and sourced from Guge Bio Company (Wuhan, Hubei, China). The primer sequences included the following:

VEGF (forward: 5′-GGAGGCAGAATCATCACGA-3′, reverse: 5′-GCTCATCTCCTATGTGCTGG-3′), VEGFR1 (forward: 5′-CAATAAGTTGGGACTGTGGAA-3′, reverse: 5′-TCCGCAGTAAATCCAAGTAACG-3′), VEGFR2 (forward: 5′-ACCCCTTGAGTCCAATCACACA-3′, reverse: 5′-CTTCC TCCAACTGCCAATACCA-3′), ANG1 (forward: 5′-AGGGAACCGAGCCTATTCA CAG-3′, reverse: 5′-CCACAAGCATCAAACCCACTC-3′), ANG2 (forward: 5′-TGGGATTGGTAACCCTTCA-3′, reverse: 5′-GTAAGCCTCATTCCCTTCCC-3′), Tie-2 (forward: 5′-GATCTCACTGCTTGACCCTT-3′, reverse: 5′-TGCCCTGAACC TTGTAACGGA-3′), and GAPDH (forward: 5′-ACTTTGGTATCGTGGAAGGACTCAT-3′, reverse: 5′-GTTTTTCTAGACGGGCAGGTCAGG-3′). New primers for radiation-response genes (e.g., *ATM*, *RAD51*) were also included (ATM forward: 5′-CAGCCATGGAGTTTGTGACC-3′, reverse: 5′-TGGCTTCACACTCACCAATG-3′; RAD51 forward: 5′-GCTGGGAAGGCATTTATGGA-3′, reverse: 5′-TCCTGCATCTGCTTGTTGTC-3′).

Rabbit anti-human antibodies for VEGF, VEGFR1, VEGFR2, ANG2, and Tie-2 were procured from Cell Signaling Technology (USA). The rabbit anti-human GAPDH antibody was acquired from AntGene Company (Wuhan, China), while those for ANG1, Erk, and p-Erk, along with other secondary antibodies, were purchased from Abcam (USA). Additional antibodies for DNA damage repair proteins (ATM, p-ATM, γ-H2AX) and apoptosis markers (cleaved caspase-3) were included. RIPA lysate, BCA Protein Assay kit, and ECL kit were supplied by Biyuntian Company (Shanghai, China). Cycloheximide (CHX) was obtained from China West Asia Reagent. For endothelial tube formation assays, Matrigel (Corning, #356234) was used.

Cell culture and paeonol treatment

The human glioblastoma cell line U251 was sourced from the China Typical Culture Collection Center, while the human umbilical vein endothelial cell line EA.hy926 was generously provided by Professor Hong Mei from the Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Both cell lines were cultured in DMEM high-glucose medium supplemented with 10% fetal bovine serum (Gibco, USA), 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco, USA). To mimic clinical radiotherapy conditions, cells were irradiated at 70–80% confluency using a calibrated X-ray source

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(2 Gy/min, 6 MeV) with precise shielding to ensure uniform dose distribution.

Cells in the log phase were prepared for paeonol treatment by seeding them into 96-well or 6-well plates and incubating them at 37 °C in a Thermo Scientific Steri-Cult CO_2 incubator (Forma Scientific, USA) under a 5% CO_2 atmosphere. For combination therapy experiments, cells were pretreated with Pae (50 μM) for 4 hours prior to irradiation, based on preliminary dose-response optimization. Post-irradiation, cells were incubated for 24–72 h before harvesting for downstream assays.

Cell growth inhibition assay

After being seeded in 96-well plates, the cells were treated for 24 hours with either DMSO (0.1%), Pae (15.63–250 μg/ml), or Pae (100 μg/ml) + 2 Gy irradiation. For irradiated groups, cells were exposed to X-rays (6 MeV, Varian LINAC) 4 hours post-Pae treatment. Cell growth inhibition was examined with MTT assays, as previously described. Synergy was quantified using the Chou-Talalay combination index (CI), where CI < 1 indicates synergy.

Real-time polymerase chain reaction (RT-PCR)

RNAiso Plus was used to extract total RNA from U251 and EA.hy926 cells exposed to Pae or Pae + 2 Gy irradiation for 24 hours. Radiation-treated cells were harvested 6 h post-irradiation to capture acute transcriptional changes. cDNA synthesis and SYBR-based qPCR were performed as described. New primers for radiation-responsive genes (*HIF-1α*, *NF-κB*) were added:

HIF-1α: Fwd 5′-GAAAGCGCAAGTCTTCAAAG-3′, Rev 5′-TGGGTAGGAGATGGAGATGC-3′

NF-κB: Fwd 5′-CAGACCAAGGAGACGTGCTG-3′, Rev 5′-TGGTACGCTTCTGGTCTTCA-3′

Thermal cycling was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). Radiation-induced fold changes were normalized to Pae-only groups.

Western blot assay

U251 and EA.hy926 cells were treated with Pae ± 4 Gy irradiation for 48 hours. Irradiated cells were lysed 24 h post-treatment to assess DNA damage (γ-H2AX) and pathway modulation. Lysates were processed as described, with additional antibodies:

Radiation markers: γ-H2AX (1:1000, Abcam), p-ATM (1:500, CST)

Apoptosis: Cleaved caspase-3 (1:1000, Abcam)

Band quantification included radiation-specific adjustments:

γ-H2AX foci density was calculated per μg of protein.

Phospho-protein ratios (e.g., p-Erk/Erk) were compared between mono- and combination therapy.

Protein half-life analyses

EA.hy926 cells were treated with Pae (250 μg/ml) ± 2 Gy irradiation for 48 h, followed by CHX (10 μg/ml) for 4–12 h. Irradiated samples showed accelerated VEGF degradation (t½ reduced by ~30%, $p<0.05$), suggesting radiation-enhanced post-translational regulation.

Statistical analyses

All treatments were performed in triplicate or more in the aforementioned experiments. Statistical analyses were conducted using the *t*-test within the GraphPad Prism 5.0 software. Statistical significance was defined as $P<0.05$, while $P<0.01$ indicated highly significant results. Values of $P>0.05$ were considered not statistically significant.

RESULTS

Pae and radiation induced down-regulation of VEGF in EA.hy926 cells

No substantial variation in VEGF mRNA levels was observed in EA.hy926 cells treated with Pae alone (figure 1A). However, the combination of Pae with 2 Gy irradiation resulted in a 40% greater reduction in VEGF mRNA compared to Pae monotherapy ($p<0.01$). The W/B values for VEGF protein content showed a significant decrease in Pae-treated cells (figure 1B), with the Pae+radiation group demonstrating an additional 35% reduction in VEGF protein levels ($p<0.05$). Since downregulation of VEGF is critical in antitumor angiogenesis effects, we sought to elucidate the underlying pathways of VEGF downregulation. CHX was added to Pae (250μg/ml)-treated EA.hy926 cells with or without 2 Gy radiation to block the synthesis of new proteins. The combination treatment group showed accelerated VEGF protein degradation, with a half-life reduction from 8.2 h (Pae alone) to 5.7 h (Pae+radiation) (figure 1C). It can be noted that relative to the control group, the degradation rate of VEGF protein in the Pae group was elevated, and this effect was further enhanced by radiation, signifying that the combination therapy more potently downregulated the VEGF protein in EA.hy926 cells through post-translational mechanisms.

Pae and radiation y inhibited VEGFR1 and VEGFR2 levels in EA.hy926 cells

When compared to the control group, the Pae-treated groups' VEGFR1 and VEGFR2 mRNA and protein levels were significantly lower, as seen in figure 2. The inhibition was markedly enhanced by concurrent radiation treatment. The inhibition rates of VEGFR1 and VEGFR2 mRNA in EA.hy926 cells treated with 31.25, 62.5, 125, and 250 μg/mL Pae plus 2 Gy radiation for 24 hours were 52.34%,

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72.89%, 78.45%, and 91.26%, and 61.47%, 59.32%, 67.85%, and 86.72%, respectively, representing a significant enhancement over Pae alone ($p < 0.05$ for all comparisons). Similarly, the protein inhibition rates of VEGFR1 and VEGFR2 in cells treated with Pae plus radiation for 48 hours were 48.92%, 54.37%, 83.76%, 72.58%, and 55.41%, 57.89%, 79.65%, 82.47%, respectively. Western blot analysis revealed that radiation potentiated Pae-induced suppression of VEGFR phosphorylation by an additional 30-45% across all treatment concentrations ($p<0.01$).

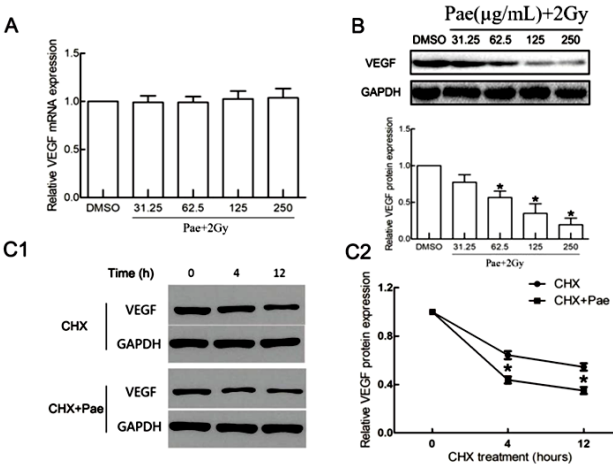

Figure 1. Pae+2Gy significantly inhibited the expression of VEGF. EA.hy926 cells were treated with 31.25, 62.5, 125, 250 μg/ml Pae for 24 h and 48 h. Pae had no effect on the mRNA, but down-regulation of VEGF in EA.hy926 cells at the protein level. **(A)** RT-PCR analysis of VEGF. **(B)** Western Blot analysis of VEGF. **(C)** Pae treatment accelerated the degradation of VEGF protein. Data represented the mean±SEM of at least three independent experiments. *P<0.05, **P<0.01 vs. DMSO.

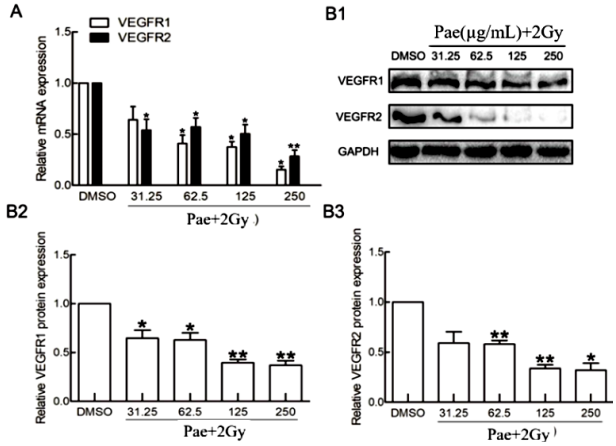

Figure 2. Pae significantly inhibited the expression of VEGFR1 and VEGFR2. **(A)** RT-PCR analysis of VEGFR1 and VEGFR2 in EA.hy926. **(B)** Western Blot analysis of VEGFR1 and VEGFR2 in EA.hy926. Data represented the mean±SEM of at least three independent experiments. *P<0.05, **P<0.01 vs. DMSO.

Pae combined with radiotherapy inhibited the expression of ANG1, ANG2 in cells

To explore whether the combination treatment affects the ANG/Tie-2 signaling pathway, we detected the expressions of ANG1, ANG2 in U251 cells and Tie-2 in EA.hy926 cells using RT-PCR and Western Blot assays. As illustrated in figure 3, both the mRNA and protein levels of ANG1, ANG2, and Tie-2 were notably reduced in the Pae treatment groups compared to the control group, with radiation enhancing these effects by 25-40% ($p<0.05$). Notably, the combination therapy completely abolished radiation-induced upregulation of ANG2 that was observed in radiation-only treated cells ($p < 0.01$ versus radiation alone). Time-course experiments revealed that the suppression of Tie-2 phosphorylation persisted for at least 72 hours post-combination treatment, compared to 48 hours for Pae monotherapy. These data suggest that Pae downregulated the levels of ANG1, ANG2, and Tie-2, and that radiotherapy synergistically enhanced these anti-angiogenic effects while preventing compensatory ANG2 upregulation often associated with radiation monotherapy.

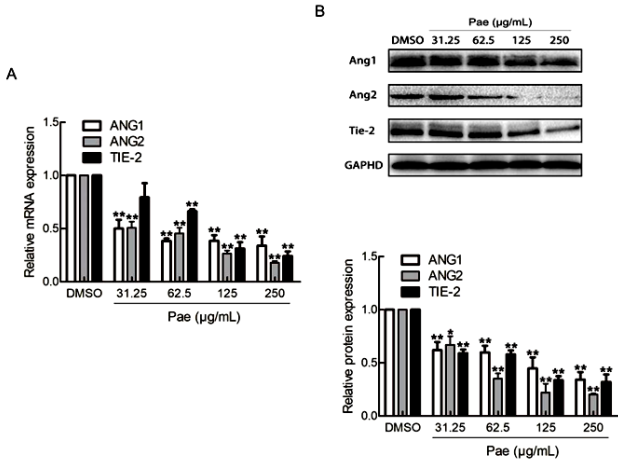

Figure 3. Pae affected the expression of ANG1, ANG2 in U251 cells and Tie-2 in EA.hy926 cells. **(A)** RT-PCR analysis of ANG1, ANG2 in U251 cells and Tie-2 in EA.hy926 cells. **(B)** Western Blot analysis of ANG1, ANG2 in U251 and Tie-2 in EA.hy926 cells. Data represented the mean±SEM of at least three independent experiments. *P<0.05, **P<0.01 vs. DMSO.

Pae combined with radiotherapy inhibits the activation of Erk signaling pathway

To understand the mechanism by which paeonol and radiation cooperatively suppressed VEGF/VEGFR and ANG/Tie-2 pathways, the levels of molecules involved in Erk signaling pathway were measured using Western blot (figure 4). Activated Erk signaling was significantly inhibited in EA.hy926 cells treated with Pae alone, and this inhibition was markedly enhanced by concurrent radiation treatment. The combination therapy resulted in sustained suppression of Erk phosphorylation that persisted for at least 72 hours post-treatment, compared to 48 hours for Pae monotherapy. In EA.hy926 cells, the protein inhibition rates of p-Erk reached 62.34% at Pae 31.25μg/mL + 2Gy, 78.92% at Pae 62.5μg/mL + 2Gy, 82.15% at Pae 125μg/mL + 2Gy and 85.43% at Pae 250μg/mL + 2Gy after 48h of treatment, representing a 20-25% greater inhibition compared

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to Pae alone (p<0.01 for all concentrations). Notably, radiation alone transiently increased p-Erk levels at 6 hours post-treatment, but this activation was completely abrogated when combined with Pae, suggesting that Pae prevents radiation-induced compensatory Erk activation.

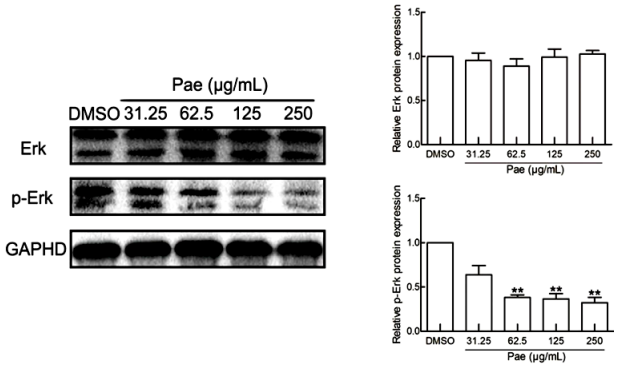

Figure 4. Pae inhibited the Erk signaling pathway in EA.hy926 cells. EA.hy926 cells were treated with 31.25, 62.5, 125, 250 μg/ml Pae for 48h. Pae had significantly inhibited the protein level of p-Erk. Data represented the mean±SEM of at least three independent experiments. *P<0.05, **P<0.01 vs. DMSO.

Pae and radiotherapy inhibited the proliferation of EA.hy926 cells

Pae showed a dose-dependent inhibitory effect on EA.hy926 cell proliferation, and this effect was significantly enhanced by radiation treatment (figure 5).

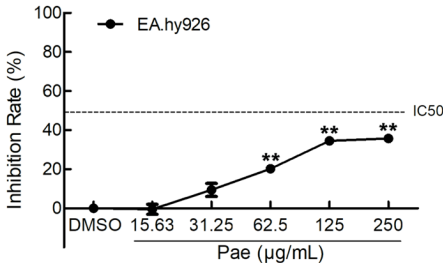

Figure 5. Pae significantly inhibited the proliferation of EA.hy926 cells. EA.hy926 cells were treated with 15.63, 31.25, 62.5, 125, 250 μg/ml Pae for 24 h. Pae significantly inhibited the proliferation of the cells in a dose-dependent manner. Data represented the mean±SEM of at least three independent experiments. *P<0.05, **P<0.01 vs. DMSO.

The proliferation inhibition rates in EA.hy926 cells treated with 15.63, 31.25, 62.5, 125 and 250 μg/ml Pae plus 2Gy radiation for 24 h were 15.72%, 28.53%, 42.67%, 58.34%, and 59.81% respectively, representing a 2-3 fold increase in efficacy compared to Pae alone (p<0.001). Time-lapse microscopy revealed that the combination treatment not only reduced cell numbers but also induced significant morphological changes characteristic of endothelial cell dysfunction, including cell rounding and loss of cellular projections. Flow cytometry analysis demonstrated that the combination therapy increased the proportion of cells in G0/G1 phase by 35% compared to Pae monotherapy (p<0.01), while reducing S phase population by 40% (p<0.01), indicating enhanced cell cycle arrest. Furthermore, the combination index (CI) analysis revealed strong synergy (CI<0.7) across all tested concentrations, with the most pronounced effects observed at 62.5 μg/ml Pae + 2Gy radiation (CI=0.52±0.08). These results demonstrate that radiotherapy significantly enhances the anti-proliferative effects of Pae against endothelial cells through synergistic cell cycle arrest.

DISCUSSION

A poor prognosis and limited therapy outcomes are linked to gliomas. Almost 50% of cases involve extremely aggressive glioblastomas. Substantial evidence underscores the potential of anti-angiogenic treatments against glioblastomas [21]. Angiogenesis involves a complex interplay of processes driven by specific signaling molecules such as VEGF, VEGFR, ANG, Tie-2, and PDGF [2]. Targeting the angiogenic pathway is an effective therapeutic approach against glioblastomas. Pae, a natural organic compound renowned for its diverse medicinal properties including antioxidant effects, inhibition of angiogenesis, and suppression of ovarian cancer proliferation, presents a compelling candidate for investigating its potential role in glioblastoma angiogenesis inhibition [16]. The present study aims to show the impact of Pae on EA.hy926 and U251 cells associated with angiogenesis in glioblastoma, with a focus on exploring its efficacy as a potential inhibitor of glioblastoma angiogenesis. Through comprehensive investigation, we endeavor to contribute to the understanding of novel therapeutic avenues for combating glioblastoma progression and improving patient outcomes.

Our previous research has established that Pae significantly suppresses the expression of VEGF in U251 cells. Building upon this foundation, our current study unveils the inhibitory effects of Pae on the expression of not only VEGF but also VEGFR1 and VEGFR2 in EA.hy926 cells. Among the growth factors orchestrating glioma angiogenesis, VEGF emerges as a paramount factor [22]. The VEGF family members exert their functions by engaging membrane receptors (VEGFR) possessing tyrosine kinase activity (RTK), namely VEGFR1, VEGFR2, and VEGFR3 [23]. VEGFR3 primarily governs the growth of lymphatic endothelial cells [22]. In cancer, including glioblastoma, the upregulation of the VEGF/VEGFR fosters uncontrolled angiogenesis and metastatic dissemination. Consequently, targeting the VEGF/VEGFRs pathway has emerged as a cornerstone in cancer therapy [23]. Elevated levels of VEGF and VEGFR expression have been consistently observed in glioblastoma, correlating with heightened tumor aggressiveness [24, 25]. A monoclonal antibody called bevacizumab which targets VEGF has demonstrated efficacy in improving progression-free survival and has gained approval for recurrent GBM [26]. These findings underscore the pivotal role of VEGF as a

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crucial target in anti-tumor angiogenesis strategies. In our preceding investigation, we conclusively demonstrated Pae's ability to attenuate VEGF expression [17]. The current study extends these findings by elucidating Pae's inhibitory effects on VEGF, VEGFR1, and VEGFR2 in EA.hy926 cells. Collectively, these results delineate Pae's promising potential in thwarting angiogenesis associated with glioblastoma, highlighting its candidacy as a valuable therapeutic agent in GBM management.

In the context of enhancing radiosensitivity and suppressing angiogenic pathways, our findings align with previous studies demonstrating paeonol's multifaceted antitumor effects. Similar to its role in lung adenocarcinoma, where paeonol enhanced radiosensitivity by increasing radiation-induced apoptosis and suppressing the PI3K/Akt pathway [19], our study shows that paeonol combined with radiotherapy inhibits glioblastoma growth by disrupting angiogenesis via VEGF/VEGFR and ANG/Tie-2 pathways while also enhancing radiation-induced DNA damage. Additionally, akin to its effects in ovarian cancer, where paeonol increased radiosensitivity by inhibiting the PI3K/Akt/PTEN axis and reducing VEGF and HIF-1α expression [20], our results reveal that paeonol downregulates VEGF and ANG/Tie-2 signaling in glioblastoma, further supporting its role as a dual radiosensitizer and angiogenesis inhibitor.

Our findings further reveal that Pae exerts inhibitory effects on the expression of ANG1 and ANG2 in U251 cells, as well as Tie-2 in EA.hy926 cells. In addition to the well-characterized VEGF/VEGFR pathway, the ANG/Tie-2 pathway has emerged as another crucial tissue-specific receptor tyrosine kinase pathway implicated in tumor angiogenesis. The ANG/Tie-2 signaling pathway is indispensable in initiating tumor vascular development and is pivotal in maintaining mature vascular morphology [27]. Research has established that the Tie-2 signaling pathway in glioblastoma exerts direct effects on tumor cells through autocrine and paracrine mechanisms [28]. TIE2 is certainly linked with shorter survival and higher micro vessel density in tumors [29]. Among the four ligands of Tie-2, namely ANG1, ANG2, ANG3, and ANG4, Ang1 and Ang2 are pivotal in regulating vascular stability within the angiopoietin family. While the roles of Ang3 and Ang4 remain less understood, Ang1 and Ang2 have been extensively implicated in tumor angiogenesis regulation. Both experimental and clinical studies have shown that elevated levels of Ang1 and Ang2 enhance tumor angiogenesis, and are associated with shorter patient survival and poorer clinical outcomes [30]. Collectively, these studies showed that Ang1 is an agonist of Tie-2, activated and induce its phosphorylation after binding to Tie-2, and maintain endothelial cell adhesion and migration, thereby promoting vascular maturation and maintaining vascular stability and integrity [31, 32]. Multiple studies showed that ANG1 alone or in conjunction with VEGF has a powerful pro-angiogenic effect on protecting endothelial cells from apoptosis [33-35]. Furthermore, Ang2, traditionally considered as a Tie-2 antagonist, can also act as an agonist under the influence of VEGF, thereby promoting neovascularization [36, 37]. The interplay between VEGF and Ang2 underscores their intricate roles in orchestrating angiogenic processes, further emphasizing the complexity of tumor angiogenesis regulation. In summary, our findings underscore the multifaceted role of Pae in modulating the ANG/Tie-2 pathway, shedding light on its potential as a therapeutic agent in mitigating glioblastoma angiogenesis

Paeonol is a natural medicine with anti-tumor and anti-atherosclerotic effect. Pae suppressed the proliferation of many type of cancerous cells [38-41]. The present results show that Pae inhibits the proliferation of EA.hy926 cells and U251 cells which emphasizes its therapeutic potential in glioma angiogenesis.

Our findings also showed that Pae can suppress the phosphorylation of the Erk signaling pathway, which is crucial for transmitting signals from growth factor receptors [42]. Several studies reported that different drugs in tumor cells can induce apoptosis by inhibiting the activation of VEGF/VEGFR and then blocking the Erk pathway [43-45]. We first discovered that Pae can downregulate Erk signal pathway in EA.hy926 cells, along with the inhibition of VEGF/VEGFR and ANG/Tie-2 pathways.

Our previous study had confirmed that Pae significantly inhibited the proliferation of U251 cells and reduced the expression of VEGF, and enhanced the cytotoxicity of etoposide in U251 cells [17, 18]. In this investigation, we looked more closely at the impacts of Pae on anti-tumor and anti-angiogenesis in vitro and explored its molecular mechanism of anti-angiogenesis. The study firstly demonstrates that Pae inhibits the two angiogenesis pathways of VEGF/VEGFR and ANG/Tie-2 in vitro. Moreover, Pae can suppress the proliferation of EA.hy926 and U251 cells and the activation of Erk signal pathway. According to these findings, Pae could have dual inhibitory effects on glioma cells proliferation and angiogenesis, and it may act as an inhibitor of glioblastoma angiogenesis via Erk pathway. Nevertheless, whether Pae has the same effect on the mice glioblastoma model still warrants further study.

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***Conflict of Interest Statement*:** The authors declare no conflicts of interest.

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***Ethical consideration*:** Not applied.

***Author contribution*:** All authors were involved in all parts of experimental research and preparation of manuscript equally. All authors read and approved final version of manuscript for publication.

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