Natural product-derived icaritin exerts anti-glioblastoma effects by positively modulating estrogen receptor β

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Abstract. Glioblastoma is the most common malignancy of the central nervous system, and patients typically have a poor prognosis. Previous studies indicate a gender bias in the development of glioblastoma; women are at a lower risk compared with men, suggesting that estrogen may confer protective effects. Icaritin, a prenyllflavonoid derivative from a Chinese herb of the Epimedium genus, selectively regulates the estrogen receptor (ER) and possesses anti-cancer properties. The aim of the present study was to investigate the protective effects of icaritin on glioblastoma and its underlying mechanisms, with a particular focus on its association with the ER. The results demonstrated that icaritin inhibited the growth of C6 and U87-MG glioblastoma cells in a dose- and time-dependent manner. At a concentration of 12.5 µM, icaritin induced apoptosis, which was characterized by the increased expression of the cleaved forms of caspases 3, 7, 8 and 9 and poly (ADP-ribose) polymerase, downregulation of BCL2 apoptosis regulator and upregulation of BCL2-associated X, apoptosis regulator expression. Additionally, icaritin inhibited the migration of C6 and U87-MG cells. The protein expression levels of matrix metalloproteinase (MMP)-2 and MMP-9 were also down-regulated following icaritin treatment. Furthermore, icaritin treatment increased the expression of estrogen receptor (ER)β and the phosphatase and tensin (PTEN) homolog oncoprotein, thus reducing the expression of downstream targets of PTEN; protein kinase B (Akt) and phosphorylated Akt. Subsequent experiments demonstrated that icaritin cooperates with 17β-estradiol to inhibit the growth of glioblastoma cells, and the inhibition of ERβ with the ERβ-specific antagonist ICI 182,780, attenuated the anti-glioblastoma effects of icaritin. In conclusion, the results of the present study demonstrate that the anti-glioblastoma effects of icaritin may be mediated by its modulation of ERβ.

Introduction

Glioblastoma is a heterogeneous group of tumor types that arise in neuroepithelial tissue, and account for 45.6% of primary malignant brain tumors in the central nervous system (1,2). Due to diffuse brain infiltration of glioblastomas and the potential risk of nervous system impairments from surgical resection, complete surgical resection is usually not feasible. Despite substantial advances in surgical strategies, radiotherapy and chemotherapy, the median survival rates for patients with high-grade glioblastoma is only 12-15 months (3,4). Due to these poor clinical outcomes, there is an urgent need for further glioblastoma research.

Epidemiological data indicates that the incidence of glioblastoma is higher in males compared with females, particularly in premenopausal females (5). This suggests that elevated levels of estrogen may serve a function in reducing the risk of glioblastoma. Endogenous estrogens primarily consist of 17β-estradiol (E2), estrone (E1) and estriol (E3). E2 is produced by the ovaries and is also a neurosteroid hormone synthesized by nerve cells (6,7). In the 1970s, Naftolin et al (9) demonstrated that E2 is derived from the activity of aromatase enzymes in the brain. The androgen hormone testosterone is catalyzed by aromatase, which aromatizes the A-ring of testosterone to form E2 (6). Cells of the central nervous system and glial cells are thought to be targets of E2. The biological effects of E2 are primarily mediated by estrogen receptors (ER) α and ERβ. Batistatou et al (10,11) reported that the expression of ERβ was detectable in 56 glioblastoma samples and paired adjacent normal brain tissues. In addition, patients with high-grade glioblastoma exhibited lower expression levels of ERβ than low-grade glioblastoma patients, as
determined by using immunohistochemical analysis (10,11). These results suggest that ERβ may serve a crucial function in the progression of glioblastoma and may be an important biomarker of patient prognosis.

The blood-brain barrier prevents the delivery of the vast majority of candidate anti-glioblastoma compounds. Icaritin (C25H20O8) has a small molecular weight (368.38) and is a hydrolytic product of Epimedium brevicornu Maxim; a member of the berberine family (12,13). Of particular note, icaritin is able to cross the blood-brain barrier and may therefore present a useful therapeutic option for patients with glioblastoma (14). It was previously reported that icaritin may exert anti-myeloma effects in nude mice (15). In addition, one previous study demonstrated that icaritin may regulate the proliferation, invasion and apoptosis of glioblastoma cells (13). However, studies investigating the mechanisms underlying the anti-glioblastoma effects of icaritin via ERβ are limited.

As a traditional Chinese medicine, icaritin is thought to exert estrogenic effects and has been used to treat impotence and infertility (16). Icaritin (Fig. 1) is a hydrolyzed product of icaritin, which is also an important metabolite of Epimedium in the human body (13). Therefore, studies investigating the effect of icaritin on the ER signaling pathway and its ability to mediate the inhibition of glioblastoma cell migration are required. Phosphatase and tensin homolog (PTEN) is a lipid phosphatase enzyme that catalyzes the phosphorylation of phosphoinositide 3-kinase (PI3K) and is a major negative regulator of protein kinase B (Akt) signaling. Loss of PTEN function is associated with the development of numerous malignant tumor types. ERβ reduces phosphorylated (p)-Akt levels by promoting the expression of PTEN, which mediates its anti-tumor effects (17).

The aim of the present study was to investigate the anti-glioblastoma effects of icaritin and the underlying mechanisms mediating these effects, with a particular relevance to ERβ. To the best of the authors' knowledge, the present study is the first to demonstrate that icaritin exerts anti-glioblastoma effects via the modulation of ERβ. This suggests that ERβ modulators, including icaritin, may be a useful therapeutic strategy for the treatment of patients with malignant glioblastoma.

Materials and methods

Cell culture and drug treatment. Human U87-MG glioblastoma cells (cat. no. HTB-14) and rat C6 glioblastoma cells (cat no. CCL-107) were obtained from the American Type Culture Collection, and U87-MG cells were authenticated with short tandem repeat profiling. The two cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Lonsera, Canelones, Uruguay) and 1% U/ml penicillin/streptomycin (Hyclone; GE Healthcare Life Sciences, Little Chalfont, UK) and cultured in an incubator with a humidified atmosphere at 37°C and 5% CO2.

Cell viability assay. The effect of icaritin on cell viability was determined using an MTT assay. U87-MG and C6 cells at a density of 2x104 cells/ml growing in logarithmic phase were seeded in 96-well plates and treated with icaritin at different doses (6.25, 12.5, 25, 50 and 100 µM) at 37°C for 24, 48 and 72 h. MTT (10 µl; 0.5 mg/ml) was added to the medium of each well and the cells were subsequently incubated at 37°C for 4 h. The medium was then removed and 100 µl dimethyl sulfoxide (Sigma-Aldrich; Merck KGA, Darmstadt, Germany) was added to each well. The optical density was read using an ELx800 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 490 nm. The effect of E2, ERβ-specific antagonist ICI 182,780 (Shanghai Amquar Biological Technology Co., Ltd.) at 37°C for 24 h and icaritin on cell viability was assessed using a cell counting Kit-8 (CCK-8) assay. CCK-8 (10 µl) was added to each well and cells were subsequently incubated at 37°C for 3 h. The optical density was then measured at 450 nm. For MTT and CCK-8 assays, a total of five replicates were prepared for each treatment.

Wound healing assay. U87-MG and C6 cells (5x104 cells/ml) were seeded in 35-mm plates and cultured until they reached confluence prior to being treated with 3.125 µM icaritin at 37°C for 24, 48 and 72 h. In each well, two perpendicular scratch-wounds with an initial width of 300 µm were generated in the confluent cell monolayer using a pipette tip. The culture medium was then refreshed with medium containing the drugs. Phase-contrast images of the cells were obtained immediately and at 24, 48 and 72 h following generation of the scratch-wound using a charge-coupled device camera connected to an Olympus fluorescence microscope (×40 magnification) (Olympus Corporation). The region imaged at the 0 h time point was marked to enable the same region to be imaged at 24, 48 and 72 h, thus allowing examination of a specific population of migrating cells. Wound closure was measured using Image-Pro Plus 6.0 (Media Cybernetics, Inc.) and data acquired from four regions of the scratch-wound on

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**Figure 1. Chemical structure of icaritin.**
each plate were averaged to obtain the mean wound-width at a given time.

**Immunofluorescence staining.** U87-MG and C6 cells were seeded at a density of 2.5x10^5 cells/well on glass coverslips placed in 6-well tissue culture plates, and allowed to adhere overnight. Cells were then treated with 25 µM icaritin at 37°C for 24 h. The following day, cells were washed with phosphate buffered saline (PBS) and fixed in 4% (v/v) paraformaldehyde for 30 min at room temperature. Fixed cells were subsequently washed with PBS and permeabilized with 0.3% (v/v) Triton X-100 in PBS for 1 h, prior to being washed again in PBS. In order to block non-specific binding sites, cells were incubated with PBS containing 5% (v/v) bovine serum albumin at room temperature for 10 min, and then incubated with a primary antibody against ERβ (1:5,000; diluted in 0.1% Triton X-100; ab3577; Abcam) overnight at 4°C. The following day, cells were washed with PBS and incubated with a secondary antibody [1:500; diluted in mixture of 1% BSA and 0.3% Triton X-100; FITC-labeled goat anti-rabbit IgG (H+L); A0562; Beyotime Institute of Biotechnology] for 1 h in the dark. Cells were then gently washed. Finally, the cell nucleus was stained with DAPI (1 µg/ml; 4083; CST Biological Reagents Co., Ltd.) at room temperature for 10 min and visualized under a DP80 fluorescence microscope (x400 magnification; Olympus Corporation).

**Western blotting.** Western blotting was performed as previously described (18). β-actin protein was used as a reference protein. U87-MG and C6 cells (1.25x10^5 cells/ml) were dissolved in SDS sample buffer (Beyotime Institute of Biotechnology) following lysis with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration was determined using a BCA kit (Beijing Solarbio Science & Technology Co., Ltd.). Equal quantities of protein (25 µg/lane) were loaded and separated by 10 or 12% SDS-PAGE (Bio-Rad Laboratories, Inc.) and electroblotted onto PVDF membranes (Merck KGaA). The membranes were incubated in 5% non-fat dry milk in tris-buffered saline/Tween buffer (1x; Epizyme, Inc., Cambridge, MA, USA) containing 0.1% Tween-20 for 2 h at room temperature, prior to incubation with the following primary antibodies. Caspase-3 (9665), caspase-7 (12827), caspase-8 (4790), caspase-9 (9508), cleaved caspase-3 (9664), cleaved caspase-7 (8438), cleaved caspase-8 (9496), cleaved caspase-9 (7237), poly (ADP-ribose) polymerase (PARP; 9542), cleaved PARP (5625), AKT (4691), p-AKT (2965), PTEN (9559), matrix metalloproteinase (MMP-2; 40994), MMP-9 (13667; all 1:1,000; CST Biological Reagents Co., Ltd.), ERβ (ab3577), Bcl2 (ab182858) and Bax (ab32503; all 1:1,000; Abcam) overnight at 4°C. ECL solution (Merk KGaA) was mixed with equal volumes of luminol reagent and peroxide solution in a clean tube. Approximately 0.1 ml of working horseradish peroxidase (HRP) substrate is required per cm² membrane area. Blots were incubated for 5 mins at room temperature. The blots were exposed using a Tanon-5200 molecular imager (Tanon Science and Technology Co., Ltd.) following incubation with a HRP-labeled secondary antibody [(HRP-labeled goat anti-mouse IgG (H+L); A0216; Beyotime Institute of Biotechnology); (HRP-labeled goat anti-rabbit IgG (H+L); A0208, Beyotime Institute of Biotechnology) at room temperature for 2 h. Image-Pro Plus 6.0 (Media Cybernetics, Inc.) was used to calculate densitometry.

**Statistical analysis.** Data are presented as the mean ± standard deviation of at least 3 replicates. SPSS 17 statistical software (SPSS, Inc., Chicago, IL, USA) was used to analyze the data, and statistical analysis was performed using one-way ANOVAs, with Fisher’s least significant difference post-hoc tests used to determine the differences between groups. P<0.05 was considered to indicate a statistically significant difference.
Results

Icaritin suppresses the growth of U87-MG and C6 cells. As presented in Fig. 2A and B, U87-MG and C6 cells were incubated with different concentrations of icaritin for 24, 48 and 72 h. The results indicated that icaritin suppressed the viability of U87-MG and C6 cells at different time points and concentrations. Notably, compared with 0 µM icaritin, U87-MG and C6 cells treated with 100 µM icaritin for 72 h showed the greatest inhibition of cell viability (P<0.001). Viability suppression of U87-MG and C6 cells was time-dependent upon treatment with 12.5 µM icaritin, and dose-dependent upon treatment with 6.25, 12.5, 25, 50 and 100 µM icaritin.

Icaritin induces apoptosis of U87‑MG and C6 cells. U87-MG and C6 cells were treated with 12.5 µM icaritin for 24, 48 and 72 h prior to Hoechst 33342 fluorescence staining being performed. As demonstrated in Fig. 3A and B, a greater fluorescence intensity was observed in the 12.5 µM icaritin group when compared with the control group. Fluorescence intensity increased in a treatment time-dependent manner. The protein expression of caspase enzymes and BCL2 apoptosis regulator (Bcl-2) in U87-MG and C6 cells treated with icaritin (25, 50 and 100 µM) for 48 h was then examined (Fig. 4A and B). Compared with the control group, icaritin treatment appeared to upregulate the protein expression of the cleaved isoforms of caspase-8, caspase-9, caspase-7, caspase-3, PARP and Bax. In addition, icaritin appeared to downregulate the protein expression of caspase-8, caspase-9, caspase-7, caspase-3, PARP and Bcl-2. The expression of these proteins were positively associated with the concentration of icaritin.

Icaritin suppresses the invasion and migration of U87-MG and C6 glioblastoma cells. U87-MG and C6 cells were treated with 12.5 µM icaritin for 24, 48 and 72 h (Fig. 5). Compared with the control group, the expression levels of extracellular MMP-2 and MMP-9 decreased significantly in a time-dependent manner (P<0.01). In addition, U87-MG and C6 cells were treated with 3.125 µM icaritin for 24, 48 and 72 h, and the number of migrating cells were observed under an inverted microscope (Fig. 6). Compared with the control group, the number of migrated cells significantly decreased in a time-dependent manner (P<0.05).

Icaritin upregulates ERβ protein expression in U87-MG and C6 glioblastoma cells. Results from immunofluorescent staining suggested that the expression of ERβ in U87-MG and C6 cells increased substantially following treatment with 25 µM icaritin when compared with the control group (Fig. 7).
Icaritin affects the protein expression levels of ERβ, PTEN, Akt and p-Akt. Compared with the 0 µM icaritin group, the protein expression levels of ERβ and PTEN in U87-MG and C6 cells significantly increased following treatment with increasing concentrations of icaritin (25, 50 and 100 µM; P<0.01) for 48 h. By contrast, the protein expression levels of p-Akt were significantly decreased (P<0.01; Fig. 8). The protein expression levels of total Akt in U87-MG and C6 cells treated with 50 µM of icaritin were decreased, although this did not reach statistical significance. However, compared with
Figure 6. Icaritin suppresses the migration of (A) U87-MG cells and (B) C6 cells in a wound healing assay (magnification, x100). The results of the wound healing assay were then quantified in (C) U87-MG and (D) C6 cells. *P<0.05 and **P<0.01 vs. the control group.

Figure 7. Immunofluorescent staining of ERβ (green) and DAPI (blue) in (A) U87-MG and (B) C6 cells (magnification, x400). DAPI staining indicates the nuclei of the cells. ERβ, estrogen receptor β; DAPI, 4',6-diamidino-2-phenylindole.
the 0 µM icaritin group, the protein expression levels of total Akt in U87-MG and C6 cells treated with 100 µM icaritin were significantly decreased (P<0.01).

Combined treatment with E2 and icaritin suppresses the viability of U87-MG and C6 cells. A total of 3.5 µM E2 exerted significant anti-glioblastoma activity compared with the control group (P<0.001). Furthermore, the combined treatment of cells with E2 and icaritin exerted significantly higher anti-glioblastoma effects when compared with the icaritin treatment alone group (P<0.05; Fig. 9), indicating the involvement of ER in the icaritin-induced viability inhibition of glioblastoma cells.

PTEN/Akt is a downstream pathway modulated by the expression of ERβ. As Fig. 11 presents, compared with icaritin alone, following the incubation of icaritin with E2 for 24 h in U87-MG and C6 cells, the protein expression of ERβ and PTEN was increased, and the protein expression of p-Akt was decreased. When ERβ was suppressed by ICI 182,780, the downregulation of ERβ and PTEN, as well as the upregulation of p-Akt, were observed.

**Discussion**

Icaritin is a flavonoid derived from a traditional Chinese herb from the *Epimedium* genus. It has a small molecular weight,
et al: ICARITIN NATURAL DERIVATIVE EXERTS ANTI-GILOBLASTOMA EFFECTS VIA ERβ and is able to cross the blood-brain barrier freely. Previous studies have demonstrated that icaritin possesses neuroprotective effects and anti-neoplastic functions in colorectal cancer, oral squamous cell carcinoma and leukemia (19-21). In addition, it has been reported that icaritin induces apoptosis, autophagy and cell cycle arrest, and inhibits the invasion and metastasis of glioblastoma cells.

Figure 9. Combination of E2 and icaritin represses the viability of (A) U87-MG and (B) C6 glioblastoma cells. ""P<0.001 vs. the control group. *P<0.05 and **P<0.01 vs. the icaritin alone group. E2, 17β-estradiol.

Figure 10. Inhibition of ERβ with the ER antagonist ICI 182,780 dampened the anti-viability effect of icaritin in (A) U87-MG and (B) C6 glioblastoma cells. """"P<0.001 and """"""""P<0.0001 vs. the control group. ER, estrogen receptor.

Figure 11. Activation or inhibition of ERβ regulated PTEN/Akt signaling pathway. (A) U87-MG and (B) C6 cells were treated with 25 µM icaritin alone or plus 3.5 µM E2 or plus 1 µM ICI 182,780 for 24 h and examined using western blotting. ERβ, estrogen receptor β; PTEN, phosphatase and tensin homolog; P-, phosphorylated; Akt, protein kinase B; E2, 17β-estradiol.
The incidence of glioblastoma is greater in males compared with females. In addition, postmenopausal women and those at a reproductive age have a survival advantage compared with males, which suggests that estrogen and the ER may serve important functions in the suppression of glioblastoma (25,26). ERα and ERβ are the two main isoforms of the ER (11). The expression of ERα in glioblastoma is low (11), and previous studies have demonstrated that ERβ functions as a tumor suppressor in various types of human malignancies, including glioblastoma (23,27,28). In addition, it has been reported that icaritin is an ER-regulator (29) and that ERβ serves a crucial function in glioblastoma tumorigenesis and prognosis (17). Therefore, the aim of the present study was to investigate whether icaritin exerts anti-glioblastoma effects by functioning as a modulator of ERβ. The immunofluorescence results demonstrated that ERβ protein expression was observed in the nucleus of glioblastoma cells. The results of the immunofluorescence staining showed that treatment of glioblastoma cells with 25 µM icaritin increased the expression of ERβ. Subsequent western blotting also demonstrated that icaritin significantly upregulated ERβ expression in glioblastoma cells in a dose-dependent manner (P<0.01 or P<0.001, compared with the 0 µM icaritin group). These results suggest that icaritin functions as an agonist of ERβ in glioblastoma.

Estrogen induces PTEN at the post-transcriptional level. E2 first induces Na+/H+ exchange regulatory factor 1 (NHERF1) expression by activating the ER. When competing with neural precursor cell expressed, developmentally downregulated 4, an ubiquitin E3 ligase, NHERF1 interacts with PTEN to inhibit its degradation through a ubiquitination-dependent signaling pathway. This subsequently results in increased PTEN expression at the protein level (30). A previous study demonstrated that E2 exerts effects on ERβ to increase the transcription of PTEN, and ERβ binds to the Sp1 region of the PTEN promoter, resulting in autophagy via inhibition of the PI3K/Akt signaling pathway (31). The results of phase II and III clinical trials investigating the ER agonist glycyrrhizin have demonstrated that it is able to cross the blood-brain barrier and target glioblastoma cells with good tolerability and low neuronal toxicity (23). In addition, glycyrrhizin was observed to enhance sensitivity to temozolomide due to the activation of multiple ERβ regulatory elements and downstream target genes, as well as the inhibition of the PI3K/Akt mechanistic target of rapamycin kinase signaling pathway (32). Therefore, the present study hypothesized that PTEN/Akt may be a downstream signaling pathway modulated by the expression of ERβ. In the present study, icaritin inhibited the protein expression of PTEN, Akt and p-Akt in a dose-dependent manner. It has been demonstrated that PTEN serves a critical functions in tumorigenesis and is an important therapeutic target. Mutations or deletions of the PTEN gene are the main genetic changes identified in glioblastomas (33). The present study demonstrated that icaritin activated the ERβ/PTEN/Akt signaling pathway and investigated whether the anti-glioblastoma effects of icaritin were dependent on the interaction between icaritin and ERβ. To achieve this, the effect of E2 on the viability of glioblastoma cells was initially assessed. A previous study had indicated that E2 activates the c-Jun N-terminal kinase/c-Jun signaling cascade and inhibits the growth of rat C6 glioblastoma and human T98G glioblastoma cells, with an half maximal inhibitory concentration of 3.5 µM (34). The results of the present study indicated that E2 treatment for 24 h reduced the viability of C6 and U87-MG cells to 71.33 and 59.28%, respectively. Treatment with 25 µM icaritin significantly increased the anti-growth effects of E2(P<0.001, compared with the 0 µM icaritin group). ICI 182,780, also known as fulvestrant, is a 7α-alkylsulfanyl analogue of E2 and is widely used as a specific antagonist of intracellular ERs (35). ICI 182,780 binds to ERs in a dose-dependent manner and inhibits essential receptor dimerization, which alters the conformation of the ER and prevents it from being transported to the nucleus (36). In the present study, ICI 182,780 was demonstrated to exert an inhibitory effect on the anti-growth activity of icaritin in glioblastoma cells. This observation supported the notion that the ER serves an important function in the anti-glioblastoma effects of icaritin. Therefore, the results indicated that icaritin exerts anti-glioblastoma effects, at least in part, via modulating ERβ.

In conclusion, the results of the present study demonstrated that icaritin may modulate ERβ and the downstream PTEN/Akt signaling pathway, resulting in subsequent suppression of glioblastoma cell growth and migration. The results also provide some evidence to suggest that icaritin may be a potential treatment for patients with glioblastoma.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
YZ and JD participated in research design. XL, WZ, LL conducted experiments. WZ and XD contributed new reagents and analytic tools. XL, JD, WZ and LL performed data analysis. YZ, JD and XL wrote or contributed to the writing of the manuscript.

Ethics approval and consent to participate
Not applicable.
Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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