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Therapeutic significance of estrogen receptor β agonists in gliomas

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Abstract

Gliomas are the most common and devastating central nervous system neoplasms. A gender bias exists in their development: females are at lower risk than males, implicating estrogen-mediated protective effects. Estrogen functions are mediated by two ER subtypes: ERα, that functions as tumor promoter and ERβ that function as tumor suppressor. We examined the potential use of ERβ agonists as a novel therapeutic to curb the growth of gliomas. Western analysis of six glioma model cells showed detectable expression of ERβ with little or no ERα. Treatment of glioma cells with ERβ agonists resulted in significant decrease in proliferation. IHC analysis of tumor tissues revealed that ERβ expression is down regulated in high-grade gliomas. We found that ERβ agonists promote both expression and tumor suppressive functions of ERβ in glioma cells. Liquiritigenin, a plant-derived ERβ agonist significantly reduced *in vivo* tumor growth in a xenograft model. Compared to control mice, animals treated with liquiritigenin had greater than 50% reduction in tumor volume and size. IHC analysis of tumors revealed a significant increase in the nuclear ERβ expression with a concomitant decrease in cell proliferation in the liquiritigenin-treated group. Our results suggest that ERβ signaling has a tumor suppressive function in gliomas. Since ERβ agonists are currently in clinical trials and are well tolerated with fewer side effects, identification of an ERβ agonist as a therapeutic agent can be readily extended to clinical use with current chemotherapies, providing an additional tool for enhancing survival in glioma patients.

Keywords

Estrogen; Estrogen receptor beta; ERβ agonists; tumor suppressor; Gliomas; liquiritigenin

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Introduction

Gliomas are the most common type of primary brain tumors that account for more than 70% of all primary brain tumors. Despite tremendous improvements in the standard therapies for patients with gliomas, patients with malignant gliomas have a survival time of approximately 12 months (1, 2) . To date, little is known about the etiology of gliomas except the high risk factor of exposure to high doses of ionizing radiation and the presence of rare genetic conditions like neurofibromatosis and tuberous sclerosis (3-5).

Recent studies suggest a possible protective role of female sex hormones in glioma progression. The incidence of developing gliomas is greater in males than in females, and females of reproductive age have a survival advantage over males and menopausal females (6-10). Estrogens are steroid hormones that play a crucial role during brain development and differentiation (11, 12), and locally synthesized estrogens from androgens by cytochrome P450 aromatase (CYP19) play a critical role in neuroprotective functions (13). Furthermore, lower glioma incidence with usage of exogenous hormones was evident in females (9, 14) . Collectively, these findings suggest that estrogens play a critical role in differentiation and survival of neural cells; yet, little is known about therapeutic significance of estrogen signaling in glioma initiation and progression.

The biological effects of estrogens are preferentially mediated through their cognate receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (15, 16). Eventhough ERα and ERβ are structurally similar, their ligand-binding domains differ enough to be selective for different ligands (17). Recent studies have shown that ERβ has quite a different function than ERα (18) and is generally considered a tumor suppressor. ERβ expression is down regulated or lost in several tumors including those of the breast, ovary, prostate, and colon (19-22). Additionally, it has been reported that overexpression of ERβ reduced cell proliferation and knockdown of ERβ enhanced cell proliferation in colon and breast cancer cells (23-25)\While the studies suggest that ERβ has tumor suppressive potential in some tumors, the role and therapeutic significance of ERβ signaling in gliomas remains elusive.

Recently, a number of selective ERβ agonists have been developed and are being investigated for therapeutic use (18). Along these lines, a novel, highly selective ERβ-agonist named liquiritigenin was recently isolated from the *Glycyrrhiza uralensis*(26). Liquiritigenin is an active compound found in MF101 (Menerba), a plant extract designed to treat vasomotor symptoms (hot flashes) associated with menopause. In a Phase II clinical trial of Menerba (27), the drug was found to be safe, well tolerated and taken with high compliance. It is being further evaluated for its therapeutic use in a Phase III clinical trial (28).

In the current study, we investigated the status and significance of ERβ signaling in gliomas through the use of both *in vitro* and *in vivo* xenograft models of gliomas, and tested its therapeutic significance using recently developed selective ERβ modulators. Our findings revealed that ERβ agonists promote both expression and tumor suppressive functions of ERβ. Liquiritigenin, a plant-derived ERβ agonist significantly reduced *in vivo* tumor growth in a xenograft model. Our results suggest that ERβ signaling plays a tumor suppressive function in gliomas, and thus ERβ agonists represent a novel class of drugs for curbing glioma progression.

Materials and Methods

Cell lines and reagents

Human glioma cell lines T98G, U87, LN229, U138, M059J, M059K, MCF7, MDA-MB-231 were obtained from the American Type Culture Collection (ATCC) and were passaged in our laboratory for less than six months. Glioma cell lines were maintained in DMEM medium, and MCF7 and MDA-MB-231 cells were maintained in RPMI-1640 medium supplemented with 10% FBS (Hyclone Laboratories Ltd, Logan, UT). DPN and PPT was purchased from Tocris Bioscience (Ellisville, MO) and MF101 was obtained from Bionovo (Emeryville, CA). Liquiritigenin was purchased from Biopurify Phytochemicals (Chengdu, China). The ERβ antibody and ERβ specific siRNA were obtained from Thermo Scientific (Waltham, MA). The ERα antibody was from Millipore (Billerica, MA). PCNA was from Cell Signaling Technology (Boston, MA). ERβ specific shRNA lentivirus, β-actin and all secondary antibodies were purchased Sigma Chemical Co (St. Louis, MO).

Cell lysis and Western blotting

Whole cell lysates were prepared from glioma cells in modified RIPA buffer (150mM NaCl, 50mM Tris-HCl, 50mM NaF, 5mM EDTA, 0.5% [wt/vol] sodium deoxycholate and 1% Triton X-100) containing phosphatase and protease inhibitors. Lysates were run on 10% SDS-PAGE. Total proteins (30 μg) were mixed with SDS sample buffer and separated on SDS-polyacrylamide gels. Resolved proteins were transferred onto nitrocellulose membranes, and the membranes were blocked with 5% non-fat dry milk solution for 1 h at room temperature and incubated overnight in the primary antibodies at 4°C. Membranes were then incubated with the respective secondary antibodies for 1 h at room temperature and immunoreactivity was detected by using an ECL kit (GE Health Care, CA). Nuclear fractionation was performed using compartmental protein extraction kit (Millipore, Billerica, MA).

Reporter gene assays

U87 and LN229 cells were seeded in 6-well plates and maintained in phenol red-free DMEM medium with 5% deactivated charcoal stripped serum. To evaluate the transcriptional activity of endogenous ERβ, cells were transfected with 1 μg of the estrogen responsive element (ERE) construct (pGL2-TATA-3XEREs-Luc) using fugene for 6 h, and 24 h after transfection the cells were treated with vehicle (0.1% DMSO), DPN, MF101 and liquiritigenin for an additional 24 h. The β-galactosidase reporter plasmid (pCMVbetaGal) (20 ng) was co-transfected and used for data normalization. Luciferase activity was measured by using the luciferase assay system (Promega, Madison, WI) and luminometer. The luciferase activity was expressed as percent of relative light units versus untreated transfected cells.

Cell proliferation and clonogenic assays

Cell proliferation rates were measured by using Cell Titer-Glo Luminescent Cell Viability Assay (Promega) in 96-well, flat, clear-bottom, opaque-wall microplates. Glioma cells were seeded in 96 well plates (2×10^3 cells/well) in phenolred-free DMEM medium containing 5% DCC serum. After an overnight incubation, cells were treated with varying concentrations of DPN, MF101 and liquiritigenin for 72 h. Total ATP content as an estimate of total number of viable cells was measured by a luminescence-based assay and an automatic Fluoroskan Luminometer. For some assays, ERβ mediated growth inhibition was determined using traditional MTT assays. Glioma cells stably expressing ERβ-shRNA were generated using human specific Lentiviral ERβ-shRNA particles. Stable clones were selected with puromycin selection (1 μg/mL) and pooled clones were used for all the

studies. Lentiviral particles expressing nontargeted short hairpin RNA (shRNA) were used to generate control cells. For the clonogenic assays, U87 and LN229 cells (500 cells/well) were seeded in 6-well plates. After an overnight incubation, cells were treated with DPN, MF101 and liquiritigenin for 72 h. The cells were washed with PBS and allowed to grow for an additional 7 days. The cells were then fixed in ice cold methanol and stained with 0.5% crystal violet solution to visualize the colonies. Colonies that contain ≥ 50 cells were counted.

Flow Cytometry

U87 and LN229 cells were seeded in 100-mm culture plates, synchronized by serum starvation for 48h and treated with liquiritigenin or 0.1% DMSO for 48 h. Cells were then trypsinized and harvested in 1× PBS, followed by fixation in ice cold 70% ethanol. Staining was done with a mixture of 50 μg/mL propidium iodide (PI) and 50 μg/mL RNase A. Then, PI-stained cells were subjected to flow cytometry by using a FACS analysis using UTHSCSA core facility.

Quantitative RT-PCR analysis

U87 and LN229 cells were treated with liquiritigenin or 0.1% DMSO for 12 h and were harvested with Trizol Reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated according to the manufacturer's instructions. Reverse transcription (RT) reactions were performed by using the Superscript III reagent kit (Invitrogen). Real-time PCR was done by using a Cepheid Smart cycler II (Sunnyvale, CA) with specific real-time PCR primers for ERβ and its target genes: ERβ: (F)GGCAGAGGACAGTAAAAGCA, (R) GGACCACACAGCAGAAAGAT; MSMB: (F)CCAGGAGATTCAACCAGGAA, (R)GAAACAAGGGTGCAACATGA; NKG2E: (F)GCCAGCATTTTACCTTCCTCAT, (R)AACATGATGAAACCCCGTCTAA; MDA-7: (F)CTTTGTTCTCATCGTGTCACAAC, (R)TCCAACTGTTTGAATGCTCTCC; Actin: (F) GTGGGCATGGGTCAGAAG, (R)TCCATCACGATGCCAGTG. Results were normalized to the β-actin transcript levels and the difference in fold expression was calculated using delta-delta-CT method.

Immunofluorescence studies

Confocal microscopy was performed as previously described (29). U87 and LN229 cells were seeded on sterile glass cover slips in 24-well plates and treated with vehicle (0.1% DMSO) or liquiritigenin for 24 h. The cells were fixed with 3.7% paraformaldehyde for 15 min followed by permeabilization with 0.2% Triton X-100 in PBS. After blocking with 5% normal goat serum (Sigma) for 1 h, the cells were incubated with the ERβ primary antibody for 1 h. The ERβ status was analyzed by phalloidin staining for 1 h at room temperature. The DNA dye 4′,6-diamidino-2-phenylindole (Invitrogen) was used to co-stain the DNA (blue). Fluorescence was captured using a Leica confocal microscope.

Tissue microarrays

The tissue microarrays (TMAs) were obtained from US BioMax (Rockville, MD). Each TMA comprised 0.6-mm cores taken from paraffin-embedded specimens that represent a total of 192 glioma tissues and 8 each of adjacent normal tissue and normal tissues.

Immunohistochemistry

Immunohistochemical analysis was performed as described (29). Tumor sections were incubated overnight with ERβ primary antibody at a dilution of 1:50. PCNA obtained from Vector Lab was used in conjunction with proper controls, visualized by DAB substrate and counterstained with hematoxylin (Vector Lab, Inc. Burlingame, CA). Proliferative index

was calculated as percentage of PCNA-positive cells in 10 randomly selected microscopic fields at 100× per slide. TUNEL analysis was done by using the *In situ* Cell Death Detection Kit (Roche, Indianapolis, IN) as per the manufacturer's protocol and 10 randomly selected microscopic fields in each group were used to calculate the relative ratio of TUNEL-positive cells.

Nude mice studies

All animal experiments were performed after obtaining UTHSCSA-IACUC approval and the animals were housed in accordance with UTHSCSA institution's protocol for animal experiments. For xenograft tumor assays, 1×10^6 U87 cells were mixed with an equal volume of matrigel and implanted subcutaneously into the flanks of 6-week-old female nude mice as described (33). Once tumors reached measurable size, mice were divided into control and treatment groups. The control group received vehicle (0.3% hydroxyl propyl cellulose), and the treatment group received liquiritigenin (20 mg/kg) subcutaneously once a day for 30 days. Tumor volumes were measured with a caliper at 5-day intervals. After the 30^{th} day, the mice were euthanized, and the tumors were isolated and processed for histological studies. Tumor volume was calculated by using a modified ellipsoidal formula: tumor volume = ½ (L × W2), where L is the longitudinal diameter and W is the transverse diameter (33). Body weight was measured at weekly intervals to rule out the drug toxicity.

Statistical analysis

SPSS software was used for all statistical analyses. A Student's *t*-test was used to assess statistical differences between control and liquiritigenin-treated groups. The level of significance was set at $P<0.05$. Statistical differences among groups were analyzed with ANOVA .

Results

Gliomas express ERβ and nuclear expression of ERβ negatively correlates with histological malignancy

Several investigations demonstrated weak or low expression for ERα in gliomas. However, very little is known on the status of ERβ in glial tumors. We used a glioma TMA to investigate whether ERβ expression correlates with the clinical grade of gliomas or adjacent normal brain tissues. We measured the expression levels of ERβ by IHC, and intensity was scored as previously described (29-31). The representative staining for each grade is shown in Fig. 1A-D. ERβ expression was higher in the normal brain tissues and in the low-grade tumors but was significantly less in the high-grade tumors. ERβ was predominantly localized in the nucleus in grade II tumors, however most of the cells in high-grade tumors had cytoplasmic staining. The percentage ERβ-expressing cells with staining in the nucleus was significantly lower in high-grade tumors than in normal tissues and low-grade tumors (Fig. 1E). These results suggest that ERβ expression was low during the progression of gliomas and that high grade gliomas express ERβ predominantly in the cytoplasm.

Glioma cells have a functional ERβ signaling pathway

To understand the significance of the ER pathway in glioma progression, we examined the status of ERα and ERβ expression in various glioma cell lines. MCF7 and MDA-MB-231 breast cancer cells were used as positive controls for ERα and ERβ, respectively (Fig. 2A). All the six glioma model cells investigated were devoid of ERα expression; however, all of them expressed detectable levels of ERβ. Transfection of either ERβ specific siRNA or shRNA into glioma cells substantially reduced the detection of ERβ band in Western blot (Supplementary Fig. S1), Results of these experiments demonstrate the specificity of ERβ

antibody used in this study. To examine the functionality of ERβ signaling in glioma cells, we used ligands that uniquely activate ERβ including DPN, MF101 and liquiritigenin. MF101 is derived from 22 herbs and is currently in clinical trials for hot flashes (28). Structure of DPN and liquiritigenin is depicted in Supplementary Fig. 2. Using reporter gene assays, we found that ERβ agonist treatment significantly enhanced the ERE-luciferase activity in U87 and LN229 glioma cell lines (Fig. 2B). To further confirm the functional activation of the ERβ transcriptional pathway, we examined the expression of ERβ target genes under conditions of ERβ agonist stimulation. Ligand stimulation enhanced the expression of the ERβ target genes *MSMB, MDA-7* and *NKG2E* (Fig. 2C and D). Collectively, these results suggest that glioma cells express ERβ and that ERβ is functionally active.

ERβ agonists reduce the proliferation of glioma cells

Emerging evidence suggest that ERβ functions as tumor suppressor. We therefore examined whether activation of ERβ pathway by agonists contribute to reduction of proliferation in four different glioma model cells. Treatment of glioma cells with MF101, DPN and liquiritigenin resulted in a significant dose-dependent reduction in cell proliferation (Fig. 3A). Knockdown of ERβ expression using either siRNA or shRNA, abolished the ability of ERβ ligands to reduce the proliferation of glioma cells (Supplementary Fig. S3). Similarly, treatment of ERα specific agonist propyl-pyrazole triol (PPT) did not showed any inhibitory effect on the proliferation of glioma cells (Supplementary Fig. S4). In cell survival assays, ERβ agonists significantly reduced the colony formation ability of glioma cells (Fig. 3B). Cell cycle analysis of glioma cells revealed that ERβ agonist treatment causes cell cycle arrest most significantly in G2/M phase in both model cells (Fig. 3C). Further, ERβ agonist also showed significant effect on S phase accumulation in addition to G2/M arrest in LN229 cells. Collectively, these results suggest that ERβ agonists have potential to block cell cycle progression of glioma cells and preferentially arrest them at the G2/M phase of cell cycle.

Liquiritigenin induces the expression and nuclear translocation of ERβ

Earlier studies suggested autoregulation of ERβ by its ligand estrogen. We therefore examined whether ERβ agonist treatment increases expression of ERβ by using RT-qPCR assay. The results revealed that liquiritigenin enhanced the expression of ERβ (Fig. 4A). In agreement with the RT-PCR results, Western analysis of cell lystaes revealed that ERβ protein expression was also significantly increased in glioma cells following liquiritigenin treatment (Fig. 4B). Since most of the ERβ staining was found in the cytoplasm in high-grade tumors, we determined whether liquiritigenin treatment promoted localization of ERβ to the nuclear compartment. Confocal microscopy revealed that most of the ERβ expression was confined to the cytoplasm in U87 and LN229 glioma cells; however, liquiritigenin treatment significantly induced the nuclear translocation of ERβ in these cells (Fig. 4C and D, upper panels). Biochemical fractionation and Western analysis also confirmed increased nuclear translocation of ERβ upon liquiritigenin treatment (Fig. 4C and D, bottom panels). These results suggest that activation of ERβ pathway via agonists has potential to increase ERβ protein expression and nuclear translocation.

Liquiritigenin reduce the growth of glioma tumors

To examine whether the ERβ agonist liquiritigenin inhibits growth of glioma cells *in vivo*, we used a nude mouse-based subcutaneous xenograft assay. Two weeks after subcutaneous implantation of U87 glioma cells and when xenograft tumors reached measurable size, liquiritigenin or vehicle was given subcutaneously at a dose of 20 mg/kg/mice/day. Tumor volume was measured for every five days. After 30 days of treatment, the mice were euthanized. As shown in Fig 5A, the rate of tumor growth was significantly reduced in liquiritigenin-treated mice. No toxicities were observed as determined by behavioral

changes, such as eating habits and mobility in animals treated with liquiritigenin, and mouse weights were not significantly different between control and liquiritigenin-treated groups (Fig. 5B). Furthermore, TUNEL analysis showed that the number of apoptotic cells was significantly higher in liquiritigenin-treated mice than in the control mice (Fig. 5C). The proliferation rate of tumor cells was significantly lower in the liquiritigenin-treated mice, which was evident from the reduced PCNA expression (Fig. 5D). ERβ expression and nuclear localization was significantly greater upon liquiritigenin treatment (Fig. 5D). Overall these results suggest that liquiritigenin can restore ERβ expression in gliomas and has potential to suppress glioma cell proliferation *in vivo*.

Discussion

Gliomas are the most common and deadliest form of primary central nervous system neoplasms. Steroid hormones play crucial roles during brain development and differentiation (11, 12) . Several lines of evidence suggest that the incidence of brain tumors is significantly higher in males than in reproductive-aged females suggesting the possible protective role of female sex hormones in the development of brain tumors (6-10). However, a molecular mechanism through which estrogen may mediate protection against the gliomas remains elusive. In this study, we examined the significance and therapeutic potential of ERβ signaling in glioma progression using ERβ-specific ligands. We found that (1) glioma cell lines uniquely expressed ERβ but not ERα, (2) ERβ agonists promoted functional activation of ERβ pathway in glioma model cells, (3) ERβ agonists enhanced ERβ expression and its nuclear localization, (4) ERβ agonists decreased glioma proliferation and (5) the ERβ agonist liquiritigenin significantly reduced glioma tumor progression in a xenograft model. Collectively, these results suggest that ERβ signaling confers tumor suppressive functions on gliomas.

Recent studies have shown that ERβ has quite a different function than ERα, and that ERβ functions as a tissue-specific tumor suppressor with antiproliferative actions (18). Evolving evidence suggests that ERβ overexpression or ligand-dependent activation results in the inhibition of proliferation of various cancerous cells and depending on cell type, activation of ERβ signaling is shown to promote either G2 or G1 arrest (23-25) . In our study, we found that ERβ agonists reduced glioma cell proliferation and colony formation. Furthermore, liquiritigenin treatment resulted in the arrest of cell cycle in G2/M phase. Our findings suggest that ERβ selective agonists such as DPN, MF101 and liquiritigenin have the potential to inhibit glioma cell proliferation and tumor growth.

ERβ is highly expressed in low-grade astrocytomas and non-neoplastic brain tissues, and its localization was preferably confined to the nucleus (32). In contrast, most of the high-grade tumors showed low ERβ expression (33). ERβ down regulation significantly correlated with the histological malignancy of gliomas (34). Recently released TCGA pilot project data ranks ERβ as top-ranking gene for gliomas (155 out of 7658 genes tested) and showed that ERβ expression decreases during glioma progression. Using TMAs, we found the presence of ERβ expression in normal brain tissue and in early stage gliomas. We also found reduced ERβ expression correlated with the higher tumor grade. We also observed that ERβ was localized in the cytoplasm in most of the high-grade tumors and glioma cell lines. ERβ overexpression is shown to promote the differentiation of tumor cells and ERβ agonist 3β-adiol was necessary for maintaining epithelial phenotype (35). Our results collaborate with recently published TMA studies that suggest reduced ERβ signaling may be a prognostic marker for gliomas (32, 33). These findings suggest agonists that increase or stabilize the ERβ expression may have clinical utility in reducing glioma tumor growth.

Currently, various ERβ-selective drugs including DPN, ERB-041, MF101, liquiritigenin are being investigated as a replacement for estrogens to treat menopausal symptoms (17, 18). Previous studies showed that ERβ agonist such as liquiritigenin did not stimulate tumor growth of breast cancer cells in nude mice studies, suggesting the lack of proliferative actions of liquiritigenin (26). Another study showed that liquiritigenin significantly reduced the growth of hepatoma tumors (36). Our results showed that liquiritigenin has the potential to inhibit glioma cell proliferation *in vitro* and also *in vivo* in xenograft-based assays. Immunohistochemical analysis revealed that liquiritigenin reduced the growth of subcutaneous tumors by decreasing proliferation of tumor cells and by inducing apoptosis. Additionally, ERβ expression was significantly greater in liquiritigenin-treated tumors. These results confirmed that liquiritigenin exhibited antitumor activity via the activation of the ERβ pathway. Further, ERβ agonists (DPN and LIQ) have good blood–brain barrier permeability and less neuronal toxicity (37, 38); hence, they are very suitable for therapeutic treatment of gliomas.

In summary, our study results demonstrated the therapeutic significance of the ERβ pathway in gliomas and suggest that functional activation of the ERβ pathway is a potential therapeutic target for gliomas. Since ERβ agonists are currently in clinical trials and are well tolerated with fewer side effects, identification of ERβ agonists as therapeutic agents can be readily extended to clinical use and ERβ agonists could represent as a novel class of drugs to treat gliomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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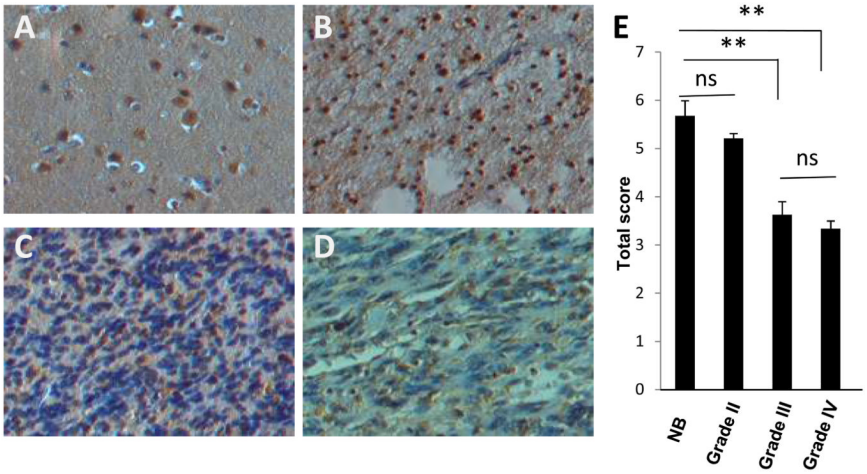

Fig. 1.

ERβ expression negatively correlates with the histological malignancy of gliomas. Glioma tissue array containing control brain (n=16) {A}, as well as grade II (n=122) {B}, grade III (n=32) {C} and grade IV (n=38) {D} glioma tumor samples were immunohistochemically stained with ERβ antibody as described in the methods section. E, Quantitation of IHC was done as described in methods section, bars, SEM. **, $p < 0.05$.

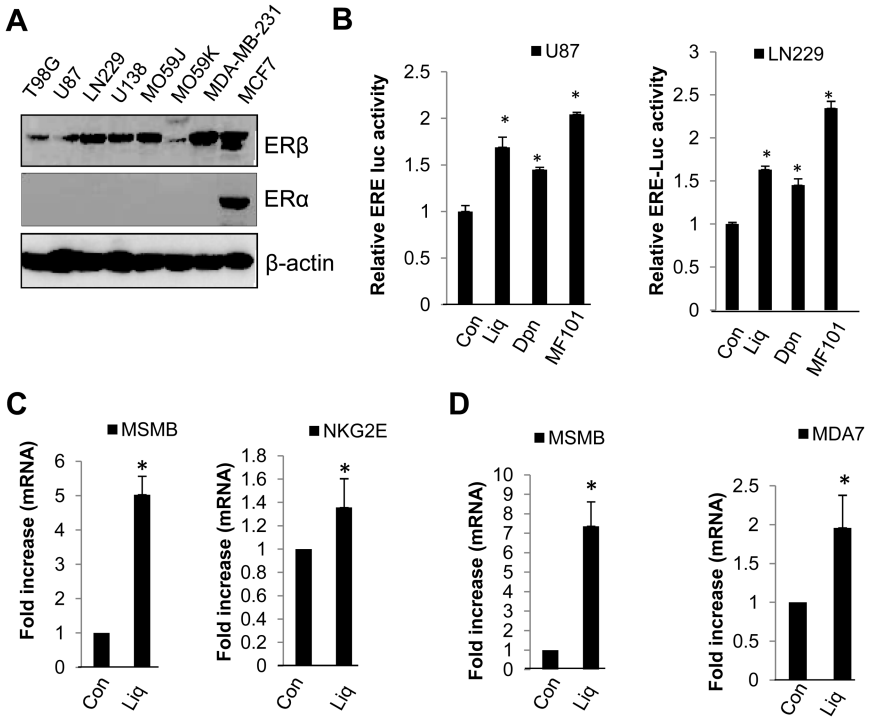

Fig. 2.

Glioma cells have functional ERβ pathway. A, Expression of ERα and ERβ protein in glioma cells was analyzed by Western blotting. Breast cancer cell lines MCF7 and MDA-MB-231 were used as positive controls for ERα and ERβ, respectively. β-actin served as loading control. B, U87 and LN229 cells were transiently transfected with the ERE-Luc reporter and 24 h post transfection, cells were treated with DPN (10 nM), MF101(10 μg) or liquiritigenin (100 μM). The reporter gene activity was measured after 24 h. C, D, Total RNA was isolated from vehicle- or liquiritigenin (100 μM) treated U87 (C) and LN229 (D) cells and subjected to real-time quantitative PCR using the primers specific for ERβ target genes. All data presented are the mean ± SEM. *, $p < 0.05$, *t* test.

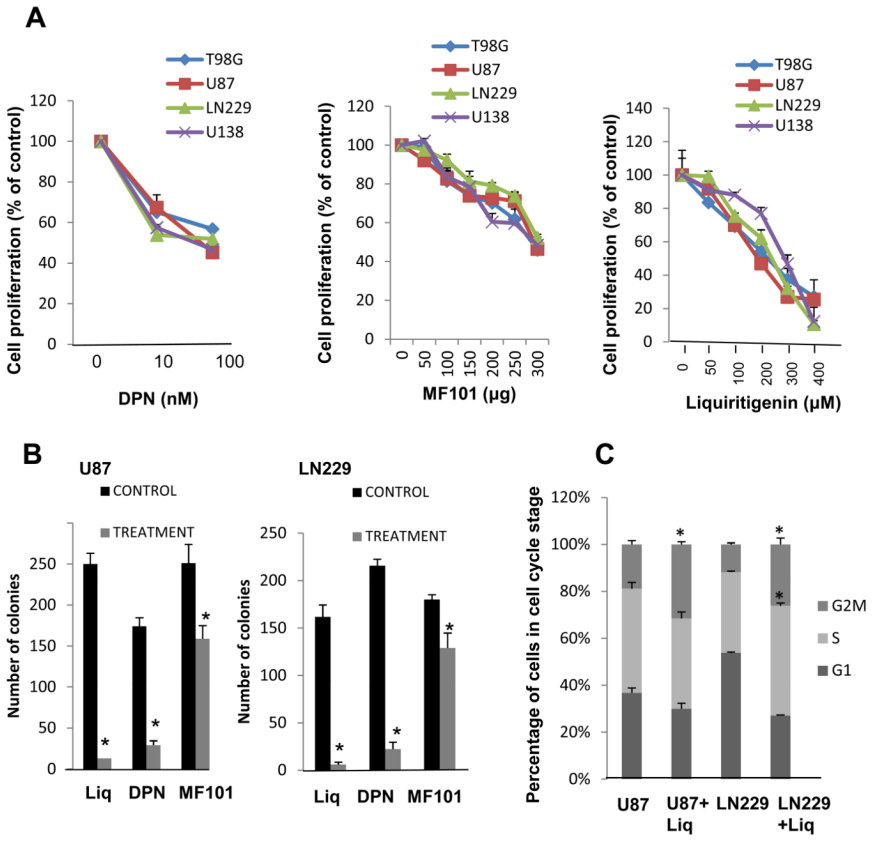

Fig. 3.

ERβ agonists inhibit the proliferation of glioma cell lines. A, T98G, U87, LN229, and U138 glioma model cells were treated with vehicle (0.1% DMSO) or indicated concentrations of DPN, MF101 and liquiritigenin for 72 h, and proliferation was measured using Cell Titer-Glo Luminescent Cell Viability Assay. B, U87 and LN229 cells were seeded in 6-well plates, and after 24 h the cells were treated with vehicle (0.1% DMSO) or DPN (1 μM), MF101 (250μg) and liquiritigenin (200 μM) for 72 h. After 7 days colonies were stained with crystal violet and colonies that contain ≥50 cells were counted. All data presented are the mean ± SEM. *, $p < 0.05$, *t* test. C, U87 and LN229 cells were treated with or without liquiritigenin (200 μM) and were subjected to flow cytometry. The percentage of cells in each cell cycle phase is shown in tabular form. All data presented are the mean of three experiments ± SEM. *, $p < 0.05$, *t* test.

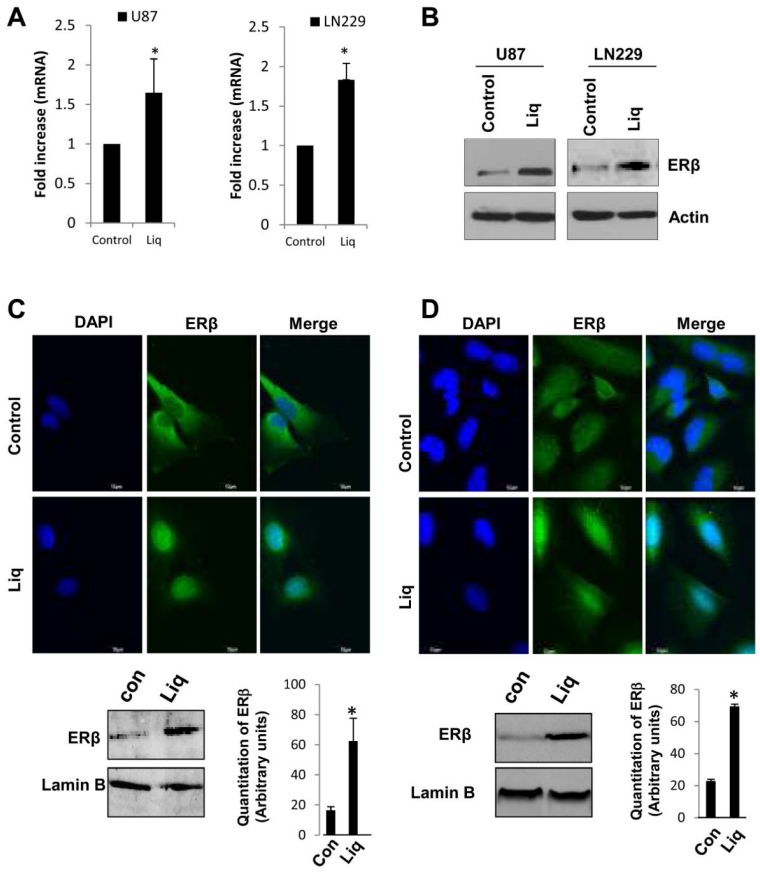

Fig. 4.

Liquiritigenin induced ERβ protein expression and nuclear translocation of ERβ. A, U87 and LN229 were treated with vehicle or liquiritigenin (100 μM) and expression of ERβ was measured by RT-qPCR. B, U87 and LN229 cells were seeded in 100-mm dishes and treated with vehicle (0.1% DMSO) or liquiritigenin (100 μM) for 24 h and ERβ protein expression was detected by Western blotting. β-actin was used as loading control. C, D, U87 and LN229 cells were seeded onto coverslips, treated with vehicle (0.1% DMSO) or liquiritigenin (100 μM) for 24 h. Cells were then fixed in 3.7 % paraformaldehyde and incubated with ERβ primary antibody and phalloidin staining (FITC conjugated from Molecular Probes) for 1 h at room temperature. Fluorescence was captured under Leica confocal microscope. DAPI was used to visualize the nuclei (upper panels). U87 and LN229 cells were treated with vehicle (0.1% DMSO) or liquiritigenin (100 μM) for 48 h, biochemical fractionation was performed to isolate nuclei and ERβ protein expression in the nuclear extracts was determined by Western analysis. Lamin B was used as an internal control. Band intensity was quantitated by densitometry and normalized to Lamin B (bottom panels). All data presented are the mean of two independent experiments ± SEM. *, $p < 0.05$, *t* test.

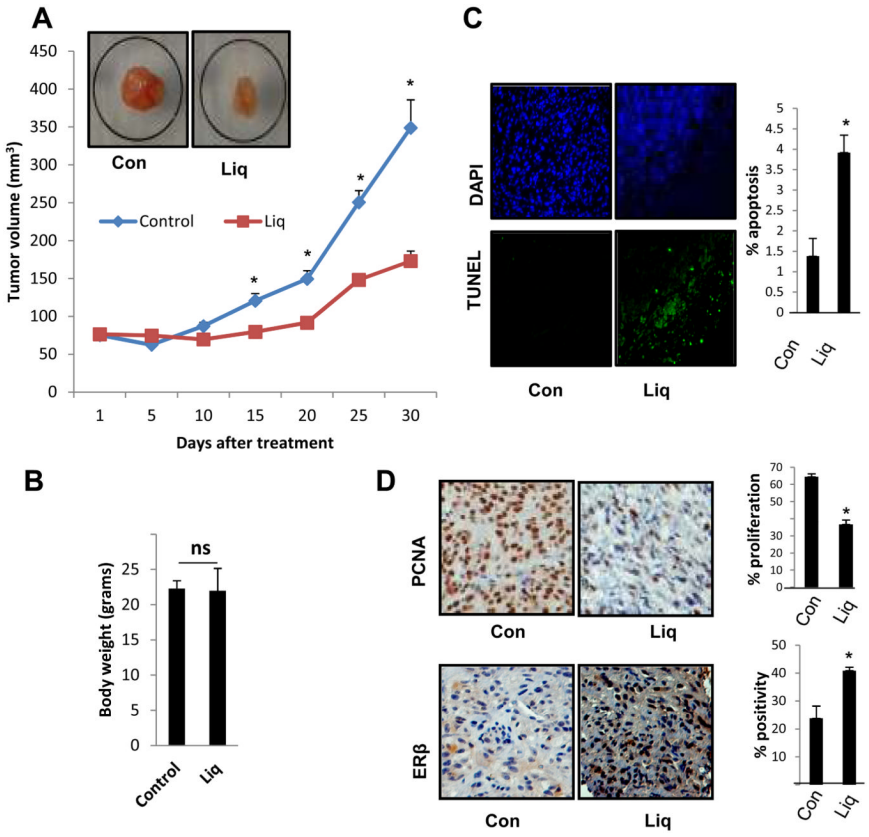

Fig. 5.

Liquiritigenin treatment reduced subcutaneous glioma xenograft tumor growth *in vivo*. A, nude mice were subcutaneously implanted with 1×10^6 U87 cells. After tumors reached measurable size, mice were treated daily with vehicle or liquiritigenin (20 mg/kg/bodyweight) for 30 days. Tumor size was measured with calipers for every 5 days. A representative picture of tumor is shown as an inset. B, Body weight of both vehicle- and liquiritigenin-treated mice was measured weekly. Column, mean body weights. C, TUNEL staining for apoptosis in control and liquiritigenin-treated tumors. Representative images are depicted (left panel). TUNEL labeling was quantified as the mean TUNEL labeling percentage based on at least 3 randomly selected high-power microscope fields per group (right panel). D, Quantitation of PCNA staining using the PCNA index is shown in upper panel. * $P < 0.05$. ERβ expression was analyzed by immunohistochemistry (IHC) in tumors treated with vehicle or liquiritigenin (lower panel); Quantitation was done as described in methods section, bars, SEM. *, $p < 0.05$.