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Abstract: Glioblastomas are a subtype of gliomas, which are the most aggressive and deadly form of brain tumours. The epidermal growth factor receptor (EGFR) is over-expressed and amplified in glioblastomas. Luteolin is a common bioflavonoid found in a variety of fruits and vegetables. The aim of the present study was to explore the molecular and biological effects of luteolin on EGF-induced cell proliferation and the potential of luteolin to induce apoptosis in glioblastoma cells. *In vitro* cell viability assays demonstrated that luteolin decreased cell proliferation in the presence or absence of EGF. Immunoblots revealed that luteolin decreased the protein expression levels of phosphorylated Akt, mTOR, p70S6K, and MAPK in the presence of EGF. Furthermore, our results revealed the ability of luteolin to induce caspase and PARP cleavages in glioblastoma cells in addition to promoting cell cycle arrest. Our results demonstrated that luteolin has an inhibitory effect on downstream signalling molecules activated by EGFR, particularly the Akt and MAPK signalling pathways, and provided a rationale for further clinical investigation into the use of luteolin as a therapeutic molecule in the management of glioblastoma.

Running title: Effects of luteolin on glioblastoma cell proliferation

Of all the cancers originating in the central nervous system, malignant gliomas are the most common and are highly invasive. Glioblastoma multiforme (GBM) is both the most biologically aggressive and common of these gliomas [1, 2]. Glioblastoma represents over 80% of malignant gliomas [3]. GBM presents with uncontrolled cellular proliferation, diffuse infiltration, resistance to apoptosis, genomic instability and a tendency for necrosis. Characteristically, GBM manifests with heterogeneity at the histologic and genetic levels, contributing even further to the complexity of the disease. Due to this complexity and aggressiveness, GBM offers a therapeutic challenge, and a diverse treatment approach is necessary. Currently, treatment involves surgical resection, radiation and chemotherapy. Still, even with diverse treatment approaches, prognosis remains poor. The median survival is generally less than two years from diagnosis. Thus, further treatment options to improve this prognosis are necessary [2, 4].

Epidermal growth factor receptor (EGFR) signalling plays an important role in many cancers including glioblastoma. EGFR, a member of the ErbB family of receptor tyrosine kinases (RTK), regulates the activation of the RTK/RAS/PI(3)K pathway. The activation of these pathways regulate key biological processes such as cell proliferation, angiogenesis, invasion and resistance to programmed cell death. Mutations such as the loss of phosphatase and tensin homologue (PTEN), an inhibitor of this pathway, are common in gliomas resulting in up-regulation of the pathway and resistance to EGFR therapies [5, 6]. In a genomic study of GBM, RTK alterations were found to occur in 67.3% of GBM while EGFR mutations were found in 57.4% of these tumours. Additionally, these EGFR mutations were associated with

significant elevations in total EGFR and its phosphorylation [7]. Due to the prevalence of these mutations, treatment options that can inhibit EGFR-mediated cell survival are needed.

Luteolin is a common flavonoid (Fig. 1), a class of secondary plant metabolites, found in a variety of fruits and vegetables. This class of compounds has demonstrated promising characteristics within the realm of cancer prevention and treatment. Flavonoids exhibit antioxidant properties, oestrogenic and anti-oestrogenic activity and antiproliferative ability. They have also been shown to induce detoxification enzymes, cell cycle arrest and the immune system [8, 9]. Additionally, some evidence points to a link between dietary flavonoids and reduced cancer risk [10]. Due to these properties, interest in these compounds as potential therapeutic agents for cancer treatment has continued to grow.

Along with its antioxidant and anti-inflammatory properties, luteolin has demonstrated anti-cancer effects in a variety of types of cancer through several mechanisms including inducing cell death, slowing cell proliferation and halting cell growth. Several studies have shown that luteolin down-regulates Akt phosphorylation in gastric, breast, brain and other cancer cell lines [11-16].

Although, the effects of luteolin have been reported in a number of cancer cell lines including GBM, detailed molecular studies of its effects on EGFR-mediated signalling in GBM cells are lacking. We hypothesized that luteolin could attenuate EGFR-mediated cell proliferation and signalling in GBM. Our findings indicated that luteolin decreases EGF-

induced cell proliferation and down-regulates downstream targets, such as phosphorylated MAPK and Akt, mediated by EGFR activation. In addition, we also observed that luteolin decreases the expression levels of Bcl-xL and induces both PARP and caspase cleavages.

Materials and Methods

Materials

Luteolin was obtained from Indofine Chemical Company (NJ, USA). Luteolin was dissolved in DMSO and then diluted with the growth medium to the final concentrations. The antibodies targeting phosphorylated ERK, mTOR, p70S6K, Akt, caspase and Bcl-xL were obtained from Cell Signaling Technologies (Danvers, MA, USA). Secondary antibodies were purchased from Jackson Immuno-Research Laboratory (West Grove, PA, USA). EGF, PARP and caspase antibodies were obtained from R and D Biosystems (Minneapolis, MN, USA). The caspase inhibitor, Ac-DEVD-CHO, was obtained from Cayman Chemical (Ann Arbor, MI, USA). MTT cell proliferation kits were obtained from Promega (Madison, WI, USA). 0.4% Trypan blue was purchased from Gibco Life Technologies (Grand Island, NY, USA). Propidium iodide, Hoechst 33342, Triton X-100, and RNAse A were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell Culture

Human glioblastoma cell lines U-87 MG and U-251 MG were obtained from Dr. Isa Hussaini, Department of Pathology, University of Virginia, Charlottesville, VA, USA. These cells were

routinely maintained and cultured in alpha-MEM supplemented with 10% foetal bovine serum (FBS) obtained from Biowest, (Riverside, MO, USA), and 1% penicillin and streptomycin (Fisher Scientific, Pittsburg, PA, USA). Cells were incubated at 37°C in a 5% humidified incubator.

Cell Transfection

Small interfering RNA (siRNA) were transfected into glioma cells as previously described by Amos *et al.* [18, 32]. The "Smart pool" siRNA targeting the EGFR and the control siRNA were purchased from Dharmacon (Lafayette, CO, USA). The targeting sequences to EGFR are as follows:

J-003114-10: CAAAGUGUGUAACGGAAUA

J-003114-11: CCAUAAAUGCUACGAAUAU

J-003114-12: GUAACAAGCUCACGCAGUU

J-003114-13: CAGAGGAUGUUCAAUAACU

The transfection of these smart pool sequences into glioma cells were done according to the manufacturer's protocol *for 48 hr. The cells were harvested and subjected to SDS page to detect the levels of EGFR*.

Cell Viability Studies

Glioblastoma cells were cultured at a density of 1×10^5 cells per well in 6-well plates, treated with various concentrations of luteolin (0-80 µM), and then incubated for one, two and three days. 100 µL of cell suspensions were obtained from these plates and counted using a hemocytometer after adding 100 µL of trypan blue dye. These experiments were performed in triplicate.

The growth inhibitory effects of luteolin were evaluated in U-87 MG and U-251 MG glioblastoma cell lines using the MTT assay. This assay evaluates the ability of cells to metabolize a yellow tetrazolium salt to a formazan product. The formation of the purple formazan can be detected using a spectrophotometer. GBM cells were seeded in a 96-well plate at a density of 5.0×10^3 cells in 100 µL of media. After a 24-hr incubation period, GBM cells were treated with various concentrations of luteolin (0- 80 µM) for 24, 48 and 72 hr. In another set of studies, GBM cells were treated with EGF (25 µg/mL) in the absence or presence of various concentrations of luteolin. Following treatments, 20 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well and cells were incubated for 4 hr at 37°C. After the 4-hr incubation period, the stop reagent was added to each well according to the manufacturer's protocol. Plates were then rocked for 30 min. in the dark at room temperature. Cellular proliferation was determined by measuring the optical density using Promega Glomax Multidetection System (Madison, WI, USA). The mean ± SEM for each experiment was calculated and plotted as a bar graph.

Cell Cycle Analysis by Flow Cytometry

U-87 MG cells were plated in 10-cm plates. Cells were serum starved and treated with varying concentrations of luteolin (0-80 μM) for 48 hr. After the 48-hr treatment, the floating cells were collected and adherent cells were trypsinized, pooled together and centrifuged at 800 x g. Cell pellets were washed three times with PBS, fixed in ice-cold 70% ethanol and stored at 4°C overnight. The fixed cells were centrifuged and washed three times with PBS, and stained with a solution consisting of PBS with 20 μg/mL of propidium iodide, 0.1% Triton X-100 and 200 μg/mL RNAse A. The cells were then kept in the dark for 1 hr. The stained cells were analysed using the BD FACScalibur equipped with CellQuest software. Cells were gated using the Cellquest software to estimate the proportion of cells distributed as a percentage in the different cell cycle compartments [17].

Apoptotic Cell Staining

This assay allows for the detection of cells undergoing apoptosis. In this assay, GBM cells were cultured in a 12-well plate. Cells from each cell line were seeded at a density of 5 x10^3 cells per well in 2 mL of media supplemented with 10% FBS. After 24 hr of incubation, the cells were treated with various concentrations of luteolin. After 48 hr, cells were washed three times with PBS and stained with 5 μg of Hoechst 33342 dye and 1 μg of propidium iodide dye. Plates were then left in the dark for 5 min. before photomicrographs of the cells were taken at a magnification of 10X under a fluorescent phase-contrast NIKON H600L Eclipse (Japan) microscope.

Western Blot Analysis

Western blot analyses were carried out as previously described by Amos *et al.* [18]. Cells were seeded in 6-well plates at a concentration of 1×10^5 cells/mL in a humidified atmosphere at 37°C for 24 hr. Following treatment with various concentrations of luteolin, cell lysates were prepared by lysing the cells in 1X lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1mM PMSF). Cell extracts were then spun at 13,000 rpm at 4°C for 10 min. Supernatants were then transferred to new tubes either for immediate analysis or storage at -80°C. Protein concentrations were quantified using a BCA™ Protein Assay Kit Thermo Fisher (Westminster, MD, USA). After protein quantification, the cell extracts were treated with sample buffer and boiled at 100°C for 5 min. Equivalent amounts of protein (20 μg) were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, gels were transferred onto nitrocellulose membranes. Nitrocellulose membranes were blocked in 1% Blotto (non-fat dry milk) for 1 hr followed by the addition of a diluted solution of the primary antibody against ERK1/2, Akt, mTOR or P70S6K. The membranes with the antibody solution were rocked at 4°C overnight. The membranes were then washed 3 times with PBST. Next, the membranes were treated with the appropriate peroxidase-conjugated secondary antibody (anti-mouse IgG or anti-rabbit IgG) for 1 hr. The membrane was washed three times with the wash buffer (PBST). Blots were then visualized using the enhanced chemiluminescence (ECL) reagents from Fisher Scientific (Milwaukee, WI, USA) as described by the manufacturer.

In sets of studies investigating the effects of PARP and caspase cleavage, glioma cell lines were treated with different concentrations of luteolin. Adherent and floating cells were pooled together and centrifuged at 800 x *g*. The cell pellet was extracted with RIPA buffer and sonicated. The cell lysate was then centrifuged at 14,000 x g for 10 min. at 4°C. Proteins (20 µg/lane) were separated by SDS-PAGE on 12% polyacrylamide gels, transferred onto nitrocellulose and then incubated with antibodies directed against PARP and caspase. Experiments were repeated three times for reproducibility. Densitometry and ImageQuant analysis were carried out using the Alpha Innotech FluorChem2 (Cell Biosciences, Santa Clara, CA, USA).

Statistical Analysis

Data are expressed as the mean ± SEM. All statistical analyses were performed with the GraphPad Prism 7 software using the one-way analysis of variance (ANOVA) followed by the Bonferroni test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effects of Luteolin on Cell Growth and Viability

To determine the effect of luteolin on glioblastoma cell growth and proliferation, GBM cell lines (U-87 MG and U-251 MG) were used. We examined the rate of growth inhibition in cells treated with luteolin at (0-80 µM) using the trypan blue exclusion assay. This assay is based on the principle that live cells possess intact cell membranes and will exclude trypan

blue, while dying cells possess compromised cell membranes and thus lack the ability to exclude the dye. We observed that both U-87 MG and U-251 MG treated with luteolin (0-80 μM) showed a dose- and time-dependent increase in trypan blue positivity (fig. 2A and 2B) compared with cells treated with control medium. Cell number was measured as a percentage of the control.

Furthermore, the viability of glioblastoma cells treated with luteolin was assessed using an MTT assay after treatment with luteolin (0-80 μM) for 24, 48 and 72 hr. This test assesses the ability of the cells to induce the metabolic conversion of a yellow tetrazolium salt to a formazan product. This conversion is considered as a measure of cell viability. Cells treated with luteolin (0-80 μM) alone for 24-72 hr showed a concentration and time-dependent decrease in cell proliferation (fig. 2C and 2D). In addition, we tested the possible inhibitory effects of luteolin on EGF-mediated increase in cell proliferation. In this study, GBM cells were pre-treated with varying concentrations of luteolin and then treated with EGF (25 ng/mL) for 24 hr. The increase or decrease in cell proliferation was measured, after the addition of the MTT dye, using a Promega Glomax Multidetection System spectrophotometer. Measurements were net changes from a baseline absorbance of 560 nm of control versus treated cells (fig. 2E and 2F).

Effects of Luteolin on the Signalling Cascade

We observed that luteolin induced a decrease in cell proliferation mediated by EGFR activation. Firstly, we established that treatment with EGF (25 ng/mL) induced a robust

phosphorylation of the EGFR (Y845). We observed that at a 60-min. time point, the phosphorylation was reduced (fig. 3A). Furthermore, we transfected GBM cells with the siRNA targeting EGFR. Our results revealed that siRNA-treated cells down-regulates the expression of EGFR compared with the control non-targeting siRNA. Our data in fig. 3B indicate that the siRNA targeting EGFR knocked down the expression of EGFR, while treatment with luteolin (40 µM) did not decrease the expression of EGFR. Similarly, the phosphorylation of EGFR (Y845) was down-regulated in the presence of the siRNA targeting EGFR (fig. 3C) and the silencing of the EGFR abrogated EGF-mediated phosphorylation of the EGFR (Y845; fig. 3D).

Thus, we decided to investigate the effects of luteolin on some downstream molecular targets mediated by EGFR activation such as MAPK, Akt, mTOR and S6K. We used western blot analyses to determine changes in the protein levels of these signalling molecules. To do this, U-87 MG and U-251 MG glioma cell lines were treated with various concentrations of luteolin as well as 25 ng/mL EGF. Our data demonstrated a decrease in p-Akt and pERK ½ in a dose-dependent manner, in the presence of EGF (fig. 4A and 4B). Additionally, the phosphorylated levels of p-mTOR, and p-p70S6K were also shown to decrease in a similar dose-dependent manner in the presence of EGF treatment (fig. 4C and 4D). This suggests that luteolin might be mediating decrease in cell proliferation and growth via attenuating key signalling molecules such as Akt and MAPK.

Effects of Luteolin on Apoptotic Signalling

Western blot analysis was used to assess the effects of various concentrations of luteolin on the apoptotic signalling cascade induced by luteolin in glioma cells. The BCL-2 family plays a vital role in regulating apoptosis and the activation of the caspase cascade leading to PARP cleavage. In this study, the protein expression levels of Bcl-xL, caspase-3 and PARP were determined. We observed an increase in the levels of cleaved caspase and PARP with increasing concentrations of luteolin (fig. 5A and 5B). Furthermore, our data in fig. 5C revealed that Bcl-xL, an anti-apoptotic protein, was decreased in a dose-dependent manner in both cell lines tested. To further explore the role of caspase-mediated PARP cleavage following luteolin treatment, cells were pre-treated with the caspase inhibitor Ac-DEVD-CHO and then treated with luteolin for 48 hr. Cell extracts were subjected to western blot analysis, and we observed that pre-treatment with the caspase inhibitor inhibited cleavage of both PARP and caspase (fig. 5D). Additionally, we further determined the biological relevance of the inhibition with the caspase inhibitor by measuring the cell number using the trypan blue exclusion assay. After treatment with both the caspase inhibitor and luteolin, we observed no statistical difference in the cell number (fig. 5E).

Detection of Apoptotic Cells and Cell Cycle Analysis

In order to further investigate the molecular mechanisms underlying the effects of luteolin in GBM cell cycle check points, we employed the technique of fluorescent activated cell sorting (FACS) to determine where in the cell cycle the cells are arrested. Analysis of the flow cytometric data revealed that luteolin potently increased the percentage of cells

arrested in the S and G2M cell cycle check points (fig. 6A). Our data showed that in the S phase (fig. 6A lower panel), 8.92% of control cells were arrested while cells treated with luteolin (10-80 μM) had an increase in arrested cells from 18.83% (10 μM luteolin) to 38.8% (80 μM luteolin). In the G2M phase (fig. 6A lower panel), there was an increase in arrested cells from 6.5% (10 μM) to 20.2% (80 μM). Additionally, we observed a concentration-dependent increase in propidium iodide staining of cells treated with luteolin (fig. 6B).

Discussion

Glioblastoma multiforme is the most lethal of the malignant gliomas. As of yet, few therapeutic treatments have been suggested for GBM due to its heterogeneity and resistance to chemotherapy and radiotherapy. As a result, the long-term prognosis for GBM is often poor. Luteolin is a common flavonoid found in several dietary sources including celery, red and green peppers, thyme, and a variety of other spices. Luteolin's anti-cancer activity has been established in a variety of cancer cell lines such as breast, gastric, prostate, osteosarcoma and leukaemia among others [11-12; 19-21]. Due to its previously established efficacy in several cancer cell lines, the authors of the present study sought to determine biological effects of luteolin EGFR-mediated signalling in glioblastoma multiforme U-87 and U-251 MG cells.

 Our data revealed that luteolin induced apoptosis, inhibited proliferation and attenuated the effects of EGFR activation in U-87 MG and U-251 MG cells. Glioblastoma cells often have mutations leading to PARP and EGFR over-expression, which causes apoptotic resistance and uncontrolled cell growth.

Akt regulates a number of several cell survival, growth and proliferation pathways. Additionally, modulations in the Akt pathway play a significant role in many types of cancers. Akt over-expression is common in cancer, thus making this protein an important target for cancer treatment [22]. We observed that luteolin also decreased p-Akt levels in these glioma cell lines. Additionally, mutations resulting in ERK over-expression are also common in cancers. The ERK signalling pathway, is known for its role in cell proliferation. It has also been shown to be involved in regulating other key biological processes, such as cellular differentiation and migration [23]. Our studies demonstrate the ability of luteolin to decrease p-ERK in cancer cell lines leading to decreased cell proliferation and growth. The combination of decreased MAPK and Akt could be responsible for the observed decreased in cell proliferation and survival [22].

Epidermal growth factor, a ligand of the EGFR is known to stimulate cell growth and proliferation. EGFR is over-expressed and amplified in glioblastoma as well as other types of cancers [1, 5]. As such, understanding the effects of luteolin on EGF activity provides a crucial look into the potential benefits of this compound. We found that luteolin inhibited cell growth and proliferation in the presence of cells pre-treated with EGF. Cells treated with luteolin were able to combat the effects of EGF, leading to more regulated growth and proliferation, and allowing for the activation of apoptosis. These observations agree with the findings of Lee *et al.* [12] and Hong *et al.* [24] which showed that luteolin decreased the EGFR mRNA in breast cancer via the inhibition of MAPK and suppression of the mTOR and Akt signalling pathways.

Luteolin has also demonstrated apoptotic effects by regulating and modifying the expression levels of proteins within the BCL-2 family. Because this family of proteins sets the threshold for apoptosis, a balance between various proteins within the family becomes important for cellular regulation and growth. In cancer, the pro-survival BCL-2 gene is often over-expressed. This prevents apoptosis, which aids the characteristics of uncontrolled growth and proliferation seen in cancer [25]. Our data showed that luteolin reduced expression of the pro-survival protein Bcl-xL similar to the findings by Wang *et al.* [20], Tsai *et al.* [26] and Chang *et al.* [27]. The BCL activates certain caspases, which play a key role in regulating apoptosis. As a family, caspase proteins provide tumour suppression. Much like with the BCL family, mutations within the genes coding for the caspase proteins are common in cancers. Through these mutations, tumour suppression is inhibited. Our data also demonstrated the potential of luteolin to increase activated caspases in two different GBM cell lines. Cleavage and activation of caspases indicates an increase in apoptosis [19, 28]. Certain caspases are also involved in the regulation of PARP, which acts as a major DNA repair mechanism. In certain mutations, this repair mechanism is intensified yielding resistance to apoptosis. Our results revealed that luteolin induced PARP cleavage similar to the findings of Lim *et al.* [29] and the use of a caspase inhibitor abrogated its potential to cause PARP cleavage. Luteolin has shown promise in increasing cleaved PARP in cancer cell lines. Cleaved PARP loses its DNA repair mechanism, without which cells more freely undergo apoptosis. Thus, PARP inhibitors are a promising and growing avenue for cancer therapy [30-31]. These data demonstrate luteolin's capacity to initiate the process of programmed cell death through the caspase family and inhibit pro-survival DNA repair through the modulation of PARP.

Collectively, our results demonstrate that luteolin decreases EGF-induced cell proliferation and some key signalling proteins such as Akt and MAPK. Luteolin also induces apoptosis as evidenced by cleaved PARP and caspase. Given luteolin's biological activity, this agent deserves to be further explored for the treatment of GBM.

Conflict of Interest

All authors declare no conflict of interest.

Authors' contributions

SA designed, performed and analysed some data and reviewed manuscripts.

DJ, RW, DA, RP, EH, RG, SL and AA performed experiments and analysed the data.

DA, TS and BD performed experiments and wrote the manuscript.

DJ and RG reviewed the manuscript.

References

1. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P et al. An integrated genomic analysis of human glioblastoma multiforme. Science 2008; 321:1807-12. doi: 10.1126/science.1164382.

2. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 2007; 21:2683-710.

3. Deangelis LM. Brain tumors. N Engl J Med 2001; 344(2):114-23.

4. Stupp R, Mason WP, Van den bent MJ, Weller M, Fisher B, Taphoorn MJ et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005; 352:987-96.

5. Padfield E, Ellis HP, Kurian KM. Current Therapeutic Advances Targeting EGFR and EGFRvIII in Glioblastoma. Front Oncol. 2015; 5:5.

6. Azuaje F, Tiemann K, Niclou SP. Therapeutic control and resistance of the EGFR-driven signaling network in glioblastoma. Cell Commun Signal. 2015; 13:23.

7. Brennan CW, Verhaak RG, Mckenna A, Campos B, Noushmehr H, Salama SR et al. The somatic genomic landscape of glioblastoma. Cell 2013; 155: 462-77.

8. Cheng WY, Chiao MT, Liang YJ, Yang YC, Shen CC, Yang CY. Luteolin inhibits migration of human glioblastoma U-87 MG and T98G cells through downregulation of Cdc42 expression and PI3K/AKT activity. Mol Biol Rep 2013; 40:5315-26.

9. Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Ther 2001; 90:157-77.

10. Neuhouser ML. Dietary flavonoids and cancer risk: evidence from human population studies. Nutr Cancer 2004; 50:1-7.

11. Ong CS, Zhou J, Ong CN, Shen HM. Luteolin induces G1 arrest in human nasopharyngeal carcinoma cells via the Akt-GSK-3β-Cyclin D1 pathway. Cancer Lett 2010; 298:167-75.

12. Lee EJ, Oh SY, Sung MK. Luteolin exerts anti-tumor activity through the suppression of epidermal growth factor receptor-mediated pathway in MDA-MB-231 ER-negative breast cancer cells. Food Chem Toxicol 2012; 50:4136-43.

13. Coleman DT, Bigelow R, Cardelli JA. Inhibition of fatty acid synthase by luteolin post-transcriptionally down-regulates c-Met expression independent of proteosomal/lysosomal degradation. Mol Cancer Ther 2009; 8:214-24.

14. Lamy S, Moldovan PL, Ben saad A, Annabi B. Biphasic effects of luteolin on interleukin-1β-induced cyclooxygenase-2 expression in glioblastoma cells. Biochim Biophys Acta 2015; 1853:126-35.

15. Sato Y, Sasaki N, Saito M, Endo N, Kugawa F, Ueno A. Luteolin attenuates Doxorubicin-induced cytotoxicity to mcf-7 human breast cancer cells. Biol Pharm Bull 2015; 38:703-9.

16. Lu J, Li G, He K, Jiang W, Xu C, Li Z et al. Luteolin exerts a marked antitumor effect in cMet-overexpressing patient-derived tumor xenograft models of gastric cancer. J Transl Med 2015; 13:42. doi: 10.1186/s12967-015-0398-z.

17. Feng X, Li L, Jiang H, Jiang K, Jin Y, Zheng J. Dihydroartemisinin potentiates the anticancer effect of cisplatin via mTOR inhibition in cisplatin-resistant ovarian cancer cells: involvement of apoptosis and autophagy. Biochem Biophys Res Commun 2014; 444:376-81. doi: 10.1016/j.bbrc.2014.01.053.

18. Amos S, Martin PM, Polar GA, Parsons SJ, Hussaini IM. Phorbol 12-myristate 13-acetate induces epidermal growth factor receptor transactivation via protein kinase Cdelta/c-Src pathways in glioblastoma cells. J Biol Chem 2005; 280:7729-38.

19. Cheng AC, Huang TC, Lai CS, Pan MH. Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells. Eur J Pharmacol 2005; 509:1-10.

20. Wang Y, Kong D, Wang X, Dong X, Tao Y, Gong H. Molecular mechanisms of luteolin induced growth inhibition and apoptosis of human osteosarcoma cells. Iran J Pharm Res 2015; 14: 531-8.

21. Chakrabarti M, Ray SK. Synergistic anti-tumor actions of luteolin and silibinin prevented cell migration and invasion and induced apoptosis in glioblastoma SNB19 cells and glioblastoma stem cells. Brain Res 2015; 1629:85-93. doi: 10.1016/j.brainres.2015.10.010.

22. Fresno vara JA, Casado E, De castro J, Cejas P, Belda-iniesta C, González-barón M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 2004; 30:193-204.

23. Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. J Cell Sci 2004; 117:4619-28.

24. Hong Z, Cao X, Li N, Zhang Y, Lan L, Zhou Y, Pan X, Shen L, Yin Z, Luo L. Luteolin is effective in the non-small cell lung cancer model with L858R/T790M EGF receptor mutation and erlotinib resistance. Br J Pharmacol 2014; 171:2842-53. doi: 10.1111/bph.12610.

25. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol 2014; 15:49-63.

26. Tsai Y, Chen H, Houng J, Lu K, Liang C, Lilang P et al. Luteolin inhibits proliferation of human glioblastoma cells via induction of cell cycle arrest and apoptosis. Journal of the Taiwan Institute Of Chemical Engineers 2013; 44:837-845.

27. Chang J, Hsu Y, Kuo P, Kuo Y, Chiang L, Lin C. Increase of Bax/ Bcl-XL ratio and arrest of cell cycle by luteolin in immortalized human hepatoma cell line. Life Sci 2005; 76:1883-93.

28. Horinaka M, Yoshida T, Shiraishi T, Nakata S, Wakada M, Sakai T. The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand. Mol Cancer Ther 2006; 5:945-51.

29. Lim DY, Jeong Y, Tyner AL, Park JH. Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin. Am J Physiol Gastrointest Liver Physiol 2007; 292: G66-75.

30. Shah GM, Robu M, Purohit NK, Rajawat J, Tentori L, Graziani G. PARP Inhibitors in Cancer Therapy: Magic Bullets but Moving Targets. Front Oncol 2013; 3:279. doi: 10.3389/fonc.2013.00279.

31. Tentori L, Ricci-Vitiani L, Muzi A, Ciccarone F, Pelacchi F, Calabrese R, et al. Pharmacological inhibition of poly(ADP-ribose) polymerase-1 modulates resistance of human glioblastoma stem cells to temozolomide. BMC Cancer 2014; 14:151. doi: 10.1186/1471-2407-14-151.

32. Amos S, Redpath GT, Dipierro CG, Carpenter JE, Hussaini IM. Epidermal growth factor receptor-mediated regulation of urokinase plasminogen activator expression and glioblastoma invasion via C-SRC/MAPK/AP-1 signaling pathways. J Neuropathol Exp Neurol. 2010 69:582-92. doi: 10.1097/NEN.0b013e3181e008fe.

Figure 1: Structure of Luteolin

Figure 2: Effects of luteolin (0-80 µM) on the cell growth of (A) U87 MG cells as measured by a trypan blue assay after 24-72 hr of treatment. (B) U251 MG cells as measured by a trypan blue assay after 24-72 hr of treatment (C). Effects of luteolin (0-80 µM) on the proliferation of U87 MG cells as measured by a MTT assay after 24-72 hr of treatment. (D) Effects of luteolin (0-80 µM) on the proliferation of U251 MG cells as measured by a MTT assay after 24-72 hr of treatment (E). Effects of luteolin (0-80 µM) on EGFR-mediated cell proliferation in U87 MG cells. (D). Effects of luteolin (0-80 µM) on EGFR-mediated cell proliferation in U251 MG. All data are expressed as the mean ± SEM of experiments performed in triplicate. *Denotes significant ($p < 0.05$) difference in proliferation of treated cells compared to untreated control cells.

Figure 3: (A). Western blot analyses of the effects of EGF (25 ng/mL) on EGFR phosphorylation (Y845) in both U87 MG and U251 MG cells. (B) Molecular silencing of EGFR using the siRNA targeting the EGFR, down-regulated the expression of EGFR. (C) Silencing of the EGFR abrogated EGF-induced phosphorylation of the EGFR (Y845).
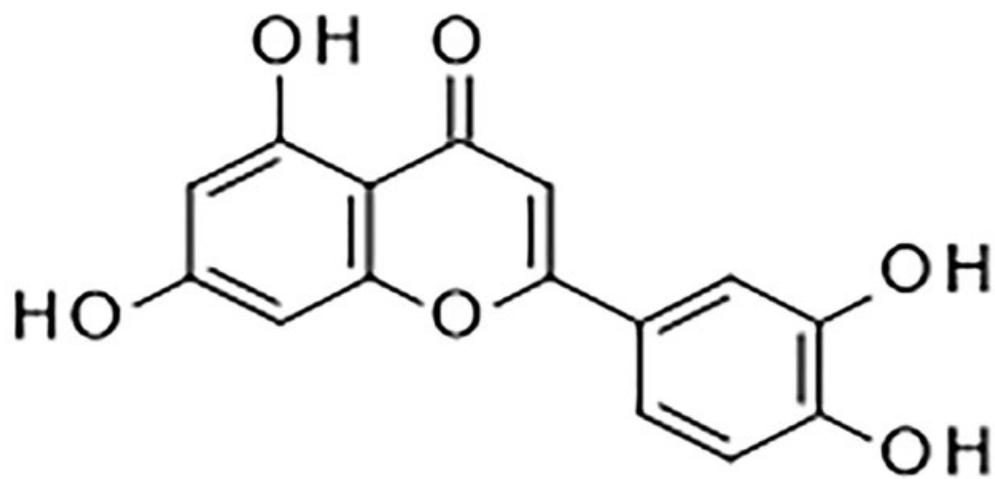
Figure 4: Western blot analyses of the effects of luteolin (0-80 µM) on the EGF-induced activation of phosphorylated Akt and phosphorylated ERK1/2 in (A) U87 MG and (B) U251 MG glioma cells. (C) Effects of luteolin (0-80 µM) on the EGF-induced activation of phosphorylated mTOR and phosphorylated p70S6K in U87 MG cells and (D) Effects of

luteolin (0-80 µM) on the EGF-induced activation of phosphorylated mTOR and phosphorylated p70S6K in U251 MG cells. All experiments performed in triplicate.

Figure 5: (A). Western blot analyses showing the effects of luteolin (0-80 µM) on the expression of cleavage of PARP in U251 MG and U87 MG cells. (B) Effects of luteolin (0-80 µM) on the expression of cleavage of caspase in U251 MG and U87 MG cells. (C) Effects of luteolin (0-80 µM) on the expression levels of bcl-xL in U251 MG and U87 MG cells. (D) Caspase inhibitor Ac-DEVD-CHO attenuates the effects of luteolin (0-80 µM) on the expression of cleavage of PARP and caspase cleavage in U251 MG and U87 MG cells. (E) Caspase inhibitor Ac-DEVD-CHO abrogates the decreased in cell viability induced by luteolin (0-80 µM) in U251 MG and U87 MG cells.

Figure 6: (A). Flow cytometry analyses for U87 MG cells treated with luteolin (0-80 µM). After 48 hr, the cells were stained with propidium iodide and subjected to flow cytometry to analyse the cell cycle distribution. Lower Panel: The percentage of cells arrested at the G0/G1, S and G2M cell cycle check points. (B) Effects of luteolin (0-80 µM) on the morphology of U251 MG cells stained with both propidium iodide and HOECHST 33342 (Magnification X10).

Figure 1

Structure of Luteolin

Figure 2.

Figure 3.

A.

EGF (25 ng/mL)

0   5   10   30   60   minutes

170 kDa-   p-EGFR (Y845)

170 kDa-   EGFR Total

U87 MG

EGF (25 ng/mL)

0   10   20   30   45   60   minutes

170 kDa-   p-EGFR

170 kDa-   EGFR TOTAL

U 251 MG

B.

Control	+	-	-	-	-
siRNA Control	-	+	-	-	-
siRNA EGFR (50 nM)	-	-	+	-	+
Luteolin (40 µM)	-	-	-	+	+

170 kDa-   EGFR TOTAL

50 kDa-   TUBULIN

U251 MG

C.

siRNA Control	+	-	-	-	-	-
siRNA EGFR (50 nM)	-	-	+	+	+	+
Luteolin (40 µM)	-	-	-	-	+	+
EGF (25 ng/mL)	-	+	-	+	-	+

170 kDa-   p-EGFR (Y 845)

50 kDa-   Tubulin

U251 MG

Figure 4

A.

LUTEOLIN (µM)   0   10   20   40   80
EGF (25 ng/mL) -   +   +   +   +   +

p-AKT (S473)

TOTAL AKT

U87 MG

LUTEOLIN (µM)   0   10   20   40   80
EGF (25 ng/mL) -   +   +   +   +   +

p-ERK 1/2

TOTAL ERK1/2

U87 MG

C.

LUTEOLIN (µM)   0   10 20 40 80
EGF (25 ng/mL) - + + + + +

pMTOR

Tubulin

LUTEOLIN (µM)   0   10 20 40 80
EGF (25 ng/mL) - + + + + +

pS6K

Tubulin

U87 MG

B.

LUTEOLIN (µM)   0   10   20   40   80
EGF (25 ng/mL) -   +   +   +   +   +

75 kDa-   p-AKT (S473)

50 kDa-   TUBULIN

LUTEOLIN (µM)   0   10   20   40   80
EGF (25 ng/mL) -   +   +   +   +   +

50 kDa-   p-ERK 1/2

50 kDa-   TUBULIN

U251 MG

D.

LUTEOLIN (µM)   0   10   20   40   80
EGF (25 ng/mL) -   +   +   +   +   +

p-mTOR

Tubulin

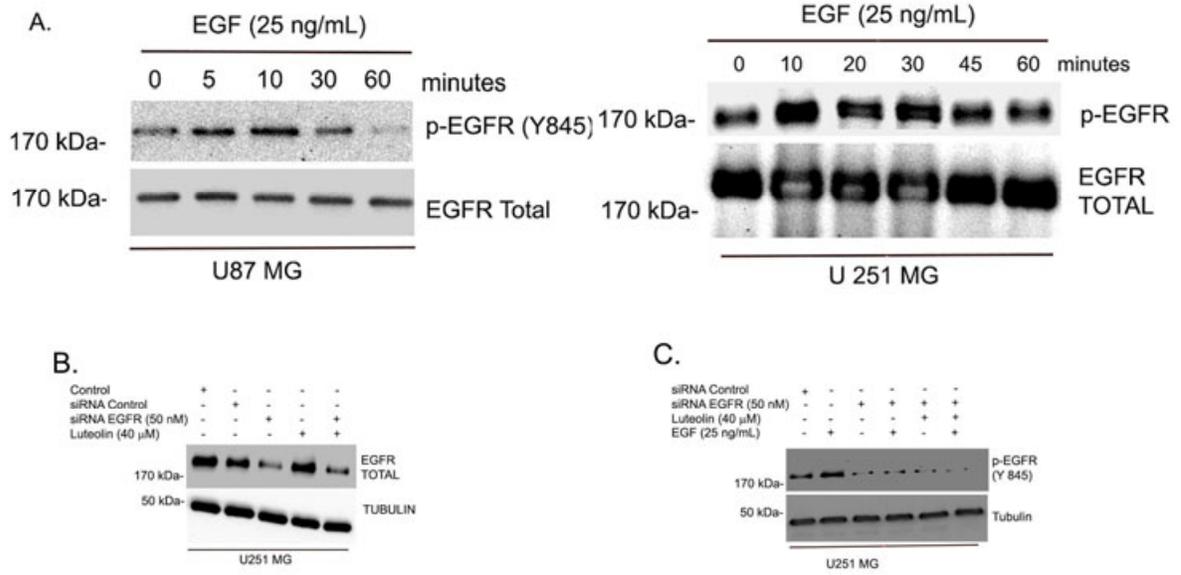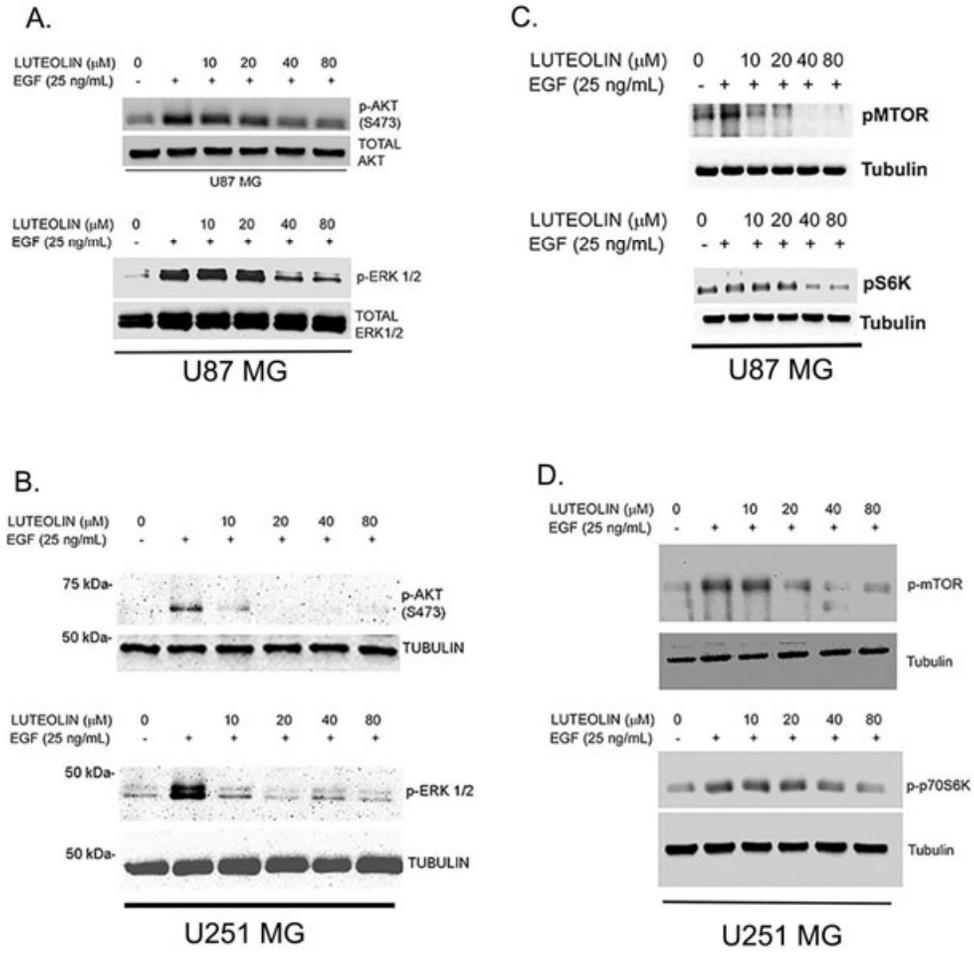LUTEOLIN (µM)   0   10   20   40   80
EGF (25 ng/mL) -   +   +   +   +   +

p-p70S6K

Tubulin

U251 MG

Figure 5.

Figure 6

Figure 6B

B.

| | HOESCHT 33332 | PROPIDIUM IODIDE | MERGE |

U 251 MG
Control

U 251 MG
Luteolin 20 μM

U 251 MG
Luteolin 40 μM

U 251 MG
Luteolin 80 μM