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Luteolin inhibits proliferation of human glioblastoma cells *via* induction of cell cycle arrest and apoptosis

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ABSTRACT

Glioblastoma multiforme (GBM) is a highly malignant brain tumor with limited therapeutic options, and high recurrence and mortality rates. In this study, the anti-tumorigenic properties of luteolin on GBM were examined *in vitro*. Luteolin was isolated from the whole plant of *Glossogyne tenuifolia* and the chemical structure was determined by its spectroscopic data. The inhibitory effect of luteolin on the proliferation of GBM 8401 and U87 human glioblastoma cells and the apoptotic effect of this compound on GBM 8401 cells were investigated. Luteolin inhibited the growth of GBM 8401 and U87 cells in a dose- and time-dependent manner. It significantly induced S and G_2/M phase cell cycle arrest and apoptotic cell death by decreasing the expression of cyclin-dependent kinase (CDK1), cyclin B1, and anti-apoptotic proteins (Bcl-2, Mcl-1). In addition, luteolin increased the expression of pro-apoptotic proteins (Bid, Bak, Bax, Bad) and activated caspase-3, with a concomitant increased in the levels of cleaved poly-ADP-ribose polymerase (PARP). Moreover, luteolin reduced the cell migration of GBM 8401 cells. Combination treatment of luteolin with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), an anticancer drug used in clinical GBM treatment, enhanced significantly the cytotoxic effect of BCNU in GBM 8401 cells. These results suggest that luteolin may be a potential candidate for GBM adjuvant therapy.
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1. Introduction

Glioblastoma multiforme (GBM, World Health Organization (WHO) grade IV) is one of the most common and aggressive malignant primary brain tumor in adults because of its high degree of cellularity, anaplasia, vascular proliferation, and necrosis. GBM causes significant neurological morbidity and mortality. For newly diagnosed patients with GBM the standard treatment is maximal surgical resection followed by radiotherapy (RT). Chemotherapy is often used during or after RT [1]. Despite advances in intensive multimodality treatment, including surgical resection, irradiation, and chemotherapy, the life expectancy of patients with GBM has remained on average 14 months [2]. Thus, there is a dire need for potentially curative therapies for this malignant brain tumor.

Apoptosis plays a pivotal role in cell development, maintenance of tissue homeostasis, and as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells [3]. Apoptosis is characterized by particular morphological changes, such as plasma membrane blebbing, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation [4]. The relationship between apoptosis and cancer has been a prominent area of research and focus in the scientific community recently. Apoptosis provides a number of useful pathways when generating effective therapies and many chemotherapeutic agents exert their anticancer effects by inducing apoptosis in cancer cells [3]. Therefore, induction of apoptosis has become a principal mechanism by which anticancer therapy is effective [5].

Glossogyne tenuifolia belongs to the Compositae family. The plant is distributed mainly in Southern Asia, Australia, New Caledonia, and in Penghu Island, which is a small island of Taiwan

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CDK, cyclin-dependent kinase; EA, ethyl acetate; FBS, fetal bovine serum; FITC, annexin-V-fluorescein isothiocyanate; GBM, Glioblastoma multiforme; PARP, poly-ADP-ribose polymerase; PBS, phosphate buffered saline; PI, propidium iodide; PVDF, polyvinylidene difluoride; RT, radiotherapy; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

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[6]. The whole plant of *G. tenuifolia* has been used in Taiwan to make daily drinks to prevent sunstroke and has been used as an antipyretic, hepatoprotective and anti-inflammatory remedy among local residents. Recent studies have demonstrated remarkable beneficial actions of *G. tenuifolia* through its antioxidant, anti-inflammatory and anticancer activities [7–9]. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a major active compound of *G. tenuifolia* [8] and it is also widely distributed in the plant kingdom [10,11]. The recommended human daily intake of luteolin is 16 mg *per* day [12]. In cellular studies, luteolin has been shown to have anti-tumorigenic, anti-inflammatory, anti-oxidant, and radical scavenger properties [13,14]. *In vitro* studies have reported that luteolin induces apoptosis in a variety of cell culture models [15–17]. However, the anticancer activities of luteolin in a human GBM cell line have not been fully elucidated.

This study investigated how luteolin affects cell growth, apoptosis and cell migration of human glioblastoma cells. The mechanism of luteolin on cell apoptosis was elucidated by analyzing expressions of apoptosis-related molecules, including caspase-3 and Bcl-2 family proteins. Finally, the adjuvant effect of luteolin on the chemotherapy by BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), which is an anticancer drug used in clinical GBM treatment, was also examined.

2. Materials and methods

2.1. Separation of luteolin

The raw materials of *G. tenuifolia* were bought from a local herb store in Penghu Island (Penghu County, Taiwan) and were deposited in the Herbarium of I-Shou University (Kaohsiung City, Taiwan). Dry whole plant materials of *G. tenuifolia* (5.3 kg) were crushed and drenched in 20 L ethyl alcohol for one day and, then, extracted three times with 20 L ethyl alcohol. After filtration by medicinal gauze, the filtrates were collected and concentrated with a vacuum evaporator. The weight of this ethanolic crude extract was 777 g, and the yield was about 14.7%. The crude extract (400 g) was further extracted by ethyl acetate (EA). The obtained dry EA extract was 71.9 g (yield = 18.0% from the crude extract). The EA extract (61.5 g) was further separated by column chromatography (760 mm length, 120 mm diameter, packed with 2.5 kg silica gel (Si-60, 40–63 μm, Merck Co.)). The samples were eluted sequentially with 1.5 L gradient of *n*-hexane/EA (from 90:10, 80:20–0:100) and followed by methanol up to 100%. After the operation, 15 fractions were collected. Luteolin was obtained from Fractions 8, 9 and 10, and its dry weight in these fractions was 2.31 g, 1.01 g and 1.13 g, respectively. Thus, the total quantity of luteolin was 4.45 g, and the yield was 7.24%.

2.2. High performance liquid chromatography (HPLC) analysis for luteolin

The purity of luteolin was determined by HPLC (L-7100, Hitachi, Tokyo, Japan). The sample was dissolved in methanol and filtered with a 0.22 μm filter. The diluted sample was analyzed by an Ascentis™ C18 column (No. 581325-U, 5 μm, 250 × 4.6 mm; Supelco, Bellefonte, PA, USA). The solution of methanol/0.05% acetic acid in water (40:60, v/v) was used as the mobile phase. The flow rate was 1.0 mL/min, and the sample injection size was 10 μL. The detection was carried out at 350 nm. The residence time of luteolin was 13.3 min.

2.3. Cell culture

The human glioblastoma cell lines GBM 8401 and U87 were obtained from Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biologicol, Israel), 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.25 μg/mL amphotericin (Biologicol, Israel). The cells were grown in 75 cm^2 tissue culture flasks in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Cell viability assay

The cell viability of GBM 8401 and U87 cells was evaluated by using the colorimetric WST-1 regent (Roche, Germany). The assay is based on the ability of viable cells to cleave the sulfonated tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases. Briefly, cells (4×10^3) were plated in 96-well plates and treated with the indicated concentration of luteolin (0, 17.5, 35, 52.5 μM) for 24, 48, and 72 h. To examine the adjuvant effect of luteolin in conjunction with an anticancer drug, we treated cells with either BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) alone or in combination with luteolin in 1% ethyl alcohol for 48 h. At the end of treatment, 100 μL of the WST-1 reagent solution (10% in culture medium) was added to each well for an additional 4 h incubation period. The plate was read in an Enzyme-Linked Immuno-Sorbent Assay (ELISA) reader at 480 nm. The WST-1 solution with culture medium acted as a blank while the ethyl alcohol (1%)-treated cells served as a control indicative of 100% survival.

2.5. Cell cycle analysis

Cells were seeded in 12 well plates and treated with luteolin at various concentrations for 48 h and alcohol (1%)-treated cells served as a control. After treatment, the media was discarded and adherent cells were washed with phosphate buffered saline (PBS). Next, 250 μL trypsin was added to cells for 5 min at room temperature to detach the cells. After centrifugation at 3500 rpm for 5 min at 4 °C, the cell pellet was resuspended with 1 mL of cold 80% ethanol at −20 °C. For analysis preparation, cells were washed again in PBS. Finally, 500 μL of propidium iodide (PI) staining solution were added to each sample. The samples were analyzed by flow cytometry (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) and the data were consequently calculated using WinMDI 2.9 software (TSRI, La Jolla, CA, USA).

2.6. Western blotting analysis

Cells were seeded in dish and incubated with various concentrations of luteolin in a humidified atmosphere (37 °C with 5% CO_2) for 48 h. Each sample (1×10^6 cells) was counted, harvested, washed with PBS and 50 μL of cell lysis buffer was added to each sample for 30 min on ice. Next, the pellet cellular debris was removed by centrifugation at 12,500 rpm for 30 min and the supernatants were then either analyzed immediately or stored at −80 °C. Protein concentrations were measured by BCA™ Protein Assay Kit (Pierce, Rockford, IL). Each sample was normalized in a final concentration of 30 μg/μL. Equivalent amounts of protein (30 μg) from total cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using precast 10% bis-tris gradient gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in blocking buffer (nonfat dried milk 5% (v/v), NaCl 150 mmol/L, Tris (pH 7.6) 25 mmol/L and 0.05% Tween 20 (v/v)) overnight at 4 °C to reduce nonspecific binding. Proteins levels were detected by incubation with various antibodies at appropriate dilutions in blocking buffer overnight at 4 °C. Unbound antibody was removed by washing with Tris-buffered saline (pH

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