Abstract. Temozolomide (TMZ) is an alkylating agent commonly used as a first-line treatment for high-grade glioblastoma. However, TMZ has short half-life and frequently induces tumor resistance, which can limit its therapeutic efficiency. In the present study, it was hypothesized that combined treatment with TMZ and acteoside has synergistic effects in glioblastoma therapy. Using cell viability and wound-healing assays, it was determined that this treatment regimen reduced cell viability and migration to a greater extent than either TMZ or acteoside alone. Following previous reports that TMZ affected autophagy in glioma cells, the present study examined the effects of TMZ + acteoside combination treatment on apoptosis and autophagy. The TMZ + acteoside combination treatment increased the cleavage of caspase-3 and levels of B-cell lymphoma 2 (Bcl-2)-associated X protein and phosphorylated p53, and decreased the level of Bcl-2. The combination treatment increased microtubule-associated protein 1 light chain 3 and apoptosis-related gene expression. It was also determined that TMZ + acteoside induced apoptosis and autophagy through the mitogen-activated protein kinase signaling pathway. These findings suggest that acteoside has beneficial effects on TMZ-based glioblastoma therapy.

Introduction

Glioblastoma is the most common type of malignant primary brain tumor and the most invasive and devastating primary brain tumor (1,2), with a median survival rate of ~18 months with aggressive multimodality therapy. Current treatment decisions are limited and include radiation, chemotherapy and surgery in combination with the alkylating agent temozolomide (TMZ) (3,4). Despite the availability of aggressive therapies, patients with glioblastoma generally have a poor prognosis (5). TMZ is the major chemotherapeutic drug used in the clinical treatment of malignant glioblastoma (6-8); as an alkylating agent in the imidazotetrazine series, it is able to penetrate the blood-brain barrier (9-11). During malignant glioblastoma progression and extensive invasion throughout the brain, steadily increasing drug resistance to TMZ decreases its therapeutic efficacy (12,13). Accordingly, novel therapeutic drugs to counter glioblastoma are urgently sought.

Previous studies have reported that glioma cells undergo cell death via autophagy, or type II programmed cell death, in response to TMZ (14,15). Autophagy can be inhibited by 3-methyladenine (3-MA) through the conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II, thus reducing glioblastoma cell death (16,17); however, the role of autophagy is dependent on the cellular context. With the exception of a cytotoxic role during TMZ action, the induction of autophagy by cellular stress is a cytoprotective process that eliminates stress-induced cytoplasmic aggregates, organelles and macromolecules in mammalian cells through the lysosomal system. In turn, cells involved in maintaining homeostasis receive energy through these catabolic processes (18,19). The complex roles of autophagy suggest that an autophagy activator may have anticancer effects in combination with TMZ treatment by inducing glioblastoma cell autophagy without...
exerting deleterious effects or providing benefits to normal tissues. Of note, TMZ has exhibited synergistic therapeutic effects in combination with vitamin D, including suppressed cell viability and enhanced autophagy in glioblastoma cells. Furthermore, the combination of TMZ and vitamin D has been shown to exert anticancer effects in vivo, including reduced tumor size and prolonged survival rate. These studies suggest that combination treatment may have synergistic anticancer effects via autophagic mechanisms in TMZ-based glioblastoma therapy.

Acteoside is a phenylethanoid glycoside that is widely distributed in several tonified traditional Chinese herbal medicines (20,21). A number of pharmacological actions, including antioxidant, anticancer, anti-inflammatory, antinephritic and antimitastatic actions, have been associated with acteoside (22-24). Previous studies have demonstrated that acteoside has protective effects against carbon tetrachloride- and D-galactosamine-induced liver injury. The mechanisms underlying the protective effects of acteoside are likely related to its capacity to inhibit P450-mediated bioactivation and free radical scavenging effects induced by carbon tetrachloride exposure (25,26).

Therefore, evidence suggests that both acteoside and TMZ induce anticancer effects through cell death. However, their association and anticancer effects in the context of glioblastoma remain to be elucidated. Therefore, the objective of the present study was to verify the synergistic anticancer effects of acteoside combined with TMZ in glioblastoma therapy. A cell viability assay was performed to analyze the anticancer effects of the combination treatment in C6 glioblastoma cells (a rat glioblastoma cell line), and the results were compared with those following treatment with TMZ alone. To further examine the mechanism of cell death caused by cotreatment with acteoside and TMZ, apoptosis- and autophagy-related genes in C6 cells were examined.

Materials and methods

Cell culture. The C6 rat glioblastoma cell line was purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured under sterile conditions at 37°C in a humid environment with 5% of CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% antibiotics and antymycotics (all Welgene, Daegu, Korea).

Plant material. Abeliiophyllum distichum Nakai [voucher no. Park1001(AHNH)] was collected in Misun-hyang Theme park, Seongbul-Mountain Recreation Forest, 78, Chungmin-rojgok-gil, Goesan-eup, Goesan-gun, Chungcheongbuk-do, Korea.

Callus induction. To induce callus formation (27), 1-cm² leaf explants were isolated from the fresh plants. The explant was cultured on Murachige and Skoog medium (4% sucrose, 0.9% agar supplemented with 1 mg/l naphthalene acetic acid and 1 mg/l 2,4-dichlorophenoxyacetic acid, pH 5.7) at 25°C. The callus was induced 20 days later. A sufficient quantity of acteoside was obtained through subculture to separate and purify acteoside from the callus (Fig. 1). Different batches of purified acteoside were used in each experiment. Each experiment was performed three times using a different batch.

Isolation and purification. The ethylacetate fractions were concentrated in vacuo and used as a sample for the purification of acteoside. Acteoside was isolated and purified by the Accelerated Chromatographic Isolation system (Isolera™ Spektro, Biotage, Uppsala, Sweden) using SNAP KPHOSPHO-SIL and SNAP Ultra Cartridges (Biotage). The acteoside purified from the callus was quantitatively analyzed using the standard acteoside by HPLC-PDA analysis. Finally, acteoside of ≥95% purity was obtained from the callus and used as a sample for chemotherapy of temozolomide-based glioblastoma.

3-(4,5-Dimethylthiazol-2-yl)–5-diphenyltetrazolium bromide (MTT) assay. To identify the TMZ half maximal inhibitory concentration (IC₅₀ value) against C6 cells, 1×10⁶ cells in single cell suspensions were seeded into individual wells of 96-well plates and incubated for 24 h at 37°C prior to TMZ treatment at the indicated concentrations (1, 5, 10, and 20 mM) at 37°C for 24 h. Following determination of the TMZ IC₅₀ value as 5 mM, the cells were treated for 24 h with 5 mM TMZ, 50 µM acteoside, or a combination of the two (5 mM TMZ and 50 µM acteoside). MTT solution (5 mg/ml) was added to each well (10 µl) and cultured at 37°C for 2 h. Subsequently, the medium was removed and DMSO was added at a volume of 200 µl each and reacted at room temperature for 30 min. The absorbance at 595 nm was measured to determine cell viability.

Wound-healing assay. A C6 cell suspension in 1 ml was cultured in a 12-well plate and grown to confluence. The cells were treated with TMZ, acteoside or TMZ + acteoside and then scratched with a sterile 10-µl pipette tip to create an artificial wound. At 0 and 24 h post-wounding, digital images of the wound healing process were captured using an inverted microscope. Cell migration was quantified by measuring the size of the scar at 0 and 24 h using the image analyzing software, ImageJ 1.43u/Java1.6.0_22 software (NIH, Bethesda, Maryland, USA). Each experiment was performed three times and the measurements were performed in triplicate.

Immunoblotting. The C6 cells were treated with TMZ, acteoside or TMZ + acteoside for 24 h. Cells were lysed on ice by the RIPA (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) lysis buffer supplemented with protease and phosphatase inhibitors cocktail (Roche, Basel, Switzerland) for 30 min. After centrifugation at 18,341 x g for 15 min at 4°C, supernatant was obtained. Protein concentration was determined using Bradford Protein Assays (Bio-rad, Hercules, CA, USA). Equal amounts of total proteins (30 µg) were loaded into single wells and fractionated via electrophoresis via a 10-15% SDS-PAGE. The proteins were electrotransferred to polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). Following incubation in blocking solution containing 5% nonfat milk in Tween/Tris-buffer saline for 30 min at room temperature. The blots were probed with 1:1,000-diluted primary antibodies (cat. no. 9662, caspase 3; Cell Signaling Technology, Inc., Danvers, MA, USA), B0-cell lymphoma 2 (sc-7382, Bcl-2),
Bcl-2-associated X protein (cat. no. 2772, Bax), phosphorylated (p)−p53 (sc-101762), total−p53 (sc-6243), β-actin (cat. no. 4970; all; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), LC-3 (L8918, Sigma; Merck KGaA, Darmstadt, Germany), Rab7 (cat. no. 9367, Cell Signaling Technology, Inc.), p62 (cat. no. 114), p−p38 (cat. no. 9211), total−p38 (cat. no. 9212), p-c-Jun N-terminal kinase (cat. no. 9251, p-JNK), total-JNK (cat. no. 9252), p-extracellular signal-regulated kinase (cat. no. 9101, p-ERK), total-ERK (cat. no. 9102; all Cell Signaling Technology, Inc.) overnight at 4°C. This was followed by incubation with horse-radish peroxidase-conjugated secondary antibodies (1:2,000; LF-SA8001A, Goat Anti-Mouse IgG-HRP) or LF-SA8002A (Goat Anti-Rabbit IgG-HRP), AB Frontier, Seoul, Korea) for 2 h at room temperature. The proteins were then visualized by exposure to Chemi-Doc (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Immunofluorescence staining.** Lysosomal-associated membrane protein 1 (LAMP1) and LC3 are markers for lysosomal and autophagy. The cells (1x10⁶ cells/well) were prepared on sterilized glass coverslips (BD Biosciences, Franklin Lakes, NJ, USA) in triplicate. Following treatment with TMZ, acteoside or TMZ + acteoside for 24 h, the cells were fixed in 4% paraformaldehyde for 30 min, blocked with 5% normal chicken serum (S-3000; Vector Laboratories, Inc., Burlingame, CA, USA), 0.1% Triton X-100 and then incubated 1 h for permeabilization and to block non-specific protein-protein interactions. The cells were then incubated with the anti-LAMP1 (1:200; sc-19992, Santa Cruz Biotechnology, Inc.) and anti-LC3 (1:400; PM036, MBL International, Woburn, MA, USA) overnight at 4°C. The cells were then washed twice with PBS and incubated for an additional 2 h in the dark with a mixture of Alexa Fluor 488 and 594 secondary antibodies (1:200; Alexa Fluor 488 (A-11008) and 594 (A-11007), Thermo Fisher Scientific, Inc., Waltham, MA, USA) and washed again with PBS. The nuclei were stained using 4,6-diamidino-2-phenylindole (Sigma; Merck KGaA), and then washed twice with PBS. The slides were then mounted and images were captured under an LSM-700 laser confocal microscope.

**Flow cytometry.** The cells were analyzed for Mitotracker Green and MitoSOX by flow cytometry using a FACSCanto II flow cytometer, as indicated by the manufacturer (BD Biosciences). Following two washes with PBS, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.25% Triton X-100 in PBS for 20 min. The cells were stained with primary antibodies for overnight at 4°C (1:200) and then with secondary antibodies for 1 h on ice. Following two washes with PBS, the cells were fixed in 4% paraformaldehyde and assayed immediately. The flow cytometry data were collected using 10,000 cells and were analyzed using FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR, USA).

**Statistical analysis.** All data were analyzed using GraphPad Prism 5.0 and are presented as the mean ± standard error of the mean. Data were analyzed with one-way analysis of variance with Tukey’s multiple-comparisons post hoc test for the comparison of mean values among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Cotreatment with TMZ and acteoside suppresses glioblastoma cell proliferation and migration.** Combined treatment with TMZ and acteoside inhibited the viability of C6 cells. TMZ reduced cell viability in a dose-dependent manner (Fig. 2A), whereas acteoside alone had no significant effect at any treatment level (Fig. 2B). Following incubation in culture medium containing TMZ with or without acteoside for 24 h, an MTT assay was performed to compare the cytotoxicities of different treatment levels. TMZ significantly suppressed cell viability, whereas acteoside did not significantly suppress cell growth. Combined treatment with TMZ and acteoside markedly reduced cell viability (Fig. 2C). Treatment with TMZ or acteoside applied alone reduced wound-healing ability (Fig. 3A). The wound distance (%) was measured using the results shown in Fig. 3A with ImageJ software. The wound distance increased significantly following combination treatment (Fig. 3B), compared with either individual treatment. These results indicate that cotreatment with TMZ and acteoside was an effective inhibitor of glioblastoma cell proliferation and migration.

**Acteoside and TMZ affect apoptosis synergistically in C6 cells.** The results of the western blot analysis revealed that the levels of cleaved caspase-3, Bax and phosphorylated p53 were higher and the levels of Bcl-2 were lower following combination treatment with TMZ and acteoside than following treatment with TMZ alone (Fig. 4A). Acteoside-mediated mitochondrial function and the generation of reactive oxygen species (ROS), which are key mediators of apoptotic signaling, were then observed in the TMZ-treated C6 cells. To verify mitochondrial mass, fluorescence-activated cell sorting analysis was performed using MitoTracker Green. Cotreatment with TMZ and acteoside resulted in a higher mitochondrial mass than treatment with TMZ alone (Fig. 4B). ROS generation was significantly higher following cotreatment with TMZ and acteoside than treatment with TMZ alone (Fig. 4C). These results suggest that ROS generation and mitochondrial function are important in apoptosis induced by treatment with TMZ + acteoside.

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**Figure 1.** Chemical structure of acteoside.
Cotreatment with TMZ and acteoside induces autophagy in C6 cells. TMZ has been reported to induce autophagy (28,29); therefore, the present study examined the extent to which autophagy was induced by TMZ + acteoside treatment. The C6 cells were treated with TMZ with or without acteoside; after 24 h, and the cells were stained for immunofluorescence to detect LAMP1 and LC3 as markers of autophagy. Higher expression levels of LAMP1 and LC3 were observed following the combination treatment (Fig. 5A). Autophagy-related protein expression was also examined, and it was found that TMZ induced the conversion of LC3-I to LC3-II, which is a signature of autophagosome generation. A higher rate of conversion of LC3-I to LC3-II was observed following cotreatment with TMZ and acteoside than treatment with TMZ alone. The expression levels of Rab7 and p62 indicated autophagy induction in the TMZ-treated cells (Fig. 5B). These findings suggest that cotreatment with TMZ and acteoside enhanced the autophagic process in glioblastoma cells.

Acteoside induces MAPK pathway gene expression in TMZ-based treatment. Previous studies have demonstrated that TMZ regulates the MAPK pathway, including p38, JNK and ERK (30). Therefore, the present study investigated whether acteoside affects TMZ-related MAPK gene expression. The C6
cells were treated with TMZ with or without acteoside. After 24 h, TMZ treatment resulted in higher expression levels of p-p38, p-JNK and p-ERK. The expression of TMZ-regulated genes was significantly higher following TMZ + acteoside treatment than treatment with TMZ alone (Fig. 6). These observations suggest that acteoside affects TMZ-based therapeutic mechanisms via the MAPK pathway.

Discussion

The results of the present study establish that the combined treatment with TMZ with acteoside offers therapeutic potential for glioblastoma treatment, and provide evidence that chemosensitization to a combination of TMZ and acteoside can occur through autophagy enhancement. As the mutation of glioblastoma cells can lead to apoptotic pathway inactivation, the induction of autophagy by TMZ + acteoside cotreatment may represent an alternative method for glioblastoma therapy. However, whether autophagy is the sole mechanism for glioblastoma therapy with TMZ + acteoside cotreatment remains to be elucidated.

Autophagy is an essential cellular mechanism for the degradation of proteins and cytoplasmic organelles. The catabolic advantage of induced autophagy may be important in stressful conditions; therefore, the induction of autophagy may be an adaptive mechanism for cell death prevention (31-33). Previous studies have reported that reduced autophagy is correlated with cancer (34-36). In addition, several proteins and signaling pathways associated with autophagy are deregulated during malignant transformation, resulting in a reduction in autophagic activity. High expression levels of LC3 have also been associated with increased...
survival rates in patients with glioblastoma with poor performance scores (37,38). Similarly, the results of the present study indicate that the restoration of normal autophagy may be a latent strategy for glioblastoma therapy, and may serve as a mechanism for the restriction of abnormal tumor cell growth.

In normal autophagy, particular cytoplasmic components are isolated within autophagosomes which then fuse with a lysosome to be degraded and recycled (39,40). When autophagy is upregulated, autophagosome formation rates surpass lysosomal degradation rates; this condition is termed autophagic stress. If stress or abnormal autophagy continues,
cell death can occur via energy depletion or changes in the beclin-1/Bcl-2 balance. Apoptosis can also be triggered by autophagosome hyperactivity, engulfing cytoplasmic organelles including the mitochondria or endoplasmic reticulum (41). It has been suggested that general autophagy can induce cell death during normal cytoplasmic and organelle turnover in healthy cells. In this process, the cell 'cannibalizes' itself from the inside, a key characteristic of type II programmed cell death. Although the mechanisms by which acteoside enhances the therapeutic effect of TMZ-based glioblastoma therapy remain to be fully elucidated, the anticancer effects exhibited by the combination treatment examined in the present study may be associated with these autophagic mechanisms.

Acteoside is a phenyl-ethanoid glycoside derived from plants (22,26). Previous studies have reported on the various biological activities of acteoside. The changes in the expression levels of cleaved caspase-3 and LC3 in the present study demonstrate that treatment with a combination of TMZ and acteoside induced apoptosis and autophagy in C6 cells (Figs. 4 and 5). The combination treatment was shown to have a synergistic effect on glioblastoma cells. Although other possible mechanisms of these synergistic effects remain to be elucidated, the present study identified the induction of autophagy as a crucial tumoricidal mechanism of acteoside chemosensitization during TMZ-based glioblastoma therapy.

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Authors' contributions
TWH and JJK drafted the manuscript and performed the experiments. TWH, DHK, DBK, TWJ, and GHK performed the experiments and data interpretation. KAY contributed critical suggestions for the manuscript, and reviewed and revised the manuscript. All authors read and approved the final manuscript.

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

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