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Original Article

Honokiol enhances temozolomide-induced apoptotic insults to malignant glioma cells via an intrinsic mitochondrion-dependent pathway

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ABSTRACT

Background: Temozolomide (TMZ) is a first-line chemotherapeutic drug for malignant gliomas. Nonetheless, TMZ-induced side effects and drug resistance remain challenges. Our previous study showed the suppressive effects of honokiol on growth of gliomas.
Purpose: This study was further aimed to evaluate if honokiol could enhance TMZ-induced insults toward malignant glioma cells and its possible mechanisms.
Methods: Human U87 MG glioma cells were exposed to TMZ, honokiol, and a combination of TMZ and honokiol. Cell survival, apoptosis, necrosis, and proliferation were successively assayed. Fluorometric substrate assays were conducted to determine activities of caspase-3, -6, -8, and -9. Levels of Fas ligand, Bax, and cytochrome *c* were immunodetected. Translocation of Bax to mitochondria were examined using confocal microscopy. Mitochondrial function was evaluated by assaying the mitochondrial membrane potential (MMP), reactive oxygen species (ROS), and complex I enzyme activity. Caspase-6 activity was suppressed using specific peptide inhibitors. The honokiol-induced effects were further confirmed using human U373 MG and murine GL261 cells.
Results: Exposure of human U87 MG glioma cells to honokiol significantly increased TMZ-induced DNA fragmentation and cell apoptosis. Interestingly, honokiol enhanced intrinsic caspase-9 activity without affecting extrinsic Fas ligand levels and caspase-8 activity. Sequentially, TMZ-induced changes in Bax translocation, the MMP, mitochondrial complex I enzyme activity, intracellular ROS levels, and cytochrome *c* release were enhanced by honokiol. Consequently, honokiol amplified TMZ-induced activation of caspases-3 and -6 in human U87 MG cells. Fascinatingly, suppressing caspase-6 activity concurrently decreased honokiol-induced DNA fragmentation and cell apoptosis. The honokiol-involved improvement in TMZ-induced intrinsic apoptosis was also confirmed in human U373 MG and murine GL261 glioma cells.
Conclusions: This study showed that honokiol can enhance TMZ-induced apoptotic insults to glioma cells via an intrinsic mitochondrion-dependent mechanism. Our results suggest the therapeutic potential of honokiol to attenuate TMZ-induced side effects.

Abbreviations: ANOVA, analysis of variance; Bax, Bcl-2-associated X protein; BrdU, bromodeoxyuridine; CI, combination index; DEVD, Asp-Glu-Val-Asp; $DiOC_6$, 3,3′-dihexyloxacarbocyanine; DAPI, 4′-6-diamidino-2-phenylindole; DMEM, Dulbecco′s modified Eagle′s medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEPES, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IETD, Ile-Glu-Thr-Asp; LEHD, Leu-Glu-His-Asp; mAb, monoclonal antibody; MMP, mitochondrial membrane potential; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; VEID, Val-Glu-Ile-Asp

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Introduction

Brain tumors comprise primary tumors of the central nervous system and secondary metastatic tumors (Seano, 2018). Gliomas that predominantly arise from transformation of astrocytes, oligodendrocytes, and ependymal cells are the most common brain tumors (Saadatpour et al., 2016; Seano, 2018). According to a grading system of the World Health Organization, gliomas are classed into low- (grades I and II) and high- (grades III and IV) grade tumors. Glioblastoma multiforme (GBM), classified as a high-grade (grade IV) glioma, is the most aggressive brain tumor. In the clinic, the recommended therapy for GBM patients is surgical resection followed by irradiation and adjuvant chemotherapy, but the median overall survival time of GBM patients is only about 12 months (Daher and de Groot, 2018). The poor outcomes are due to uncontrolled tumor proliferation, infiltrative growth, angiogenesis, and resistance to apoptosis (Furnari et al., 2015). Temozolomide (TMZ), a DNA-alkylating agent, is the chief chemotherapeutic drug for treating GBM patients (Hottinger et al., 2016). TMZ can freely pass through the blood-brain barrier (BBB) and subsequently induces apoptosis of glioma cells by alkylating guanine at the O^6 site (Hottinger et al., 2016; Seano, 2018). Unfortunately, TMZ can lead to various side effects, such as nausea, vomiting, constipation, headaches, fatigue, loss of appetite, mouth sores, and hair loss, as well as drug resistance (Omuro and DeAngelis, 2013). These complications reduce therapeutic effects of TMZ and quality of life of GBM patients. As a result, discovering new drugs that can improve TMZ's capacity while reducing its side effects is urgent and necessary.

Honokiol (2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol), one of the main physiologically bioactive constituents of the traditional Chinese medicine Houpo (*Magnolia officinalis Rehd. et Wils.*), exhibits diverse anti-inflammatory, antimicrobial, antithrombotic, and anxiolytic effects (Hahm et al., 2008; Pan et al., 2016). Previous studies showed that honokiol can be used for treating diverse diseases such as anxiety, nervous disturbances, thrombotic stroke, typhoid fever, and dead muscles (Lo et al., 1994; Fried and Arbiser, 2009). Moreover, honokiol possesses antitumor activities against leukemia, breast cancer, pancreatic cancer, and oral squamous cell carcinoma cells due to induction of cell cycle arrest and cell apoptosis (Bonner et al., 2016; Bilia et al., 2017). We conducted consecutive studies in our lab on antitumor activities of honokiol against brain tumors. At first, we demonstrated that honokiol can pass through the BBB *in vitro* and *in vivo* (Lin et al., 2012). Then, we showed the safety of honokiol to brain normal cells and its ability to kill neuroblastoma cells and glioma cells via an apoptotic mechanism (Lin et al., 2016a,b). Furthermore, we reported the autophagic effects of honokiol on glioma cells and neuroblastoma cells (Lin et al., 2016b; Yeh et al., 2016). Recently, we demonstrated that honokiol can kill drug-sensitive and -resistant glioma cells (Chio et al., 2018). Therefore, honokiol may have the potential to be clinically applied for combined treatment with TMZ in order to reduce its side effects and drug resistance.

Apoptosis, a process of programmed cell death, is characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms (Aubrey et al., 2018). When discovering *de novo* drugs for cancer therapy, we generally design such drug candidates so that they can specifically induce apoptosis of tumor cells (Mills et al., 2018). A variety of intrinsic and extrinsic factors contribute to cell apoptosis (Goyal, 2001). The extrinsic apoptosis pathway is initiated following binding of death Fas ligand to death Fas receptor, contributing to activation of caspase-8 (Bao and Shi, 2007). In contrast, caspase-9 is activated via an intrinsic mitochondrion-dependent pathway (Bao and Shi, 2007; Chen et al., 2013). In an intrinsic apoptotic mechanism, translocation of proapoptotic Bax protein from the cytoplasm to mitochondria can permeabilize the outer membrane, which then disturbs the mitochondrial membrane potential (MMP) and triggers release of cytochrome *c* and reactive oxygen species (ROS) (Franklin, 2011; Chang et al., 2016). Afterward, cytochrome *c* can stimulate cascade activation of caspases-9, -3, and -6 that can cleave key cellular proteins and consequently fragment genomic DNA (Goyal, 2001). Our previous studies showed that honokiol can induce apoptotic insults to neuroblastoma and glioma cells via an intrinsic pathway. Recently, we reported the effects of hypoxia-induced autophagic death for treating human malignant glioma cells (Cheng et al., 2017). Furthermore, we also demonstrated the capacity of honokiol to induce autophagic apoptosis in brain tumors cells (Lin et al., 2016b; Yeh et al., 2016). Thus, this study was aimed to further verify if honokiol could enhance TMZ-induced insults to malignant glioma cells and its possible mechanisms.

Materials and methods

Cell culture and drug treatment

The human glioma U87 MG and U373 MG cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Murine GL261 glioma cells were a kind gift from Dr. Rong-Tsun Wu (Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan). Glioma cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, and 1 mM non-essential amino acids at 37 °C in a humidified atmosphere of 5% CO_2. Cells were grown to confluence before drug treatment.

Honokiol and TMZ were purchased from Sigma (St. Louis, MO, USA) and freshly dissolved in dimethyl sulfoxide (DMSO). The purities of honokiol and TMZ used in this study were >98%. Cells were exposed to honokiol, TMZ, and a combination of honokiol and TMZ at different concentrations for various time intervals.

Cell morphology and cell survival assays

Cell survival was assayed using a trypan blue exclusion method as described previously (Lin et al., 2018). Glioma cells (2×10^4 cells) were seeded in 24-well tissue culture plates. After drug treatment, morphologies of glioma cells were observed and photographed (Nikon, Tokyo, Japan). Then, cells were trypsinized with 0.1% trypsin-EDTA (Gibco-BRL). Following centrifugation and washing, glioma cells were suspended in phosphate-buffered saline (PBS), containing 0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, and stained with trypan blue dye (Sigma). Fractions of dead cells with a blue signal were visualized and counted using a reverse-phase microscope (Nikon).

Quantification of DNA fragmentation

DNA fragmentation in glioma cells was quantified using a cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Indianapolis, IN, USA) as described previously (Chuang et al., 2011). Briefly, glioma cells (2×10^5 cells) were seeded in 24-well tissue culture plates and labeled with bromodeoxyuridine (BrdU) overnight. Cells were harvested and suspended in DMEM. One hundred microliters of a cell suspension was added to each well of 96-well tissue culture plates. Glioma cells were treated with TMZ, honokiol, and a combination of honokiol and TMZ for different time intervals at 37 °C in a humidified atmosphere of 5% CO_2. Amounts of BrdU-labeled DNA in the cytoplasm were quantified using a microplate photometer (Anthos Labtec Instruments, Lagerhausstrasse, Wals/Salzburg, Austria).

Measurement of apoptotic cells

Apoptotic cells were determined by detecting cells which were arrested at the sub-G_1 stage as described previously (Chio et al., 2013).