Original Article

Anti-tumor effects of jaceosidin on apoptosis, autophagy, and necroptosis in human glioblastoma multiforme

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Abstract: Glioblastoma multiforme (GBM) is the most aggressive and common malignant neoplasm. Nevertheless, a 5-year survival rate of patients with GBM has remained below 5%. Artemisia princeps PAMPANINI, used as a food and traditional medicine, have shown beneficial properties including anti-inflammatory, anti-oxidative, and anti-cancer activities. Thus, this study aimed to investigate biological mechanism of a bioactive compound, jaceosidin (JAC), isolated from A. princeps in human GBM T98G cells. Herein, as a result of analysis in terms of cancer survival and death, we found that JAC significantly reduced cell survival against T98G cells. In addition, JAC increased apoptotic cell death via changes on morphological and molecular phenotypes in T98G cells as evidenced by cellular shapes and DNA fragmentation. The apoptotic cell death was confirmed by the cleavage of caspase-3 and PARP, the downregulation of survivin and Bcl-2. Moreover, JAC decreased the expression of cyclinD1 and Cdk5 and increased the phosphorylation of EKR, JNK, and p38 MAPKs. Specifically, JAC suppressed the PI3K/AKT signaling and its downstream molecules including p70S6, GSK3β, and β-catenin. In addition, as a result of analysis in terms of metastasis using wound healing and Boyden chamber assays, JAC showed anti-migrative and anti-invasive activities. Finally, we analyzed in terms of autophagy and necroptosis that are modes of programmed cell survival and death different from apoptosis in T98G cells. We found that JAC inhibited autophagic regulatory proteins including Beclin-1, Atgs, and LC3A/B, thereby reducing autophagic-mediated cell survival, whereas JAC did not affect phosphorylation of key proteins in necroptosis, especially MLKL. Given these findings, our results provided novel evidences on the biological mechanisms of JAC in T98G cells, suggesting that JAC may be a therapeutic agent for patients with GBM.

Keywords: Apoptosis, Artemisia princeps, autophagy, GBM, JAC, necroptosis

Introduction

Glioblastoma multiforme (GBM) is the most aggressive and common malignant neoplasm that arises from glial cells in the brain and spinal cord, accounting for 60% of brain cancers in adults, and the global incidence of GBM is more than 10 per 100,000 and continues to increase [1]. Current treatments for patients with GBM begin with surgical resection, where applicable and safe, followed by radiation therapy and concurrent chemotherapy, and 83% of patients with GBM who received chemotherapy were associated with improved survival [2]. Although these interventions have advanced in recent years, the prognosis of patients with GBM is still very poor, with an average patient survival of only 12-15 months due to its anatomical location, heterogeneous feature, rapid growth, and invasive nature [3]. Therefore, it is necessary to discover new compounds that inhibit cell growth, migration and invasion and to study their biological mechanisms for therapy of patients with GBM.

The Artemisia princeps PAMPANINI (A. princeps), which is locally called Sajabalssuk, is distributed on Ganghwa Island in Korea contains a
lots of constituents of phenolic compounds, especially including jaceosidin (JAC), eupatilin, and caffeoylquinic acids, which have a wide range of bioactivities such as anti-inflammatory, anti-cancer, and anti-obesity properties [4-7]. The protective effect of A. princeps can be realized by adding directly to food or food products during processing [8]. However, the efficacy of active compounds from A. princeps on cancer survival and death through apoptosis, autophagy, and necroptosis in GBM is still unknown.

In the present study, we demonstrated the biological mechanisms of JAC (99% purity), an active compound isolated from the dry aboveground part of A. princeps, by examining its anti-cancer effects on programmed cell death modes, migration and invasion in human GBM T98G cells.

Materials and methods

Plant material

The dry aboveground part of A. princeps was purchased at the commercial herbal medicine market. A voucher specimen (P399) has been deposited in the Natural Products Bank, National Institute for Korean Medicine Development (NIKOM). The nuclear magnetic resonance (NMR) spectra were obtained on a Jeol ECX-500 spectrometer (JEOL Ltd., Tokyo, Japan) operating at 1H (500 MHz) and 13C (125 MHz). High performance liquid chromatography (HPLC) was performed using Agilent 1260 series (Agilent Technologies, CA, USA). Column chromatography was conducted using ODS-A (s-75 μm; YMC Co., Kyoto, Japan) and silica gel 60 (70-230 mesh/230-400 mesh ASTM, Merck, Darmstadt, Germany).

Extraction and isolation of active compound from the dry aboveground part of A. princeps

The dry aboveground part of A. princeps (2.4 kg) was extracted with 80% MeOH (15 L, 2 times) at room temperature for 1 day. The crude extract (137.0 g) was suspended in distilled water (DW), and then solvent partitioned using EtOAc, and n-BuOH. The EtOAc soluble fractions (47.0 g) was subjected to a silica gel (70-230 mesh) open column chromatography eluted with a gradient of Hexane-EtOAc (7:1→1:1, v/v) to yield fractions (1-20). The fraction 16 was purified by silica gel (230-400 mesh) open column chromatography eluting with (CHCl3-MeOH, 30:1, v/v) to give 10 fractions. The fraction 16-6 was purified by ODS-A gel open column chromatography eluting with (MeOH-H2O, 2:1, v/v) to give 12 fractions. The active compound (120 mg) was obtained from fraction 16-6. The structure of jaceosidin was identified by comparing the spectral data using before literature (Ryu et al., 2004).

Jaceosidin

Yellow powder, EI-MS m/z 330.29 [M]+, Molecular formula C17H14O7; 1H-NMR (500 MHz, CD3OD) 7.44 (1H, dd, J=8.2, 2.2 Hz, H-6'), 7.39 (1H, d, J=2.2 Hz, H-2'), 6.91 (1H, d, J=8.2 Hz, H-5'), 6.54 (1H, s, H-8), 6.52 (1H, s, H-3), 3.93 (3H, s, OCH3-6), 3.87 (3H, s, OCH3-3'); 13C NMR (125 MHz, CD3OD) 184.0 (C-4), 165.9 (C-2), 158.4 (C-7), 154.4 (C-5), 151.8 (C-9), 149.2 (C-3'), 148.4 (C-4'), 132.6 (C-6), 123.4 (C-1'), 121.5 (C-6'), 116.5 (C-5'), 110.3 (C-2'), 105.8 (C-10), 103.6 (C-3), 95.2 (C-8), 60.9 (OCH3 at C-6), 56.3 (OCH3 at C-3').

Cell culture

Human GBM T98G cells, MG63 cells, and YD-10B cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Dulbecco’s Modified Eagle Medium (WELGENE Inc., Gyeongsangbuk-do, Korea) that is supplemented with 10% fetal bovine serum and 1 X Gibco® Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Cancer toxicity assay

Cell toxicity was analyzed using a MTT assay as previously described based on published methods [9]. Absorbance was measured at a 540 nm wavelength using the Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Terminal deoxynucleotidyl transferase-mediated FITC-dUDP nick-end labeling assay

Terminal deoxynucleotidyl transferase-mediated FITC-dUDP nick-end labeling (TUNEL) assays
were accessed using an in-situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) to detect apoptotic DNA fragmentation as previously described based on published methods [9].

Western blot analysis

Western blot analysis was carried out as previously described based on published methods [10]. Briefly, the total protein concentration of cell lysates was measured using Bradford reagent (Bio-Rad, Hercules, CA, USA). 20 μg concentration was resolved on sodium dodecyl-polyacrylamide gel electrophoresis were transferred to PVDF membrane (Millipore, Bedford, MA). An enhanced chemiluminescence kit (Millipore) in the ProteinSimple detection system (ProteinSimple Inc., Santa Clara, CA, USA) was used to detect the protein signal in the membrane.

Immunofluorescence assay

Cells were washed in 1× PBS and fixed in 10% formalin for 10 min at room temperature. The fixed cells were washed three times in 1× PBS and permeabilized by 0.2% Triton X-100 for 15 min. The following procedure was performed as previously described based on published methods [9].

Wound healing assay

Cells were wounded by using a 200 μl pipette tip and cell debris was washed in 1× PBS. The cells were treated with or without JAC, and the following procedure of the cell migration assay was performed as previously described based on published methods [9].

Boyden chamber assay

Invasion of T98G cells was carried out using a Boyden chamber assay as previously described based on published methods [9]. Invasion cells were detected using a motic light microscope.

Statistical analysis

Data are shown as mean ± standard errors of the means (S.E.M.) for all analyses, and statistical significance (‘P<0.05) was tested using Student’s unpaired t test in the GraphPad Prism version 5 program (GraphPad Software, Inc., San Diego, CA).

Results

JAC suppresses cell survival in human GBM T98G cells

In order to investigate anti-cancer effects of A. princepsin in GBM, JAC was isolated as a phenolic compound from its dry aboveground part (Figure 1A). The 1H and 13C nuclear magnetic resonance spectra (NMR) and high-performance liquid chromatography (HPLC) of JAC are further shown in Figure 1B-D. To examine the cytotoxicity of OGAL against GBM T98G cells and other cancer cells (MG63 cells and YD-8 cells), JAC (10-50 μM) was treated in the cells and cell viability was analyzed by detecting NADH-dependent dehydrogenase activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed that JAC significantly reduced the survival of T98G cells compared to MG63 cells and YD-8 cells (Figure 1E and 1H).

JAC leads to apoptotic cell death in human GBM T98G cells

Based on the anti-survival efficiency of JAC in T98G cells, we further examined whether JAC led to apoptotic cell death in T98G cells. JAC (10-50 μM) was treated in GBM T98G cells, and morphologic alterations were detected using a light microscope. The results showed that JAC decreased cell size and altered them into a round single cell type, which are the morphological phenotype of apoptotic cells (Figure 2A). Moreover, the apoptotic cell death was validated by monitoring apoptotic DNA strand breakage using a terminal deoxynucleotidyl transferase-mediated FITC-dUDP nick-end labeling (TUNEL) assay. As shown in Figure 2B, JAC (10-50 μM) increased TUNEL positive signals in the nucleus of T98G cells as observed in a fluorescence microscope, compared with the control. In detail, JAC-induced apoptosis was biochemically investigated using a Western blot analysis. The results showed that JAC (10-50 μM) induced an increase in the cleavage of poly ADP-ribose polymerase (PARP) and cleaved cysteinyl-aspartate protease (caspase)-3 proteins, while induced loss of full length PARP.
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A

Jaceosidin (JAC)
$C_{17}H_{14}O_{7}$ (> 99% purity)

B

$^1$H-NMR

C

$^{13}$C-NMR

D

E

MG63 cells

Cell viability (%)

JAC (µM): 0 10 30 50

F

YD-8 cells

Cell viability (%)

JAC (µM): 0 10 30 50

G

T98G cells

Cell viability (%)

JAC (µM): 0 10 30 50

H

T98G cells

Cell viability (%)

JAC (µM): 0 10 30 50
Figure 1. Isolation of JAC from A. princeps and its effects on cytotoxicity in human GBM T98G cells. (A) Chemical structure of JAC isolated from dry aboveground part of A. princeps. (B, C) $^1$H NMR (B) and $^{13}$C NMR (C) spectra of JAC. (D) HPLC chromatogram of JAC. (E, F) Osteosarcoma MG63 cells (E) and oral squamous cell carcinoma YD-8 cells (F) were treated with JAC at doses of 10, 30, and 50 μM for 24 h, and then cell viability was analyzed using an MTT assay. (G, H) GBM T98G cells were treated with JAC at doses of 10, 30, and 50 μM for 24 h (G) and 48 h (H), and then cell viability was analyzed using an MTT assay. Data represent the results of three independent experiments. Asterisk (*) indicates statistically significant difference compared to the control ($P<0.05$).
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Figure 2. Effects of JAC on alternations of apoptotic cell morphology, DNA fragmentation, and protein levels in human GBM T98G cells. (A) The indicated doses of JAC were treated for 24 h, and morphological changes were monitored at 4× and 10× using light microscopy. Scale bar: 100 μm. (B) TUNEL (green)- and DAPI (blue)-positive cells were monitored using fluorescence microscopy. Scale bar: 50 μm. (C, D) Western blot analysis was performed to determine the protein levels for PARP, cleaved PARP, procaspase-3, and cleaved caspase 3 (C), or Survivin and Bcl-2 (D). β-actin was used as a control for the equal amount of lysates. Data represent the results of three independent experiments.

Figure 3. Effects of JAC on cell cycle molecules and MAPKs in human GBM T98G cells. (A, B) The indicated doses of JAC were treated for 24 h, and whole cell lysates were analyzed using Western blot analysis to determine the protein levels for Cyclin D1, Cdk4, and Cdk6 (A), or Cdk5 (B). β-actin was used as a control for the equal amount of lysates. (C) 30 μM JAC was treated for 0, 5, 15, 30, 45, and 60 min, and p-ERK, ERK, p-JNK, JNK, p-p38, and p38 were analyzed using Western blot analysis. Data represent the results of three independent experiments.

JAC suppresses cell cycle molecules and activates mitogen-activated protein kinases in human GBM T98G cells

Cyclin D1 is a key regulator of cell cycle progression and cell proliferation through complex formation with cyclin-dependent kinase (Cdk) 4 and Cdk6. To further examine the effects of JAC on cell cycle molecules, the expression levels were analyzed in T98G cells. The results showed that JAC (10-50 μM) suppressed the expression of Cyclin D1, cdk4, and cdk6 in a dose-dependent manner (Figure 3A). In addition, we found that JAC (10-50 μM) reduced the expression of Cdk5, an atypical Cdk (Figure 3B). Since the mitogen-activated protein kinases (MAPKs) are key regulators of cancer progression, we also examined whether the activities of p38, JNK, and ERK1/2 were regulated by JAC in T98G cells. Western blot analysis showed that JAC treatment enhanced the phosphorylation levels of p38, JNK, and ERK1/2 MAPK in a time-dependent manner in T98G cells (Figure 3C).

JAC inhibits the AKT pathway involved in cell survival, migration, and invasion in human GBM T98G cells

To specifically investigate the biological mechanisms on JAC-mediated anti-cancer effects, we focused on the AKT pathway in T98G cells. Western blot analysis showed that JAC (10-50 μM) suppressed the phosphorylation of PI3K and AKT in T98G cells (Figure 4A). The results were also confirmed by detecting the phosphorylation of AKT using a fluorescence microscope (Figure 4B). We also analyzed the downstream molecules of PI3K/AKT signaling. As shown in Figure 4C-E, JAC (10-50 μM) dephosphorylated p70S6K protein that promotes cell proliferation by increasing components of protein synthesis, as well as dephosphorylated GSK3β and β-catenin proteins that regulate cell migration and invasion.
Figure 4. Effects of JAC on AKT signaling molecules in human GBM T98G cells. (A) Whole cell lysates were subjected to Western blot analysis to determine the protein levels for p-PI3K, PI3K, p-AKT, and AKT. (B) Cells were immunostained with an anti-p-AKT antibody and a secondary antibody conjugated to Alex Fluor-568 (red), followed by staining with DAPI (blue). The images were monitored using fluorescence microscopy. Scale bar: 50 μm. (C-E) Whole cell lysates were subjected to Western blot analysis to determine the protein levels for p-p70S6K and p70S6K (C), p-GSK3β and GSK3β (D), or β-catenin and β-actin (E). Data represent the results of three independent experiments.

JAC inhibits the migration and invasion of human GBM T98G cells

The anti-metastasis effect of JAC on T98G cells was analyzed by wound healing assay. In comparison to the control, 10 μM JAC significantly attenuated cell migration in a dose- and time-dependent manner after 24 and 48 hours (Figure 5A and 5B). At higher concentrations of JAC (30 and 50 μM), cell migration was not only completely inhibited but also expanded beyond the wound area compared to the wounded area (Figure 5A and 5B), suggesting that this phenomenon is due to the marked cell death of T98G cells by JAC. We further confirmed the anti-metastasis effect of JAC using a Boyden...
Figure 5. Effects of JAC on metastatic phenotypes of human GBM T98G cells. (A, B) The indicated doses of JAC were treated and cell migration was monitored at 0, 24, and 48 h using light microscopy. Scale bar: 100 μm (A). Migration rate (%) was shown as a bar graph normalized to that of 0 h (B). (C, D) Cell invasion was performed using Boyden chamber assay and monitored under light microscopy. Scale bar: 50 μm (C). Invasion rate (fold) was shown as a bar graph normalized to that of 0 μM JAC (D). Data represent the results of three independent experiments. Asterisk (*) indicates statistically significant difference compared to 0 h of the control (P<0.05). Sharp (#) indicates statistically significant difference between 24 and 48 h of the control group and 24 and 48 h of each dose of JAC (P<0.05).

chamber assay, showing that JAC significantly suppressed cell invasion across the Matrigel-coated membrane in a dose-dependent manner compared with the control (Figure 5C and 5D).

JAC inhibits autophagy but does not affect necroptosis in human GBM T98G cells

To investigate whether JAC has effects on other modes of cell survival and death in addition to apoptosis, we monitored the molecular machinery of autophagy within T98G cells using Western blot analysis. The results showed that JAC treatment reduced the expression levels of Beclin-1, autophagy related gene (Atg) 5, Atg7 and microtubule-associated protein light chain 3A/B (LC3A/B), suggesting that JAC blocked the survival of T98G cells through autophagy (Figure 6A). Finally, we monitored the molecular machinery of necroptosis within T98G cells, and Western blot analysis showed that JAC treatment did not affect the phosphorylation levels of receptor-interacting serine/threonine-protein kinase (RIP) 1, RIP3, and mixed lineage kinase domain like pseudokinase (MLKL), suggesting that JAC-induced cell death was not associated with necroptosis (Figure 6B).

Discussion

Traditional medicine has been practiced worldwide for hundreds or thousands of years, and various bioactive compounds isolated from plants that have been used in traditional medicine have attracted great attention to treat various diseases and to improve general health and wellbeing [11, 12]. The theoretical background and technological advancements in life sciences have enabled a clearer understanding of the bioactive compounds in traditional medicine [13]. More recently, natural products continue to enter clinical trials, or provide clues to compounds that have entered
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and 4-parvifuran, phenolic compounds, obtained from *Dalbergia odorifera* heartwoods [15, 16]. In the present study, we demonstrated the anti-cancer effects and biological mechanisms of JAC, a phenolic compound, isolated from the dry aboveground part of *A. princeps* in the cell survival, death, migration, and invasion of human GBM.

Genetic instability of GBM induces aggressive cell growth by preventing apoptotic cell death through the upregulation of anti-apoptotic proteins and the downregulation of pro-apoptotic proteins [17]. The aggressive growth is a key feature of cancer cells, and thus most anti-cancer drugs are primarily aimed at inducing apoptosis and arresting cell division [18]. As is well established, the major signals of apoptosis are the activation of caspase 3 enzyme mediated through cellular changes, such as the Bcl-2 family proteins and the inhibitor of apoptosis family proteins, including Bcl-2 and Survivin [19, 20]. The activated caspase-3 cleaved PARP, a nuclear enzyme that responds to DNA strand breaks, and this step is necessary for apoptosis to prevent futile repair of DNA strand breaks during apoptotic cell death [21, 22]. In the present study, we found that JAC suppressed cell growth in GBM with findings showing that JAC increased caspase-3 and PARP cleavages, while decreased Survivin and Bcl-2 expression levels. Our results also demonstrated that JAC increases the apoptotic DNA strand breaks. It was reported that Cyclin D1 plays a central role in regulating cell growth, apoptosis, and tumourigenesis, and it interacts with Cdk4 and cdk6 to form active kinases that promote the transcription of genes required for cell cycle progression [23, 24]. In the present study, we found that JAC inhibits Cyclin D1, Cdk4, and cdk6 in GBM. In addition, JAC reduced the expression of Cdk5 in GBM. Cdk5 has been reported to contribute to cancer growth, migration, invasion, and chemotherapy resistance [25]. Therefore, our results suggest that JAC has anti-cancer activities on cell growth through apoptotic cell death in GBM.

AKT is a prime point in pathways that induce the amplification of growth signals and prevent apoptosis, and thus inhibition of the AKT pathway is a potential treatment target against patients with GBM [26]. It has been reported that the AKT pathway is mainly deregulated in...
GBM, and there is a correlation between the high level of AKT phosphorylation, the histopathological grade of GBMs, and the poor prognosis of patients with GBM, while AKT phosphorylation is rarely detected in healthy tissues [27, 28]. In the present study, we demonstrated that JAC inactivates constitutively active AKT and its signaling proteins, PI3K, p70S6K, GSK3β, and β-catenin in GBM T98G cells. In addition to tumorigenesis, the AKT pathway also contributes to GBM metastasis and is considered potential drug targets for inhibiting lethal metastatic events [29]. In the present study, we examined the anti-metastasis effect of JAC, demonstrating that JAC induced anti-migrative and anti-invasive effects in GBM T98G cells. It was also reported that Ginsenoside Rh2, one of the major bioactive ginsenosides isolated from Panax ginseng, suppresses the migration and invasion of GBM through inhibiting the AKT pathway [30]. Given that apoptosis also acts as inhibitory process in metastatic events of malignant cancers [31], our result suggest that JAC is a potential agent that inhibits metastasis with apoptosis induction through inhibiting the AKT pathway in GBM.

Although cancer cells can be eliminated through apoptosis by anti-cancer drugs, cancer cells evolves into a means of circumventing apoptotic cell death, leading to the problems of poor prognosis among cancer patients [31]. To this end, it is essential to investigate the different modes of programmed cell death that control cancer-associated cell death such as autophagy and necroptosis. Accumulating evidences suggest that autophagy plays critical roles in therapeutic strategy to suppress tumorigenesis, metastasis, and chemoresistance [32]. Several research provided evidences that reduction of autophagy enhances the apoptotic response of cancer cells to chemotherapy [33]. Consistent with these reports, our data showed that JAC inhibits the autophagic pathway, leading to apoptotic cell death. Recently, necroptosis also plays an important role in the regulation of cancer biology, indicating that the molecular machinery of necroptosis is reduced in cancer cells, allowing cancer cells to evade necroptosis for tumor initiation, promotion, and progression [34]. It was reported that the key regulator proteins associated with necroptosis are significantly higher in tumor tissue of GBM as in comparison to normal tissue, suggesting that the dysregulation of necroptosis has a poor prognosis in patient with GBM [35]. In the present study, we demonstrated that JAC has no effect on necroptosis-pathway-associated proteins. Therefore, our findings suggest that JAC possesses anti-cancer effects by targeting autophagy and apoptosis in GBM but not necroptosis.

In conclusion, as the first study, we found that JAC inhibits the growth, survival, migration, and invasion of human GBM T98G cells by enhancing apoptotic cell death with reduction of the autophagic pathway regardless of necroptosis. Our data also suggest that JAC has a potentially therapeutic effect in patients with GBM.

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Disclosure of conflict of interest
None.

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