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Original Articles

Isolinderalactone regulates the BCL-2/caspase-3/PARP pathway and suppresses tumor growth in a human glioblastoma multiforme xenograft mouse model

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common malignant brain tumor, which remains incurable. Plant extracts are a potential source of potent anticancer medicines. In this study, we investigated the effect of isolinderalactone from *Lindera aggregata* on tumor growth using U-87 human glioblastoma cells. Treatment with isolinderalactone inhibited cell viability and promoted apoptotic cell death. In addition, intraperitoneal injection of isolinderalactone significantly inhibited tumor growth in a human GBM xenograft mouse model. To identify the proteins involved in the induction of apoptosis in isolinderalactone-treated cells, we performed a human apoptosis proteome array analysis and western blotting. Isolinderalactone suppressed the expression of B-cell lymphoma 2 (BCL-2), as well as of survivin and X-linked inhibitor of apoptosis protein (XIAP), known as apoptosis inhibitors, and increased the level of cleaved caspase-3. In addition, isolinderalactone treatment increased cleaved poly(ADP-ribose) polymerase (PARP) and DNA damage. In xenograft tumor tissues, we observed high immunofluorescence of cleaved caspase-3 and TUNEL in isolinderalactone-treated group. Taken together, isolinderalactone enhances U-87 GBM cell apoptosis *in vitro* and *in vivo* and retards tumor growth, suggesting that isolinderalactone may be a potential candidate for anti-glioblastoma drug development.

1. Introduction

Glioblastoma multiforme (GBM) is the most aggressive and malignant primary brain tumor, characterized by high invasiveness and strong resistance to chemotherapy [1,2] Surgical resection of the primary tumor, followed by radio- and chemotherapy, is the current standard of care; however, the mean overall survival of GBM patients is only 14.6 months [1,3]. Furthermore, recurrent GBMs change to more malignant forms, which are resistant to radio- and chemotherapy [4]. Malignant gliomas show aberrant proliferation and apoptosis. The signaling pathways stimulating cell proliferation are constitutively activated, whereas proapoptotic pathways are inactivated in malignant gliomas, or cell survival genes are overexpressed to overcome cell death [3]. Tumor resistance to treatment may correlate with physiological changes making tumors less sensitive to apoptosis induction; therefore, more effective therapeutic agents against GBMs should be developed.

Traditional medicines, based on natural products, are a promising source for developing new conventional medicines. Many herbs have been applied as cancer treatments, such as complementary medicines, health supplements, or nutrients [5]. We have previously selected 29 medicinal herbs through text mining for the treatment of ischemic brain injury and reported their effects on the prevention of blood-brain barrier breakdown and brain edema [6]. We hypothesized that some of the 29 medicinal herbs might affect the highly vascularized brain tumor, glioblastoma [1], because these 29 medicinal herbs were selected for the treatment of brain injury caused by blood vessel problems.

We selected a root extract of *Lindera aggregata*, which was most effective in inhibiting glioblastoma cell growth, then screened eight

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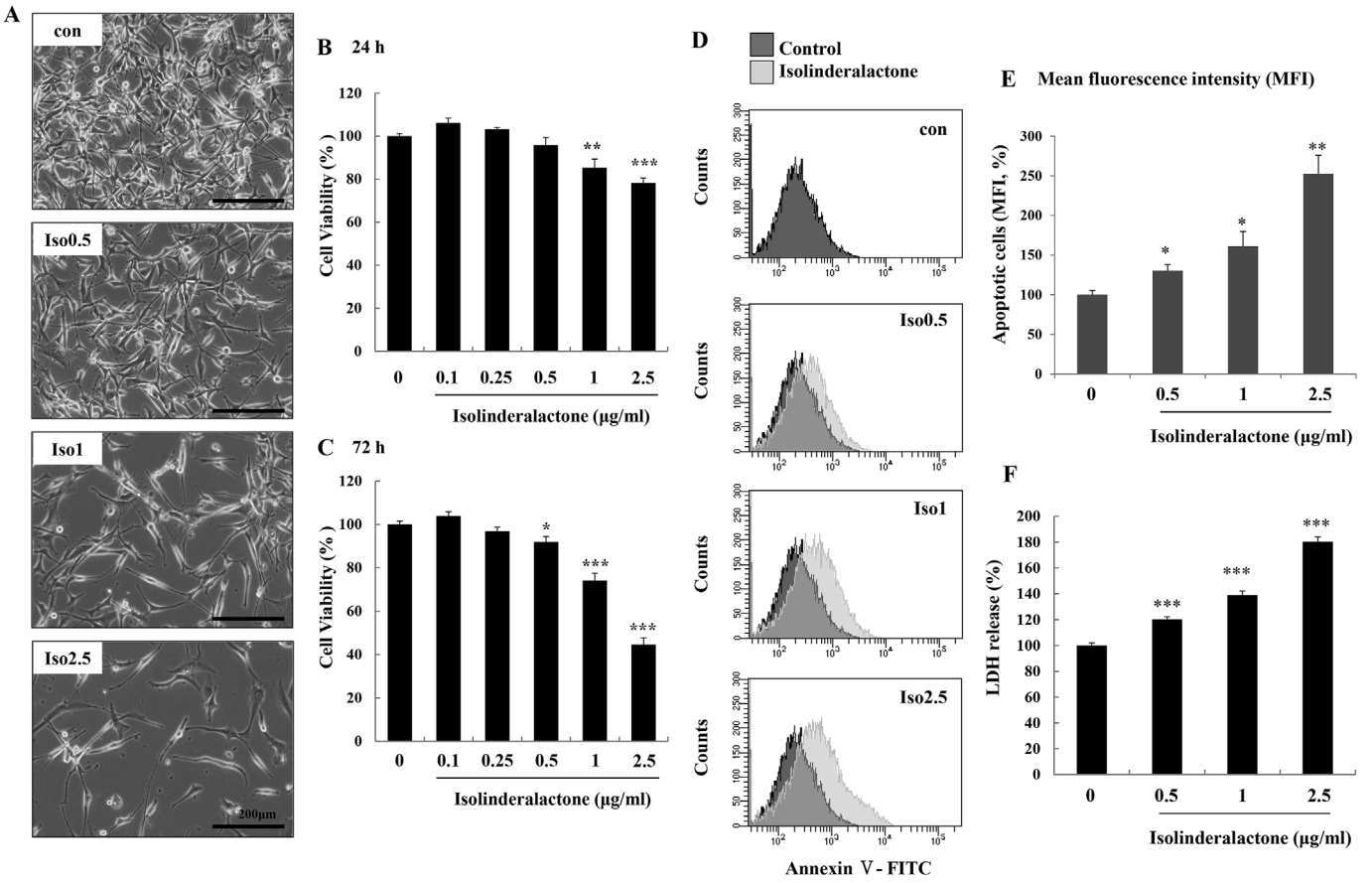

Fig. 1. Isolinderalactone treatment induces apoptosis in U-87 GBM cells. (A) U-87 cells were treated with the control (0.1% DMSO) and different concentrations of isolinderalactone. Cell morphology is shown after 72 h of isolinderalactone treatment: Iso0.5 (0.5 μg/mL), Iso1 (1 μg/mL), and Iso2.5 (2.5 μg/mL). Magnification: × 100; scale bar = 200 μm. **(B, C)** Cell viability was determined by the CCK-8 assay at 24 and 72 h of treatment (n = 6). Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 versus the control group. **(D, E)** U-87 GBM cells were treated with isolinderalactone at concentrations of 0.5, 1, and 2.5 μg/mL for 72 h. Annexin V–FITC staining was analyzed by flow cytometry. ***(D)*** Flow cytometry histograms show U-87 GBM cells treated with isolinderalactone (light shading) compared with those treated with the vehicle control (dark shading). ***(E)*** Graphs show the mean fluorescence intensity (MFI) of Annexin V–FITC (n = 3). *p < 0.05 and **p < 0.01 versus the control group. **(F)** LDH assay was performed after 72 h of treatment of U-87 GBM cells with isolinderalactone (n = 6). Data are presented as the mean ± SEM. ***p < 0.001 versus the control group.

commercially available compounds from the *L. aggregata* extract, and chose isolinderalactone as a potential GBM-suppressive compound because it showed high efficacy at a low concentration. Isolinderalactone is one of sesquiterpenes from the root extracts of *L. aggregata* [7]. Few studies have reported the inhibitory effect of isolinderalactone on cancer cells [8–10]. The compound was shown to inhibit proliferation of non-small-cell lung cancer cells [8,9] and to suppress MDA-MB-231 breast cancer cells [10]; however, the effect of isolinderalactone on glioblastoma has not been reported yet. In this study, we investigated whether isolinderalactone induces U-87 GBM cell apoptosis *in vitro*, suppresses xenograft tumors *in vivo*, and its underlying mechanisms. We here propose that isolinderalactone may become a potential candidate for developing a medicine against malignant brain tumors.

2. Materials and methods

2.1. Cell culture and reagent

The human U-87 glioblastoma cell line was purchased from the American Type Culture Collection (Manassas, VA USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO2 incubator. Isolinderalactone was purchased from ALB Technology (ALB-RS-6003; Hong Kong) and was dissolved in dimethyl sulfoxide (DMSO; Duchefa Biochemie, Haarlem, Netherlands).

2.2. Cell Counting Kit −8 assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay according to the manufacturer's instructions. U-87 GBM cells were seeded on a 96-well plate (1×10^4 cells/well) and incubated overnight for cell attachment. On the next day, the cells were treated with various concentrations of isolinderalactone or 0.1% DMSO for the control group and further incubated for 24–72 h. Then, the CCK-8 solution (10 μL per well) was added, and the plate was incubated at 37 °C for an additional 2 h. Absorbance was measured at 450 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.3. Detection of apoptosis using annexin V-FITC assay

U-87 GBM cells (3×10^5) were seeded on a 60-mm culture dish and incubated overnight. The cells were then treated with isolinderalactone and the vehicle control for 72 h, trypsinized, and suspended in phosphate-buffered saline (PBS). Annexin V binding assays were performed using an Annexin V-FITC apoptosis assay kit (BD Biosciences, San Jose, CA, USA). Stained cells were analyzed using a BD FACS Canto™ II flow cytometer (BD Biosciences), and data were analyzed using the FACSDiva software (BD Biosciences).

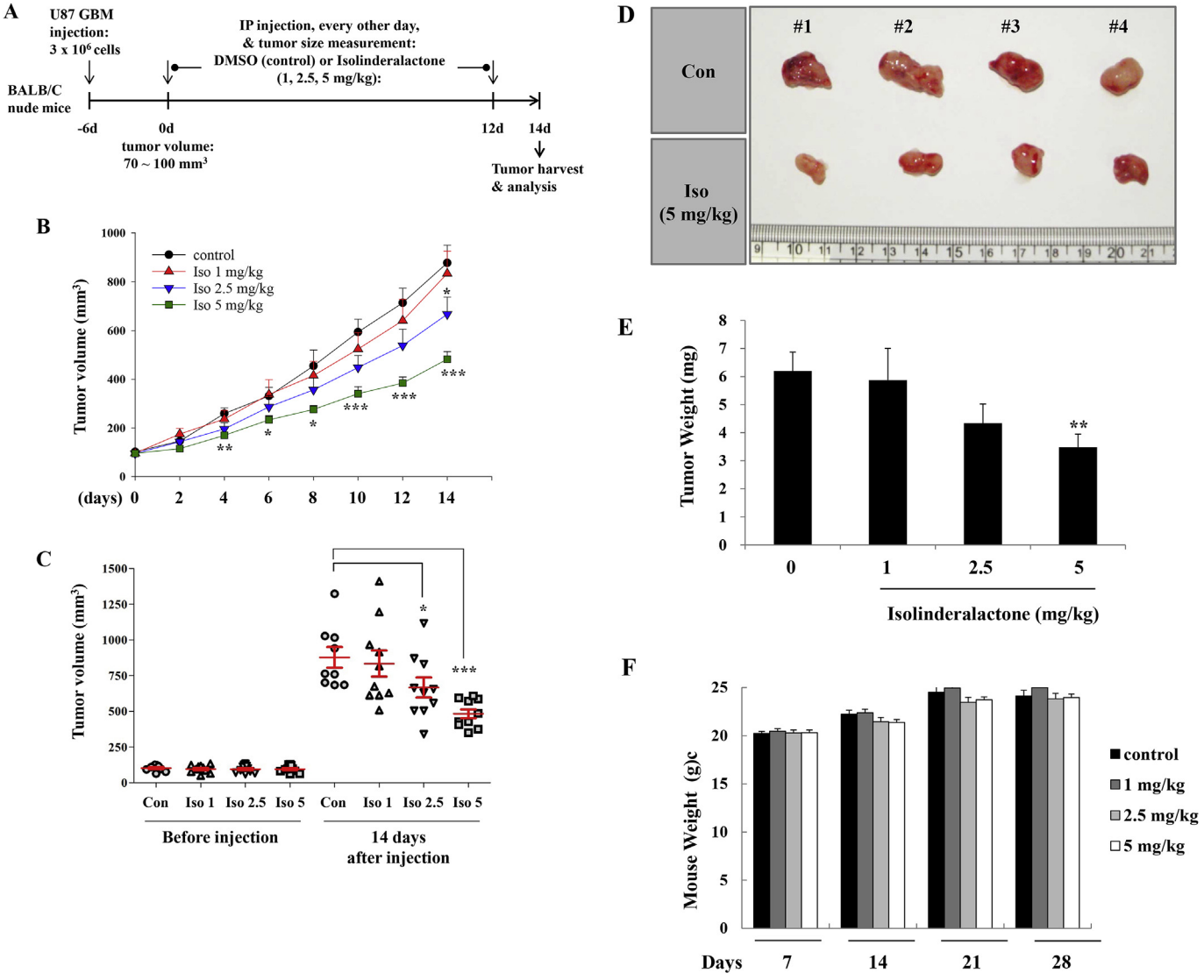

Fig. 2. Isolinderalactone suppresses tumor growth in a human GBM xenograft mouse model. (A) Schematic diagram displaying the time course of tumor induction and treatment in mice. **(B, C)** Isolinderalactone inhibited tumor growth, as measured by the tumor volume, compared with that in the vehicle control (mean ± SEM). *p < 0.05, ***p < 0.001 versus the control group. Control, n = 9; isolinderalactone-treated groups, n = 10 each. **(D)** Representative tumor images are shown for four mice from each group, control and isolinderalactone (5 mg/kg). **(E)** Tumor weight (mean ± SEM). **p < 0.01 versus the control group. Control, n = 9; isolinderalactone-treated groups, n = 10 each. **(F)** Body weight was monitored for 4 weeks.

2.4. Lactate dehydrogenase assay

U-87 GBM cells were seeded on 96-well plates (1×10^4 cells/well), incubated overnight, and then treated with isolinderalactone and the vehicle control in 100 μL of the medium. The media were collected after 72 h of treatment, and cytotoxicity was determined using a lactate dehydrogenase (LDH) cytotoxicity detection kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The plate was read at a wavelength of 490 nm using a SpectraMax 190 spectrophotometer (Molecular Devices).

2.5. In vivo xenograft model

All animal protocols were implemented in accordance with the Pusan National University Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Review Board of Pusan National University (PNU-2017-1523). Four-week-old male BALB/c nude mice were purchased from Nara Biotechnology (Seoul, Korea) and adapted for 1 week. Thereafter, U-87 GBM cells (3×10^6 cells/100 μL of serum-free DMEM) were subcutaneously implanted into the right flank of the mice. When tumors grew to approximately 70–100 mm^3 (approximately 6 days after implantation), the mice were divided into four groups (n = 9–10 per group). Mice in three groups were intraperitoneally injected isolinderalactone (1, 2.5, and 5 mg/kg, respectively) every other day. The control group was intraperitoneally injected an equal volume of DMSO. The tumor volume was measured with calipers and calculated according to the following formula: Tumor volume (mm^3) = (length × width^2) × ½, as previously reported [11]. The mice were sacrificed after 14 days of treatment, and whole tumor tissues were harvested, weighed, and photographed.

2.6. Human apoptosis proteome array

The expression levels of apoptosis-associated proteins were analyzed in U-87 GBM cell lysates using a proteome profiler human apoptosis array kit (R&D Systems) according to the manufacturer's instructions. Briefly, 300 μg of total protein from U-87 GBM cells, cultured with or without isolinderalactone (2.5 μg/mL), was mixed with a

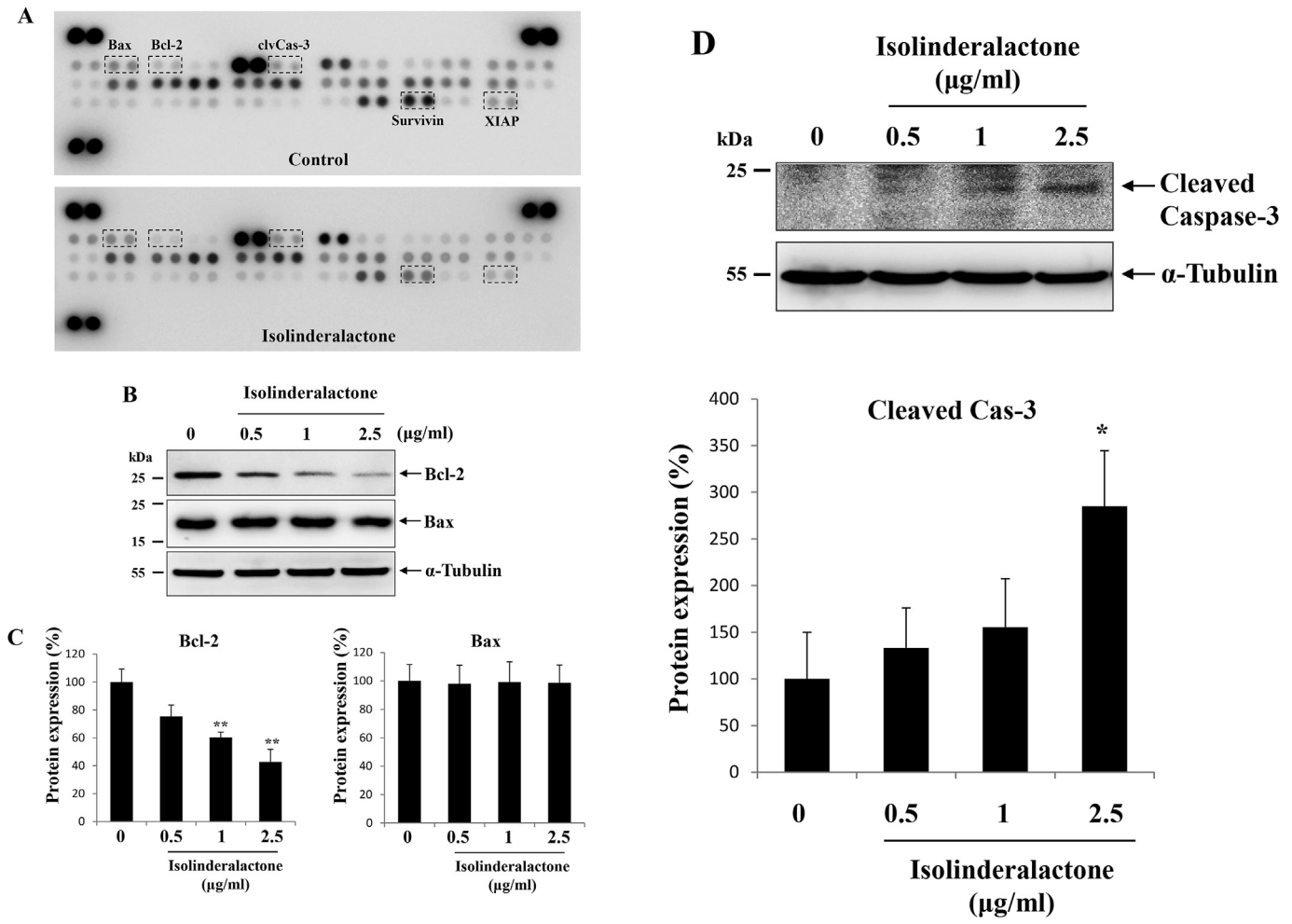

Fig. 3. Effects of isolinderalactone on the expression of apoptosis-related proteins. (A) Total protein lysates from the vehicle control (top) and isolinderalactone (2.5 μg/mL)-treated cells (bottom) were analyzed by a human apoptosis proteome array. **(B)** U-87 GBM cells were treated with isolinderalactone for 48 h, and expression of BCL-2 and BAX was detected by western blotting. **(C)** Quantitative data for BCL-2 and BAX (n = 5). **p < 0.01 versus the control group. **(D)** Expression of cleaved caspase-3 was detected by western blotting (top) and quantified (bottom). *p < 0.05 versus the control group (n = 4).

cocktail of biotinylated detection antibodies and incubated with proteome array membranes overnight at 4 °C. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 1 h at room temperature. Specific signals were detected using the SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA), and blot images were obtained with an ImageQuant LAS 4000 imaging system (GE Healthcare Life Sciences, Uppsala, Sweden).

2.7. Western blotting

U-87 GBM cells were washed with PBS and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). The extracted total protein (~20 μg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Amersham, Little Chalfont, UK), and immunoblotted with specific antibodies against BCL-2 (1:500, sc-7382, Santa Cruz Biotechnology, Dallas, TX, USA), BCL-2-associated X protein (1:1000, 2772, BAX; Cell Signaling Technology), cleaved caspase-3 (1:200, 9661, Cell Signaling Technology), survivin (1:1000, NB500-201, Novus Biologicals, Littleton, CO, USA), X-linked inhibitor of apoptosis protein (1:1000, NB100-56183, XIAP; Novus Biologicals), PARP (1:1000, 9542, Cell Signaling Technology), and α-tubulin (1:3000, T5168, Sigma–Aldrich, St. Louis, MO, USA). After incubation with an HRP-conjugated secondary antibody (Enzo Life Sciences, Farmingdale, NY, USA) at room temperature for 1 h, immunoreactive bands were visualized by luminescence detection using the SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific), and images were obtained using an ImageQuant LAS 4000 imaging system (GE Healthcare Life Sciences). Band intensities were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to that of α-tubulin.

2.8. Fluorescence staining

Excised tumors were fixed in 4% paraformaldehyde at 4 °C, equilibrated in a sucrose gradient (5% sucrose, 4 h at room temperature; 15% sucrose 4 h at room temperature; and 30% sucrose, overnight at 4 °C), and then embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). Sections (10-μm thickness) were stained with anti-cleaved caspase-3 (1:200, 9661, Cell Signaling Technology) at 4 °C overnight, followed by incubation with an Alexa Fluor 594-conjugated secondary antibody (Life Technologies) for 2 h in total darkness. The nuclei were stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Eugene, OR, USA). Apoptotic cells were detected in tumors using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay according to the DeadEnd™ Fluorometric TUNEL System (Promega) protocol. Fluorescence images were taken with a Zeiss LSM 700 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) and quantified using the iSolution full image analysis

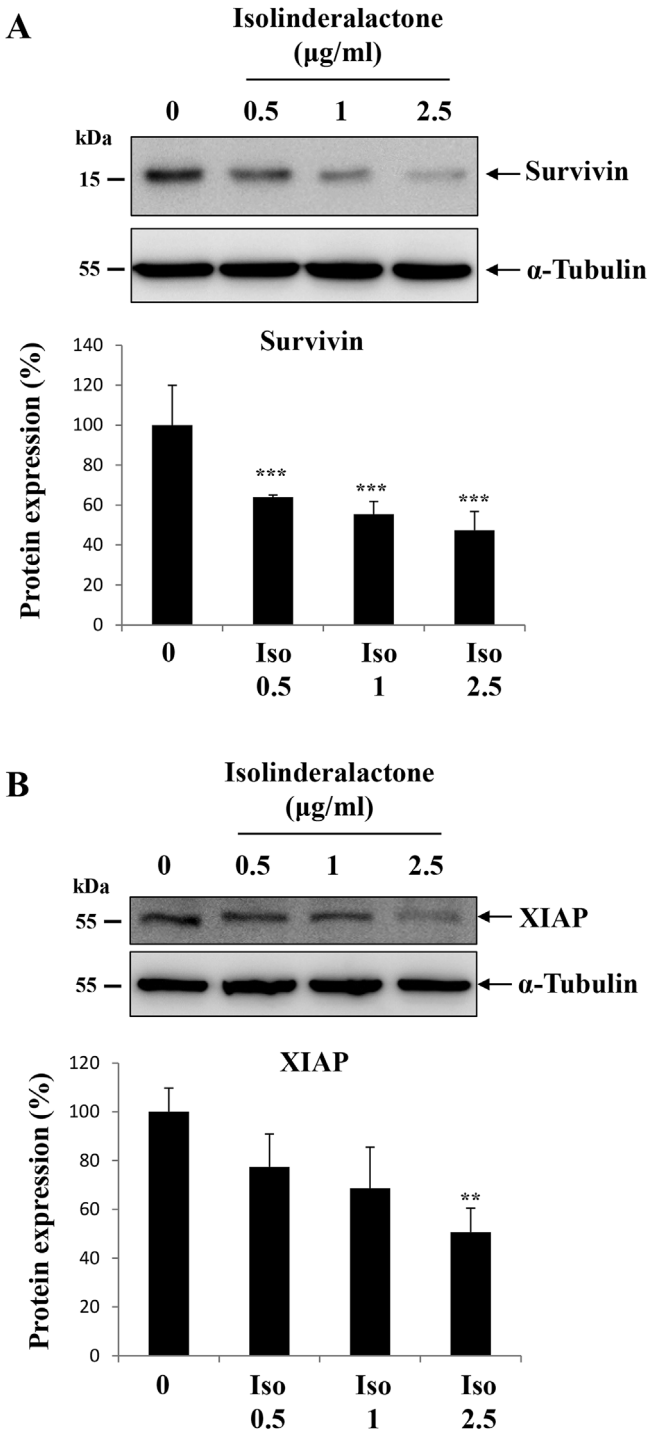

Fig. 4. Isolinderalactone treatment decreased inhibitors of apoptosis. Expression levels of the pro-survival protein survivin **(A)** and anti-apoptotic protein XIAP **(B)** were analyzed by western blotting (n = 5) in lysates of U-87 GBM cells treated with isolinderalactone for 48 h. Quantitative data are presented as the mean ± SEM. **p < 0.01, ***p < 0.001 versus the control group.

software (Image & Microscope Technology, Vancouver, BC, Canada).

2.9. Immunocytochemical analysis

U-87 GBM cells (6×10^4 cells/well) were seeded into a 4-well chamber (NUNC, Roskilde, Denmark), incubated overnight, and treated with 2.5 μg/mL isolinderalactone for 48 h. The cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min, followed by permeabilization with 0.3% Triton X-100 in PBS for 10 min. The cells were then incubated in 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h to block non-specific signals and labeled with an antibody against phosphorylated histone 2AX (1:2000, ab11174, γ-H2AX, phospho-S139; Abcam, Cambridge, UK) overnight at 4 °C, followed by incubation with an Alexa Fluor 594 fluorescent dye-conjugated secondary antibody (Life Technologies). The nuclei were stained with DAPI (Molecular Probe). Fluorescence images were taken with a Zeiss LSM 700 laser scanning confocal microscope (Carl Zeiss) and quantified using the iSolution full image analysis software (Image & Microscope Technology).

2.10. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Comparisons between two groups were performed using a Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Root extract of L. aggregata and isolinderalactone inhibits U-87 glioblastoma cell growth

We first measured the inhibition of U-87 GBM cell growth after treatment with the 29 medicinal herbs and selected the most effective one, a root extract of *L. aggregata*. As shown in Supplementary Fig. S1, the *L. aggregata* extract significantly reduced cell viability in a dose-dependent manner. We then tested eight commercially available compounds from the *L. aggregate* extract for their effects on viability of U-87 GBM using the CCK-8 assay (data not shown). Confluence of isolinderalactone-treated cells was reduced compared with that of the control cells (Fig. 1A), and isolinderalactone significantly inhibited cell viability in a dose-dependent manner (Fig. 1B and C). In addition, isolinderalactone treatment increased the Annexin V–FITC(+) population compared with that in the vehicle control (Fig. 1D and E), and the LDH assay showed that isolinderalactone treatment increased the LDH release in a dose-dependent manner (Fig. 1F). These results indicate that isolinderalactone may induce U-87 GBM cell death through apoptosis.

3.2. Isolinderalactone inhibits tumor growth in a human GBM xenograft mouse model

Because isolinderalactone inhibited U-87 GBM cell growth and induced cell death *in vitro*, we investigated whether the compound would inhibit tumor growth in an *in vivo* animal xenograft model (Fig. 2). U-87 GBM cells were injected subcutaneously into nude mice, and once the tumor volume reached a range from 70 to 100 mm^3, the mice were injected with the vehicle control or isolinderalactone (Fig. 2A). Administration of isolinderalactone (5 mg/kg) significantly reduced tumor volume (Fig. 2B and D, Supplementary Fig. S2) and weight (Fig. 2E). Body weight was also monitored during the entire 4 weeks of treatment, and the data showed no differences among the groups (Fig. 2F), which indicated the absence of systemic toxicity of isolinderalactone treatment.

3.3. Isolinderalactone regulates apoptosis-associated proteins

To identify the proteins involved in the induction of apoptosis in isolinderalactone-treated cells, we performed a human apoptosis proteome array analysis (Fig. 3A). Various apoptosis-related proteins, including BCL-2, cleaved caspase-3, survivin, and XIAP, were regulated in GBM cells by isolinderalactone treatment (2.5 μg/mL). We further confirmed the expression of these selected proteins by western blot

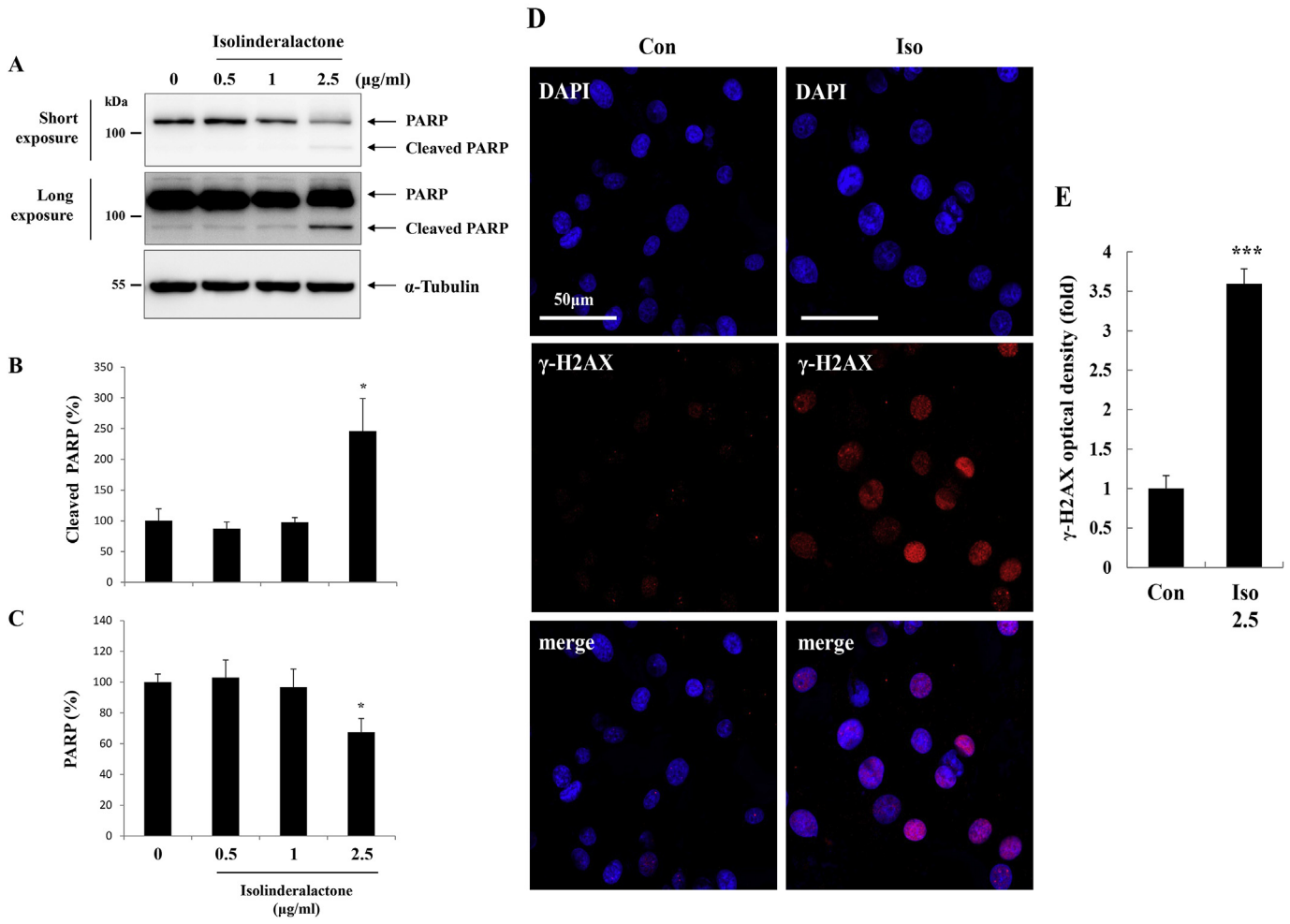

Fig. 5. Isolinderalactone increases cleavage of PARP and DNA breakage. (A) U-87 GBM cells were treated with the control (0.1% DMSO) and isolinderalactone for 48 h. Expression of the PARP protein was determined by western blotting. **(B, C)** Quantitative data for cleaved PARP and PARP are shown as the mean ± SEM (n = 4). *p < 0.05 versus the control group. **(D)** Immunofluorescence of γ-H2AX (red) after isolinderalactone (2.5 μg/mL) treatment. Nuclear DNA was counterstained with DAPI (blue). Magnification: × 400; scale bar = 50 μm. **(E)** Quantitative data for the intensity of γ-H2AX fluorescence. At least five to six randomly selected fields were analyzed from three independent experiments. Data are shown as the mean ± SEM. ***p < 0.001 versus the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

analysis. BCL-2, known as a cell survival protein that inhibits apoptosis, significantly decreased in a dose-dependent manner, whereas the level of BAX remained unchanged (Fig. 3B and C). Furthermore, the level of cleaved caspase-3 significantly increased at 2.5 μg/mL isolinderalactone (Fig. 3D). Moreover, the levels of survivin and XIAP, known to be highly correlated with a poor prognosis [12], significantly decreased, depending on the concentration of isolinderalactone (Fig. 4). These results suggested that isolinderalactone activated the apoptotic pathway via decreasing BCL-2, survivin, and XIAP expression, followed by an increase in cleaved caspase-3.

3.4. Isolinderalactone increases cleaved PARP and DNA damage

Cleaved caspase-3 catalyzes the cleavage of PARP to an inactive form, thus blocking DNA repair, which results in apoptosis [13,14]. Therefore, we investigated the PARP protein by western blotting and observed a significant increase in cleaved PARP at 2.5 μg/mL isolinderalactone (Fig. 5A–C). In addition, we examined the γ-H2AX, a marker for DNA damage (Fig. 5D and E). Immunofluorescence staining of untreated U-87 GBM cells showed insignificant and weak nuclear γ-H2AX staining. After 48 h of treatment with isolinderalactone (2.5 μg/mL), U-87 GBM cells showed predominantly bright γ-H2AX nuclear staining. Thus, the data suggest that isolinderalactone increases DNA breakage and thereby induces cell apoptosis.

3.5. Isolinderalactone induces apoptosis in a GBM xenograft mouse model

To confirm the apoptosis-inducing effect of isolinderalactone *in vivo*, we evaluated the expression of cleaved caspase-3 by immunofluorescence and also used TUNEL staining in xenograft tumor tissue (Fig. 6). Cleaved caspase-3 fluorescence was approximately 1.5-fold higher in the isolinderalactone-treated group than in the control group (Fig. 6A and B). In addition, detection of apoptosis by TUNEL staining showed a significantly higher number of TUNEL-positive cells in the isolinderalactone group than in the control group (Fig. 6C and D). These results indicate that isolinderalactone treatment induces apoptosis and thus increases tumor cell death *in vivo*.

4. Discussion

In this study, isolinderalactone was found to decrease the viability and increase the apoptosis of U-87 GBM cells. Isolinderalactone

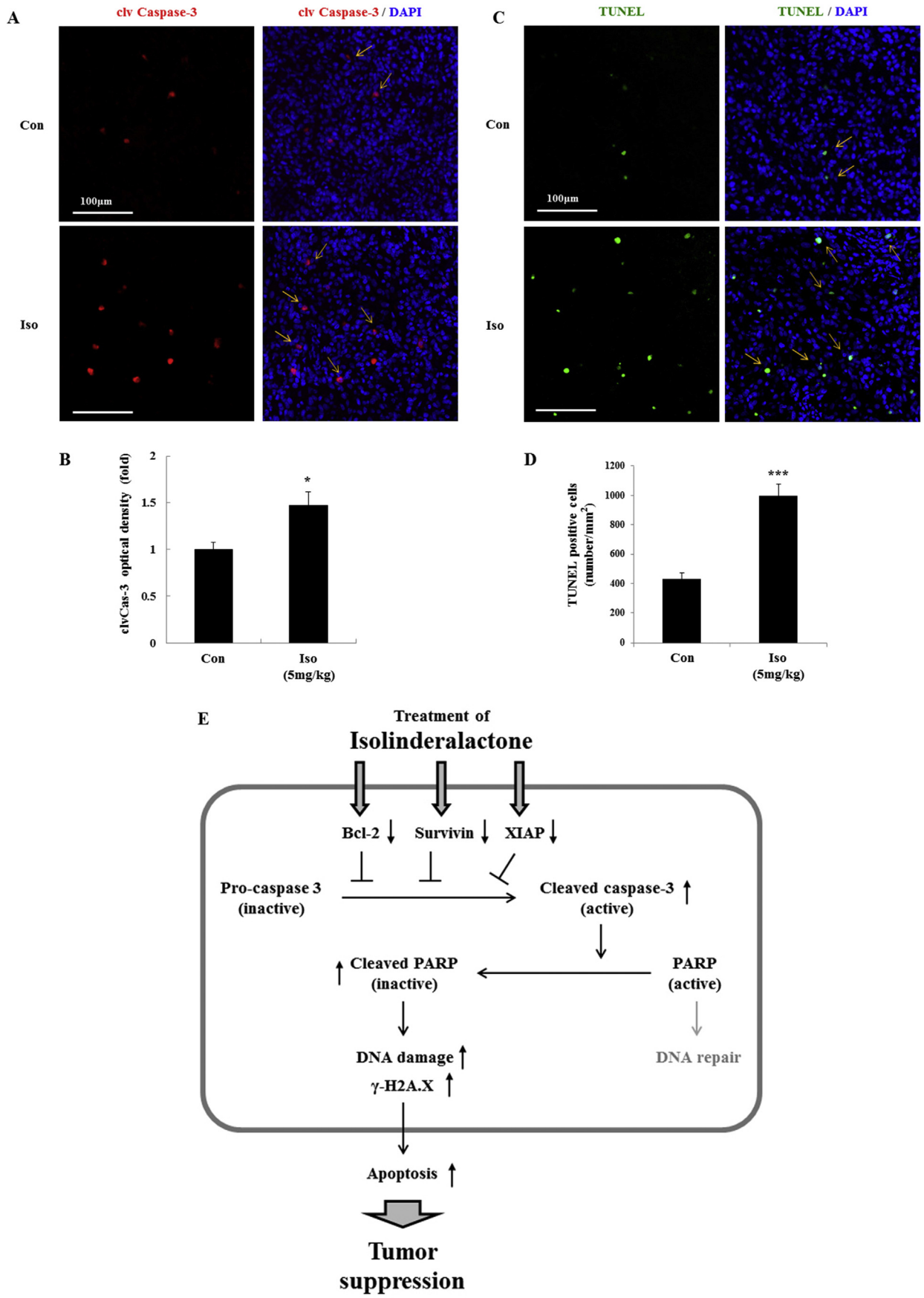

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Fig. 6. Isolinderalactone induces apoptosis in a xenograft mouse model. (A) Cleaved caspase-3 staining (red; arrows). The nuclei were counterstained with DAPI (blue). Magnification: × 200; scale bar = 100 μm. **(B)** Immunofluorescence intensity of cleaved caspase-3. Data are presented as the mean ± SEM. *p < 0.05 versus the control group. At least five randomly selected fields from one mouse were analyzed from five different mice. **(C)** Tumor tissues were stained with TUNEL (green; arrows) and DAPI (blue). Magnification: × 200; scale bar = 100 μm. **(D)** TUNEL-positive cells were quantified in tumor tissue. Data are shown as the mean ± SEM. ***p < 0.001 versus the control group. At least five randomly selected fields from one mouse were analyzed from five different mice. **(E)** Schematic diagram of the potential mechanism of isolinderalactone-induced apoptosis and GBM tumor growth inhibition. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

suppressed the expression of BCL-2, an antiapoptotic protein, and also decreased the expression of the prosurvival proteins, survivin and XIAP. Moreover, isolinderalactone increased the level of cleaved caspase-3, which can cleave PARP to an inactive form, thereby disrupting DNA repair and resulting in apoptosis. Isolinderalactone treatment also suppressed tumor growth, likely by inducing apoptosis. A schematic representation of the potential mechanism of isolinderalactone's effects on GBM is shown in Fig. 6E.

The root extract of *L. aggregata* has been used as a traditional herbal medicine, and has been reported to show cytotoxicity against human small-cell lung cancer cells [15,16]. Radix Linderae is the dried root of *L. aggregata* (Sims) Kosterm. [syn. *L. strychnifolia* (Sieb. & Zucc.) Fern.], and total alkaloids from Radix Linderae ameliorated inflammation and prevented joint destruction in a rheumatoid arthritis animal model [17]. In addition, these alkaloids were shown to inhibit the production of inflammatory cytokines by suppressing nuclear factor-κB (NF-κB) and the activation of mitogen-activated protein kinases in RAW 264.7 cells [18]. Root extracts of *L. aggregata* improve insulin sensitivity, slow the progression of diabetic nephropathy, and decrease the renal expression of transforming growth factor-β1 [15,19,20].

Isolinderalactone is one of the components of the root extract of *L. aggregata* [7], and previous studies have reported the effects of isolinderalactone on cancer cells [8–10,21]. It induced cell cycle arrest and FAS ligand-mediated apoptosis in A549 non-small-cell lung carcinoma cells [8,9], prevented the invasion and migration of A549 cells through the inhibition of MMP-2 [21], as well as induced apoptosis through the suppression of signal transducer and activator of transcription 3 (STAT3) signaling in MDA-MB-231 breast cancer cells [10]. However, the effect of isolinderalactone on glioblastoma has not been investigated yet. Interestingly, isolinderalactone exhibited a suppressive effect on glioblastoma; the compound suppressed tumor growth (Fig. 2), induced apoptosis *in vitro* and *in vivo* (Figs. 1 and 6), and stimulated the BCL-2/caspase-3/PARP pathway (Figs. 3–5), suggesting that isolinderalactone may be a potential candidate for antiglioblastoma drug development.

Malignant gliomas show genetic inactivation of proapoptotic pathways or activation of gene expression that can promote cell survival and overcome cell growth inhibition [3]. Thus, genetic inactivation has been reported for caspases and other proteins directly involved in the apoptotic pathway [22]. Another mechanism for escaping cell death is overexpression of genes that can activate cell survival. The BCL-2 protein family plays an important role in apoptosis [23]. Proapoptotic proteins such as BAX, BCL-2 homologous antagonist/killer (BAK), and BCL-2-related ovarian killer (BOK) are activated, while prosurvival proteins such as BCL-2, BCL-extra large (BCL-XL), and BCL-2-like protein 2 (BCL-W) are inhibited. A low level of BCL-2 is associated with mitochondrial dysfunction and the release of intermembrane proteins, such as cytochrome *c*, which activate caspases [24]. Overexpression of the antiapoptotic protein BCL-2 significantly increases tumor cell resistance against chemotherapies such as paclitaxel, vincristine, and doxorubicin, as well as against gene therapy [23]. In this study, we showed that isolinderalactone treatment decreased the expression of BCL-2 in U-87 GBM cells, without affecting BAX expression (Fig. 3).

As members of the inhibitor of apoptosis (IAP) family, survivin and XIAP are also important regulators of apoptosis and act by inhibiting caspase activation [25]. When survivin binds to caspase-9, the apoptosis pathway is deactivated. Therefore, procaspase-3 is not cleaved and does not, in turn, cleave PARP. As a result, PARP maintains an active state and continues DNA repair, resulting in cell survival [13,14,26]. The expression of survivin and XIAP is associated with a poor prognosis and tumor recurrence in many cancers [12,27]. Inhibition of survivin using nanoparticles and a survivin-targeting treatment suppressed tumor growth and promoted apoptosis [28,29]; thus, survivin is considered a new target for apoptosis-based cancer therapy. In our study, isolinderalactone treatment significantly decreased the expression of both survivin and XIAP (Fig. 4) and increased DNA damage, as indicated by the expression of γH2AX in U-87 GBM cells (Fig. 5).

It is unclear as to how isolinderalactone regulates the expression of BCL-2, survivin, and XIAP. One possible mediator that is overexpressed in cancer is the transcription factor NF-κB. NF-κB is a well-known transcription factor in inflammation and immune responses, and has an essential role in cancer initiation and progression [30]. It has been reported that NF-κB activates the IAP family members (c-IAP, XIAP, and survivin). Furthermore, the target gene of the NF-κB signaling pathway is BCL-2 [30]. In addition, total alkaloids from Radix Linderae decrease the production of inflammatory mediators through suppression of NF-κB and MAPK activation in RAW 264.7 cells [18]. In conclusion, while further studies are required to investigate the mechanisms underlying the inhibitory effects of isolinderalactone on the expression of BCL-2, survivin, and XIAP, our data showed that isolinderalactone activates the apoptosis pathway in U-87 GBM cells and inhibits tumor growth *in vitro* and *in vivo*. Therefore, isolinderalactone could be a potential candidate for developing antiglioblastoma therapeutics.

Authors' contributions

Participated in the research design: Seo-Yeon Lee and Hwa Kyoung Shin.

Conducted the experiments: Ji Young Hwang, Jung Hwa Park, and Min Jae Kim.

Performed data analysis: Seo-Yeon Lee, Ki-Tae Ha, Woo Jean Kim, and Byung Tae Choi.

Wrote or contributed to the writing of the manuscript: Ji Young Hwang, Seo-Yeon Lee, and Hwa Kyoung Shin.

All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.027.

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