β-Elemene inhibits proliferation through crosstalk between glia maturation factor β and extracellular signal-regulated kinase 1/2 and impairs drug resistance to temozolomide in glioblastoma cells

TING-ZHUN ZHU, XIAO-MING LI, LI-HAN LUO, YING-HUI XU, PENG CAO, YANG LIU and GUO-BIAO LIANG

1Department of Neurosurgery, The General Hospital of Shenyang Military Region, Shenyang, Liaoning 110840; 2Health Care Centre, Shenyang Entry-Exit Inspection and Quarantine Bureau, Shenyang, Liaoning 110016; 3Department of Neurosurgery, The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011, P.R. China

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Abstract. β-elemene, a plant-derived drug extracted from Curcuma wenyujin, has demonstrated marked antiproliferative effects on glioblastoma, while toxicity remains low. However, the underlying molecular mechanisms of the antitumor activity of β-elemene remain to be elucidated. Previously, it was identified that the glia maturation factor β (GMFβ)/mitogen-activated protein kinase kinase (MAPK) 3/6/p38 pathway participates in the antiproliferative activity of β-elemene on glioblastoma. In the present study, in order to illustrate the association of GMFβ and the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, U87 and U251 cells were treated with β-elemene at various doses and for different durations, and the expression of phosphorylated ERK1 (p-ERK1/2), ERK1/2, B-cell lymphoma 2 (Bcl-2), Bcl2-associated X and survivin was examined by western blot analysis. Following treatment with β-elemene and the ERK1/2 inhibitor PD98059, U87 cell viability was evaluated using a Cell Counting Kit-8 (CCK-8) assay, and the expression levels of Bcl-2 and survivin were examined by western blot analysis. GMFβ was then downregulated by RNA interference in β-elemene-treated U87 cells, and the effect of this on the expression of ERK1/2 and p-ERK1/2 was determined by western blot analysis. Finally, the chemosensitisation of U87 cells to temozolomide (TMZ) through β-elemene was examined using the CCK-8 assay. The results demonstrated that β-elemene inhibited the proliferation of U87 glioblastoma cells through the GMFβ-dependent inactivation of the ERK1/2-Bcl-2/survivin pathway. Furthermore, inhibition of ERK1/2 by PD98059 enhanced the antitumor effect of β-elemene and impaired the expression levels of Bcl-2 and survivin. β-elemene also increased the sensitivity of U87 glioblastoma cells to the chemotherapeutic TMZ, which was synergistically enhanced by PD98059. In conclusion, these results suggested that GMFβ-dependent inactivation of the ERK1/2-Bcl-2/survivin pathway mediated the antiproliferative effect of β-elemene on glioblastoma. Therefore, β-elemene is a promising chemosensitizer or adjuvant therapeutic for TMZ against glioblastoma brain tumors.

Introduction

Glioblastoma is the most common and aggressive subset of primary brain tumors, accounting for ~52% of all primary intracranial tumors. Glioblastomas are lethal tumors, characterized by high chemotherapy resistance and diffuse infiltration into the brain tissue. Despite the numerous advances in the development of cancer therapeutics in recent years, the prognosis for patients with glioblastoma remains discouraging. An effective antitumor medicine is the extract from Curcuma wenyujin, and this essential oil contains a mixture of β-, γ- and δ-elemene (1). β-elemene (1-methyl-1-ethenyl-2,4-isopto-penyl-cyclohexane, molecular formula: C14H22; molecular weight: 204.34), the major active anticancer component in the elemene mixture, has demonstrated a strong antiproliferative effect and induces apoptosis in various types of tumor, including glioma, breast, liver, laryngeal, leukemia and ovarian cancer (2-6). Previous studies by our group identified that β-elemene inhibited the proliferation of glioblastoma cells and induced cell apoptosis in vitro and in vivo (6-9). Furthermore, β-elemene exerted significant chemosensitisation effects in combination with cisplatin treatment of glioblastomas (8). However, the mechanisms underlying the antiproliferative effect of β-elemene in glioblastoma remain to be elucidated.

Glia maturation factor β (GMFβ), also known as GMF, is a 17 kDa intracellular regulator of stress-associated signal transduction and is predominantly expressed in astrocytes in the brain. GMFβ is necessary for the growth and maturation of brain glial cells and neurons (10,11). GMFβ is able to inhibit the growth of rat C6 and human HG-1 glioblastoma cells through...
G0/G1 cell cycle arrest in vitro, reducing tumor volume and increasing the number of cells expressing glial fibrillary acidic protein (GFAP, a marker for normal astrocytes) in the tumor tissue of glioblastoma-bearing mice (12). Cellular behavioural responses to extracellular stimuli (e.g., growth factors and hormones) are also regulated by the mitogen-activated protein kinase (MAPK) pathway, which participates in the cascade reaction of MAPK kinase kinase (MKKK)-MAPK kinase (MKK)-MAPK (13). MAPK family members include extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and stress-activated c-Jun N-terminal protein kinase (JNK). The ERK1/2 cascade is an evolutionarily conserved pathway and is critical in regulating multiple fundamental cellular processes, including proliferation, cell survival, differentiation, tumorigenesis and development. Constitutive activation of the ERK1/2 pathway is functionally important for cell proliferation and drug resistance in glioblastoma and ovarian cancer (14,15).

It was previously identified that β-elemene is able to arrest U87 and C6 glioblastoma cells in G0/G1 phase of the cell cycle and inhibit cell proliferation through the activation of GMFβ-MKK3/6-p38 and the downregulation of phosphorylated ERK1/2 (p-ERK1/2 (6,8,16). Overexpression of GMFβ activates p38 and simultaneously inhibits the activity of ERK1/2 in C6 cells (17,18). Therefore, it was hypothesized that there may be a potential association between the activation of GMFβ and inactivation of the ERK1/2 pathway in the antiproliferative action of β-elemene on glioblastoma cells.

In the present study, the regulatory action of GMFβ on the ERK1/2 pathway in the antiproliferative effect of β-elemene on glioblastoma was examined. The effect of β-elemene on the ERK1/2/B-cell lymphoma 2 (Bcl-2)/survivin pathway in association with GMFβ was examined. Furthermore, the effect of GMFβ silencing by transfecting small interfering (si)RNA into glioblastoma on the phosphorylation levels of ERK1/2 was assessed. In addition, the effect of β-elemene on the sensitivity of U87 glioblastoma cells to the chemotherapeutic temozolomide (TMZ) was tested. The results indicated that GMFβ-ERK1/2-Bcl-2/survivin pathway may be a putative target for novel molecular therapeutic strategies against glioblastoma in the future.

Materials and methods

Reagents, antibodies and cell culture. β-Elemene (98% purity) was obtained from Jingang Pharmaceutical Co., Ltd. (Dalian, China). TMZ was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against p-ERK1/2, ERK1/2, GMFβ, survivin, Bcl-2, Bcl-2-associated X protein (Bax) and GAPDH and PD98059 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). siRNAs for GMFβ and a negative control were obtained from Shanghai GenePharma Co., Ltd. (Pudongxinqu, Shanghai, China). A reverse transcription polymerase chain reaction (RT-PCR) kit was purchased from Takara Bio, Co., Ltd. (Dalian, China). The Lipofectamine 2000 transfection reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The Cell Counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). All of the other reagents were obtained from Sigma-Aldrich. The human U251 and U87 glioblastoma cell lines were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences, maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (Invitrogen Life Technologies), 50 IU/ml penicillin (Invitrogen Life Technologies) and 50 µg/ml streptomycin (Invitrogen Life Technologies) and grown at 37°C in a humidified atmosphere with 5% CO₂.

Cell proliferation assay. Cell viability was evaluated by a CCK-8 assay. The cells in exponential growth phase were cultured in a 96-well culture plates and treated according to the study design. Then, 10 µl of CCK-8 was added to each well and the mixture was incubated for 4 h at 37°C. The optical density (OD) of each well was measured at 450 nm using a spectrophotometric microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Five replicate wells were designed for each cell sample. Cells were examined using an inverted microscope (ECLIPSE TE2000-U; Nikon, Tokyo, Japan).

Western blot assay. The cells were lysed with radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 150 mM NaCl, 1 mM aprotinin, 1 mg/ml phenylmethanesulfonyl fluoride, 1 µg/ml pepstatin and 1 µg/ml leupeptin]. The concentrations of total protein in the cellular extracts were measured using the bicinchoninic acid assay kit from Keygen Biotech, Co., Ltd. (Nanjing, China). Following separation by 12% SDS-PAGE, the membranes were transferred to nitrocellulose filter membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with Tween-20 at 4°C overnight and probed with various primary antibodies at 4°C overnight. This included primary antibodies against p-ERK1/2 (goat polyclonal IgG), ERK1/2 (rabbit polyclonal IgG), GMFβ (mouse monoclonal IgG), survivin (mouse monoclonal IgG), Bax (rabbit polyclonal IgG) and GAPDH (mouse monoclonal IgG) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Followed by incubation with horseradish peroxidase-conjugated secondary antibodies (donkey anti-goat IgG, goat anti-mouse IgG and mouse anti-rabbit IgG; Santa Cruz Biotechnology, Inc.) at 37°C for 3 h. The membranes were exposed to an enhanced chemiluminescence system (Amersham Biosciences, Uppsala, Sweden) and fluorescence was detected by exposing the membrane to X-ray film (Fujiﬁlm Co., Ltd., Tokyo, Japan). The results were scanned with the Image Quant 5.2 software (Amersham Biosciences) and the gray bands were semi-quantitatively evaluated using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Rockville, MD, USA). The gray values were normalized to GAPDH.

RNA silencing. The cells were plated at a density of 4x10⁴ cells/well in six-well plates or 4x10⁵/well in 96-well plates and cultured for 24 h. siRNA oligonucleotides were transfected into glioblastoma cells with Lipofectamine 2000 according to the manufacturer’s instructions. Post-transfection (24 h), the cells were treated with β-elemene for 24 h. siRNA oligonucleotides were obtained from Shanghai GenePharma Co., Ltd. and specific sequences identical to those used in a previous study by our group (8) were as follows: Human
GMFβ (NCBI reference sequence: NM_004124.2), siRNA sense 5'-GCUCAUUGUGUAAGUATT-3' and antisense 5'-UAACUAUACAAUGAGCTT-3'. The negative control oligonucleotide sequences are as follows: Sense, 5'-UUUCGGAACGUGUCAGGUTT-3' and antisense, 5'-ACGUGACACGCUCCGAGAATT-3'. A Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD, USA) search was performed to ensure that the selected GMFβ siRNA sequences only targeted the GMFβ gene. The efficiency of RNA interference was determined by western blot analysis.

**Statistical analysis.** Values are expressed as the mean ± standard deviation of at least three independent experiments. Statistical analysis was performed using Student's t-test. A level of P<0.05 was considered to indicate a statistically significant difference between groups and P<0.01 was considered to indicate a highly significant difference. Statistical analysis was performed with SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA).

**Results**

### β-elemente inhibits the proliferation of human U87 and U251 glioblastoma cells

To evaluate the antitumor effect of β-elemente on glioblastoma cells, human U87 and U251 glioblastoma cells were treated with β-elemente at variable doses or for different durations. Cell viability was measured with a CCK-8 assay. It was identified that the viability of human U87 and U251 glioblastoma cells treated with β-elemente evidently decreased as the drug dose (Fig. 1A and C) or treatment time (Fig. 1B) increased. These results suggested that β-elemente inhibited the proliferation of human glioblastoma cells in a dose- and time-dependent manner.

### β-elemente decreases the phosphorylation levels of ERK1/2 and impairs the expression of Bcl-2 and survivin

To investigate the role of the ERK1/2 signalling pathway in the antiproliferative effect of β-elemente on glioblastoma cells, the expression levels of p-ERK1/2, ERK1/2, Bcl-2, and survivin were examined by western blot analysis in β-elemente-treated U87 cells (Fig. 2). β-elemente decreased the expression of p-ERK1/2, Bcl-2 and survivin in human U87 cells. By contrast, β-elemente did not affect the expression levels of total ERK1/2 and Bax.

### Inhibition of ERK1/2 enhances the antiproliferative effect of β-elemente on glioblastoma in vitro

To further confirm the role of ERK1/2 inactivation in the anti-proliferative effect of β-elemente on glioblastoma cells, ERK1/2 was pretreated with 30 µM PD98059 for 1 h in U87 cells. Following treatment with 80 µg/ml β-elemente for 24 h, U87 cell viability was measured by the CCK-8 assay (Fig. 3A) and the expression levels of Bcl-2 and survivin were detected using western blot analysis (Fig. 3B and C). The antiproliferative effect of β-elemente on glioblastoma was enhanced by PD98059, and the inhibition of ERK1/2 further decreased the expression of Bcl-2 and survivin in human U87 glioblastoma cells.

### Downregulation of GMFβ decreases the inactivating activity of β-elemente on the ERK1/2 pathway

A previous study by our group reported that β-elemente inhibited the proliferation of U87 cells via activation of the GMFβ-MKK3/6-p38 signalling pathway (6-8). Several studies have
demonstrated that the overexpression of GMFβ simultaneously activates p38 and inhibits the activity of ERK1/2 in C6 glioblastoma cells (17,18). To investigate the correlation between the GMFβ and the ERK1/2 pathway in the antiproliferative effect of β-elemene, RNA interference was performed using GMFβ siRNA to downregulate GMFβ expression in the U87 cells. The interference efficiency was determined by western blot analysis (Fig. 4A and C). U87 cells were then treated with 80 µg/ml β-elemene for 24 h and the expression levels of p-ERK1/2 were detected by western blot analysis (Fig. 4B and D). Transfection with 53 nM GMFβ siRNA for 24 h significantly decreased the expression of GMFβ. The p-ERK1/2 levels were increased in the GMFβ siRNA group as compared with those in the control group. Silencing of GMFβ decreased the inactivating action of β-elemene on the ERK1/2 pathway. In conclusion, the inactivation of ERK1/2 depends on GMFβ activation and mediates the antiproliferative effect of β-elemene on glioblastoma.
Treatment with PD98059 promotes the chemotherapy sensitising effect of β-elemene. As previously reported, the ERK1/2 signalling pathway is associated with drug resistance in numerous types of cancer, including glioblastoma and ovarian cancer (14,15). Earlier studies identified that β-elemene, as a chemosensitizer, increased the sensitivity of glioblastoma cells to cisplatin-induced cytotoxicity. To confirm whether treatment with β-elemene sensitized glioblastoma cells to TMZ-induced cytotoxicity, U87 cells were plated in 96-well plates and cultured for 24 h. Five groups were designed: a, not treated with TMZ, β-elemene or PD98059; b, treated with 80 µg/ml β-elemene for 24 h; c, treated with 300 µM TMZ for 24 h; d, treated with 80 µg/ml β-elemene combined with 300 µM TMZ for 24 h and e, treated with 80 µg/ml β-elemene combined with 300 µM TMZ and 30 µM PD98059 for 24 h. (B) Cell viability was measured by a CCK-8 assay and quantified. The results demonstrated that the viability of the cells in group e was lower than that in groups a, b, c or d. Treatment with β-elemene increased the sensitivity of U87 cells to TMZ-induced cytotoxicity and this chemotherapy sensitising effect was enhanced by PD98059. Values are presented as the mean ± standard deviation (**P<0.01). TMZ, temozolomide; CCK-8, Cell Counting Kit-8.

Discussion

Glioblastoma is the most common and lethal type of brain tumor. In spite of improvements in cancer therapeutics, the prognosis for patients with glioblastoma remains poor. The majority of chemotherapeutics are not effective in treating glioblastoma due to frequent drug resistance and the severity of the various associated side effects (19). Elemene has demonstrated potent antitumor activity against various tumor types in vitro and in vivo. β-elemene is able to arrest non-small cell lung cancer cells at the G2/M phase and induce apoptotic cell death (4). Elemene inhibited the growth of HEp-2 laryngeal cancer cells and induced cell apoptosis with decreased expression of eukaryotic translation initiation factors 4E, 4G, basic fibroblast growth factor and vascular endothelial growth factor (20). Furthermore, β-elemene decreased the expression levels of the Bel-2 protein, increased cytochrome c release, and activated poly ADP-ribose polymerase and caspase-3, -7, -9 and -10 in prostate cancer cells. Concurrently, the apoptotic percentage of prostate cancer cells was increased by β-elemene in a dose- and time-dependent manner (21).

In the past ten years, numerous clinical and basic studies have been conducted on the antiproliferative effect of elemene on glioblastoma carcinoma. Elemene is able to significantly reduce the size of tumors and prolong the lifespan of patients, without serious side effects. Simultaneously, elemene has demonstrated strong antitumor activity on glioblastoma cell lines from humans, rats and glioblastoma-bearing nude mice by inducing tumor cell apoptosis, inhibiting cell proliferation and arresting cell cycle processes (6-9,22,23). However, the molecular mechanisms of the antitumor activity of β-elemene are not well defined, and this hinders its application in the clinical treatment of glioblastoma.

GMFβ is an intracellular protein primarily localized in the mammalian central nervous system and is important in regulating the growth and development of glial cells and neurons. GMFβ has been identified to mediate apoptosis in glioblastoma cells and the development of an inflammatory response (24-26). Several studies have suggested that GMFβ interacts with ADF/cofilin to promote the remodelling
and/or disassembly of brain cortical actin structures (27). Furthermore, GMFβ inhibited the proliferation of rat C6 and human HG-1 glioblastoma cells and restored cell contact inhibition (12). One particular study identified that the overexpression of GMFβ in N18 neuroblastoma cells increases caspase-3 activity and causes cytotoxicity and a loss of cell viability (28). It is widely recognized that a complex crosstalk exists between GMFβ and MAPK signalling pathways. For example, overexpression of GMFβ triggered an inflammatory response through the GMFβ-p38/necrosis factor-κB/granulocyte macrophage colony-stimulating factor/tumor necrosis factor-α, interleukin (IL)-1β and IL-6 signalling pathways in astrocytes (10). Furthermore, the overexpression of GMFβ is able to activate p38 and simultaneously induce the dephosphorylation of ERK1/2 (17,18).

In recent years, numerous basic studies on the anti-glioblastoma molecular mechanism of β-elemene have been performed. It has previously been reported that β-elemene is able to arrest cells at G0/G1 in the cell cycle and activate the GMFβ-MKKβ/p38 pathway to inhibit the growth of human U87, U251 and rat C6 glioblastoma cells (6-8). In the present study, it was identified that the GMFβ-dependent downregulation of p-ERK1/2-Bcl-2/survivin mediates the anti-glioblastoma proliferation effect of β-elemene. The mechanism underlying the adverse regulation of GMFβ of the ERK1/2 and p38 MAPK pathways remains elusive. GMFβ can be phosphorylated at threonine 26/serine 82 by protein kinase (PK)A, at serine 71 by PKC, at threonine 26 by p90 ribosomal S6 kinase and at serine 52 by casein kinase II. The generation of various phosphorylation sites on GMFβ may explain its adverse modulatory actions on different MAPK members (29). According to this study, it is hypothesized that β-elemene inhibits the proliferation of human glioblastoma cells, induces cell apoptosis and causes cell cycle arrest in G0/G1 phase by activating GMFβ, which activates MKK3/6-p38 and simultaneously inhibits the ERK1/2-Bcl-2/survivin pathway.

TMZ is a monofunctional alkylating agent widely used in the clinic as a first-line chemotherapeutic agent against newly diagnosed or recurrent glioblastoma (30,31). However, glioblastoma cells from numerous patients often exhibit strong chemoresistance to TMZ. In the present study, it was identified that β-elemene, as a chemosensitizer, is able to increase the sensitivity of glioblastoma cells to TMZ. Furthermore, the chemosensitisation effect of β-elemene is enhanced by the ERK1/2 inhibitor PD98059, which suggests that the constitutive activation of ERK1/2 may be a drug-resistant factor in U87 glioblastoma cells. Several studies have demonstrated that ERK1/2 regulates numerous multidrug resistance proteins, including p53, O6-methylguanine-DNA-methyltransferase, multidrug resistance-associated protein-1, p-glycoprotein and glutathione S transferase π, to mediate chemoresistance in glioblastoma, hepatocellular, gastric, ovarian and lymphoma carcinoma types (32-35,16).

In conclusion, the present study suggested that the antiproliferative effect of β-elemene on glioblastoma proceeds via the inactivation of the ERK1/2-Bcl-2/survivin pathway, which is dependent on GMFβ activation. The GMFβ-ERK1/2-Bcl-2/survivin pathway may be a putative target for molecular therapy against glioblastoma. In addition, β-elemene should be investigated further, to facilitate the development of a combined therapeutic regimen with TMZ for a more efficacious strategy in the treatment of glioblastoma primary brain tumors.

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