

Original Article

A high-throughput drug screen for identifying NF- κ B inhibitors to suppress proliferation of malignant brain glioma cells

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Abstract: Purpose: Malignant glioma is the most common brain tumor and one of the most devastating human diseases. The goal of this study was to identify drugs that inhibit the proliferation of malignant brain glioma both *in vivo* and *in vitro*. Method: A commercial kinase inhibitor library containing 464 drugs was screened. Using Western blotting, quantitative Polymerase Chain Reaction (qPCR) and Enzyme-linked immunosorbent assay (ELISA), the inhibitory effects of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) inhibitors on the growth of malignant brain glioma cells was tested. An *in vivo* glioma tumor-bearing mouse model was used to investigate the effect of NF- κ B inhibitors *in vivo*. Results: Three NF- κ B inhibitors, namely caffeic acid phenethyl ester (CAPE), JSH-23, and sodium 4-aminosalicylate (S4-A)-significantly suppressed proliferation of three malignant brain glioma cell lines and significantly suppressed primary malignant glioma cell proliferation. Pharmacological NF- κ B inhibition led to decreases in phospho-p65 levels and in TNF- α expression at both the transcriptional and translational levels. *In vivo* experiments showed that CAPE exerted strong antitumor activity in an *in situ* tumor-formation mouse model. The NF- κ B pathway activation may be a crucial step in the growth of malignant brain glioma cells. Conclusion: This research identifies a potential new treatment for malignant brain glioma.

Keywords: Glioma, drug screen, proliferation, brain

Introduction

Malignant glioma is the most common brain tumor and one of the most devastating human diseases [1]. The histogenetic origin of malignant glioma is unclear, and due to its location in the brain, clinical treatment is complex. A multi-regimen of radiotherapy and chemotherapy is the most commonly used treatment to achieve optimal results from tumor-debulking surgery [2]. Unfortunately, the survival rate of glioblastoma is poor, and most patients die within 1-1.5 years despite aggressive management by surgery, radiation, and chemotherapy [3]. Indeed, as surgery, chemoradiation and adjuvant immunotherapy cannot reduce the high rates of recurrence after treatment, new treatment modalities, such as targeted therapy and immune checkpoint therapy, must be developed [4]. The most common and lethal malignant glioma is WHO grade IV glioblastoma, which has a medi-

an survival of 15 months with the standard multi-regimen. Gliomas are also highly resistant to cytotoxic therapies: treatment with temozolomide improves survival only 2.5 months beyond that with radiation and surgery alone [5].

As an emerging approach, manipulation of immune checkpoints to treat malignancy has provided new opportunities for therapy for malignant glioma, though autoimmune toxicity may impede their development. CTLA-4 and PD1/PD-L1 inhibitors are two FDA-approved therapeutics that target negative regulatory pathways of T cells, and combination therapy involving CTLA-4 and PD-1 blockade may be more efficacious than blockade of either mediator alone. A preclinical study in mice suggested that PD-1 inhibition was more effective than PD-L1 inhibition, which, in turn, was more effective than CTLA-4 inhibition, and that the combination of CTLA-4 and PD-1 inhibition was 75%

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effective [6]. Overall, the development of targeted therapies has substantially broadened with advances in genetic sequencing and gene expression profiling [7, 8]. Moreover, scientists are attempting to clarify the molecular mechanisms of glioma pathogenesis. For example, glioma can be treated by targeting the genes involved, and the most relevant signaling associated with glioma are the p53, RTK/PI3K/MAPK, and Rb pathways [9]. However, no clinical trials targeting these pathways using PDGFR, mTOR, VEGF, or VEGFR inhibitors have demonstrated significantly extended patient survival [10]. Redundancy of signal transduction, pathways between tumor cells, genomic heterogeneity and blood-brain barrier protection of infiltrating cells are the main causes of poor prognosis [11, 12]. Thus, the goal of this study was to identify drugs that can target the key pathways mediating glioma growth, thus providing new drugs for use in the clinical treatment of glioma in the near future.

In the present study, a kinase inhibitor library containing over 464 compounds was screened using the U87 cell line and validated the results in M059K, U251 and primary malignant glioma cells (**Figure 1**). Unexpectedly, among the tested compounds, three NF- κ B inhibitors (caffeic acid phenethyl ester (CAPE), JSH-23 and sodium 4-aminosalicylate (S4-A)) strongly suppressed the glioma cell growth. Then, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway was shown to be a critical regulator of growth of glioma cells. Furthermore, Both *in vitro* and *in vivo* studies show that three NF- κ B inhibitors-CAPE, JSH-23, and S4-A, have the potential to serve as therapeutic drugs for malignant glioma.

Materials and methods

Cell lines

U87, M059K and U251 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and $1 \times$ penicillin-streptomycin. Cell lines were maintained in a 5% CO₂ incubator at 37°C and routinely tested to exclude mycoplasma contamination.

Drug screening and cell viability assay

U87, M059K, U251 and primary malignant glioma cells were seeded into 96-well plates at a

density of 5000 cells per well. Cells were treated with compounds (10 μ M) from the Target Selective Inhibitor Library (#L3500, Selleck Chemicals) for 24 hours. Subsequently, 100 μ l of fresh medium containing 10 μ l of Cell Counting Kit-8 (CCK-8) solution (Dojindo Laboratories, Tokyo, Japan) was added to the cells and incubated for 1 h (37°C, 5% CO₂). Absorbance was measured at 450 nm using a microplate reader (Tecan, Morrisville, NC, USA).

Isolation and culture of primary human malignant brain glioma cells

The Institutional Review Board of the Army Medical University of China approved the use of human brain tumor tissue for this study. Patient consent for participation in the study or for tissue use was obtained. Brain tumor tissues were collected from 5 patients (3 males and 2 females) with newly histologically confirmed malignant brain glioma. Tumors (100 to 200 mg wet weight) were placed in 10 ml sterile high-glucose DMEM supplemented with 2% B27 (Invitrogen, Carlsbad, CA) and 0.5 mM glutamine (Invitrogen) in a 50 ml tube at 4°C. Tissues were minced by ophthalmic scissors and digested for 30 minutes at 37°C with dispase (50 U/ml, BD Biosciences) and 0.01% DNase I. The isolated cells were cultured in high-glucose DMEM supplemented with 10% FBS, 2% B27, penicillin-streptomycin and 0.5 mM glutamine.

qPCR and ELISA

Cells were treated with 10 μ M NF- κ B inhibitors (CAPE, JSH-23 and S4-A) for 24 hours. Total RNA was isolated from the transfected cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. cDNA was prepared using reverse transcriptase (Takara Biotech) and an oligo-dT primer and then purified using a PCR purification kit (Takara Biotech). qPCR was performed using a SYBR ExScript RT-PCR kit (Takara Biotech) on a quantitative PCR system (Bio-Rad, Hercules, CA). The presence of human tumor necrosis factor alpha (TNF- α) in cell supernatants was analyzed by ELISA (Endogen, Woburn, MA) according to the manufacturer's instructions.

Western blot analysis

For Western blot analysis, cells were lysed in RIPA buffer supplemented with protease in-

NF-κB inhibitors suppress proliferation of glioma cells

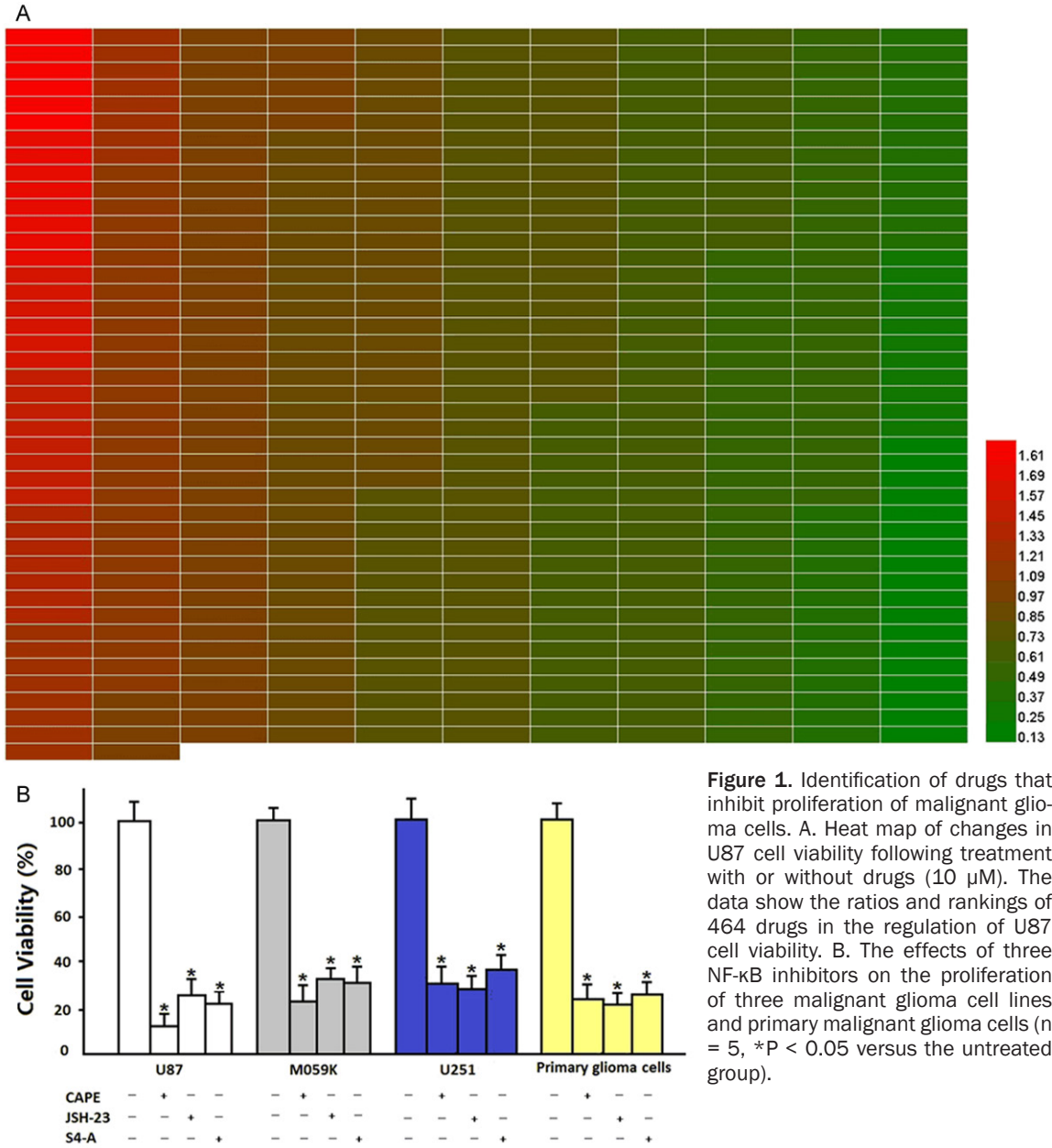


Figure 1. Identification of drugs that inhibit proliferation of malignant glioma cells. A. Heat map of changes in U87 cell viability following treatment with or without drugs (10 μM). The data show the ratios and rankings of 464 drugs in the regulation of U87 cell viability. B. The effects of three NF-κB inhibitors on the proliferation of three malignant glioma cell lines and primary malignant glioma cells (n = 5, *P < 0.05 versus the untreated group).

inhibitor cocktail (Sigma, P8340), phosphatase inhibitor cocktail (Sigma, P5726), and 1 mM Na₃VO₄ using sonication. The supernatant was centrifuged, and proteins were separated by SDS-PAGE (GenScript, M42012CS) and transferred to PVDF membranes (Sigma, 03010040001). The membranes were blocked with Tris-buffered saline with Tween 20 (TBST) containing 5% skim milk for 1 hour at room temperature and then incubated with the indicated primary antibodies (1:2000) overnight at 4°C. After washing with TBST, the membranes were incubated with an HRP-linked anti-mouse IgG

secondary antibody or an HRP-linked anti-rabbit IgG secondary antibody (1:2000) (Cell Signaling Technology; #7076 for anti-mouse, #7074 for anti-rabbit) for 1 hour at room temperature. The membranes were washed three times in TBST and then analyzed with a ChemiDoc Touch Imaging System (Bio-Rad, USA). The band intensities were evaluated with Image Lab software. Antibodies against the following proteins were obtained from Cell Signaling Technology: phospho (p)-p65 (S536) (#3033P), p65 (#6959), p-STAT1 (Tyr701) (#9167), STAT1 (#14994), and β-actin (#3700).

NF- κ B inhibitors suppress proliferation of glioma cells

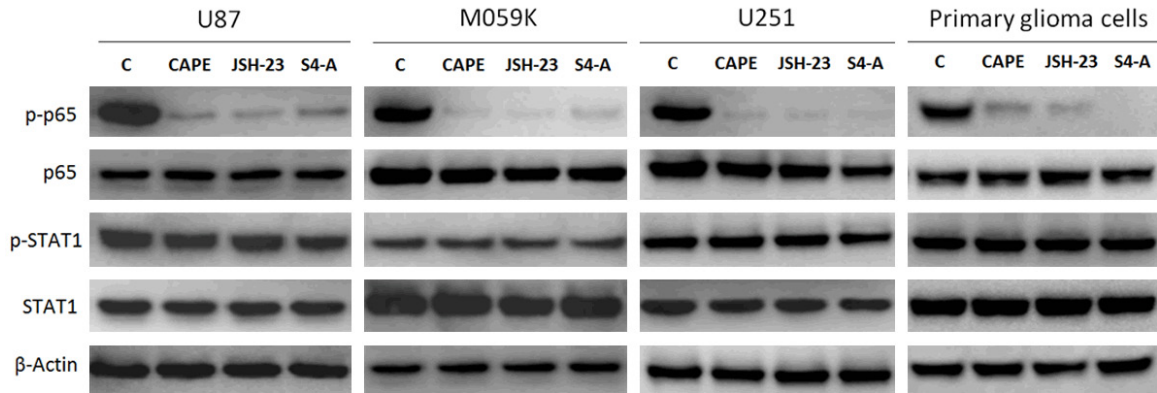


Figure 2. NF- κ B inhibitors inhibit p-p65 phosphorylation in glioma cells in vitro. Western blot analysis of expression of indicated proteins of the NF- κ B and STAT1 signaling pathways in three malignant glioma cell lines and primary malignant glioma cells.

Animal studies

All animal experiments were approved by the Army Medical University Committee on Research Animal Care and performed in accordance with their guidelines and regulations. Six- to eight-week-old male athymic nude mice were anesthetized with a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine in 0.9% sterile saline. U87 cells resuspended in PBS were intracranially injected at a rate of 0.4 μ l/min using a Micro 4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, FL) attached to a Hamilton syringe with a 33-gauge needle (Hamilton, Reno, NV) into the mid-left striatum at the following coordinates (in mm) from the bregma: +0.5 anterior-posterior, +2.0 mediolateral, and -2.5 dorsoventral. Each mouse was injected with 10^6 U87 cells in 10 μ l of PBS.

Athymic nude mice were imaged by a 7.0 T small animal magnetic resonance detector to confirm the formation of in situ tumors. One week after the U87 cell injection, fifty nude mice were randomly divided into two groups (twenty-five mice per group). The experimental group was intraperitoneally injected with CAPE dissolved in DMSO at a dosage of 0.1 mg/kg/day for four consecutive weeks. The control group was injected with an equal volume of DMSO. Magnetic resonance imaging (MRI) was used to detect the growth of intracranial tumors in the nude mice, and the tumor volumes and survival times were recorded and analyzed.

Statistical analysis

GraphPad Prism v5.0 software was used for statistical analysis of all data. A *p*-value of less

than 0.05 was considered statistically significant. To analyze differences between multiple groups, one-way analysis of variance (ANOVA) was performed. An unpaired two-tailed *t*-test was used for comparisons between two samples. Survival was analyzed using Kaplan-Meier curves.

Results

Identification of drugs that inhibit the proliferation of malignant glioma cells

To identify drugs that could inhibit the proliferation of glioma cells, a commercial kinase inhibitor library from Selleck Chemicals containing 464 compounds was used. Several chemicals significantly inhibited the proliferation of U87 cells. The top 5 chemicals were CAPE (cell viability = 0.161), cryptotanshinone (cell viability = 0.191), PF-5274857 (cell viability = 0.197), pifithrin- μ (cell viability = 0.201) and tenovin-6 (cell viability = 0.202). CAPE is an NF- κ B inhibitor. Although not among the top 5 chemicals, two other NF- κ B inhibitors, JSH-23 (cell viability = 0.271) and S4-A (cell viability = 0.252), also exhibited a strong inhibitory effect on the proliferation of U87 cells (**Figure 1**). To verify this effect, the inhibitory effect of the three NF- κ B inhibitors was assessed in two other glioma cell lines (M059K and U251) and primary malignant glioma cells (**Figure 1B**). All three NF- κ B inhibitors significantly inhibited glioma cell proliferation ($P < 0.05$).

NF- κ B inhibitors inhibit p-p65 phosphorylation in glioma cells in vitro

Activation of the transcription factor NF- κ B and signal transducer and activator of transcription

NF-κB inhibitors suppress proliferation of glioma cells

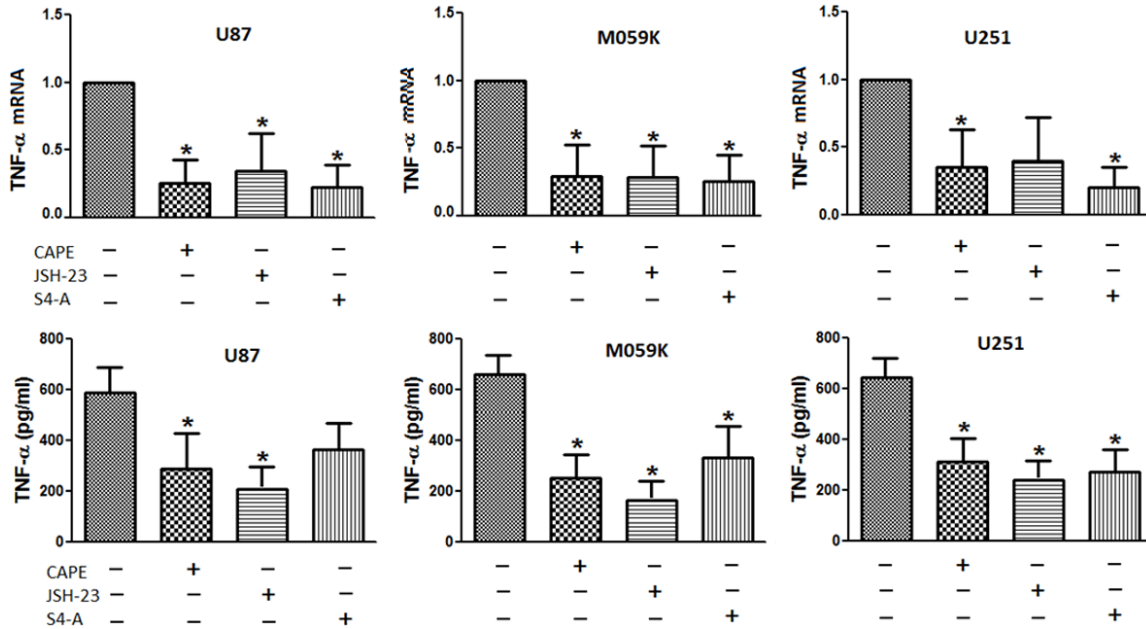


Figure 3. Effect of NF-κB inhibitors on TNF-α expression at the transcriptional and translational levels. Pharmacological inhibition of NF-κB leads to reduced expression of TNF-α at both the transcriptional and translational levels (n = 3, *P < 0.05 versus the control group).

(STAT) signaling pathways has been shown to promote the proliferation and survival of brain cancer cells, a hallmark of poor prognosis in patients diagnosed with glioma. Thus, the expression of key proteins in these two signaling pathways after treatment with NF-κB inhibitors was investigated in U87, M059K, U251 and primary malignant glioma cells. The most important proteins in the NF-κB and STAT signaling pathways are p-p65 and p-STAT1, respectively, and the three brain glioma cell lines and primary malignant glioma cells highly expressed both p-p65 and p-STAT1 (Figure 2). However, when treated with the three NF-κB inhibitors (CAPE, JSH-23 or S4-A), expression of p-p65 but not total p65 was significantly reduced. In contrast, expression of p-STAT1 and total STAT1 did not significantly change in any tested brain glioma cell type (Figure 2). These results show that the three NF-κB inhibitors suppress the phosphorylation of p65, thus inhibiting activation of the NF-κB pathway.

NF-κB inhibitors inhibit TNF-α expression at both transcriptional and translational levels in glioma cells *in vitro*

Furthermore, the expression of TNF-α mRNA and the release of the TNF-α protein in three glioma cell lines was examined by real-time

PCR and ELISA, respectively. The cell lines were treated with 10 μM NF-κB inhibitors (CAPE, JSH-23, or S4-A) for 24 hours, and expression of TNF-α mRNA was significantly lower in treated cells than in nontreated cells (*P < 0.05) (Figure 3). Consistent with the level of TNF-α transcription, release of TNF-α was also significantly reduced by treatment with CAPE, JSH-23 or S4-A (P < 0.05, Figure 3).

Antitumor activity of NF-κB inhibitors *in vivo*

Because CAPE can cross the blood-brain barrier, CAPE was used in the *in vivo* study. Tumor-bearing nude mice were administered 0.1 mg/kg CAPE via intraperitoneal injection once daily for four consecutive weeks. The tumor volumes and animal survival were recorded and analyzed. The tumor volume curve is shown in Figure 4B. Two weeks after the administration of CAPE, the tumor volume began to decrease (*P < 0.05). Three weeks after the administration of CAPE, the tumor volume was significantly reduced (**P < 0.01), indicating that CAPE can inhibit glioma growth *in vivo*. Moreover, the Kaplan-Meier curve shows that CAPE administration was associated with significantly increased overall survival (**P < 0.01, Figure 4C). Thus, the results from this *in vivo* experiment show that CAPE has strong antitumor

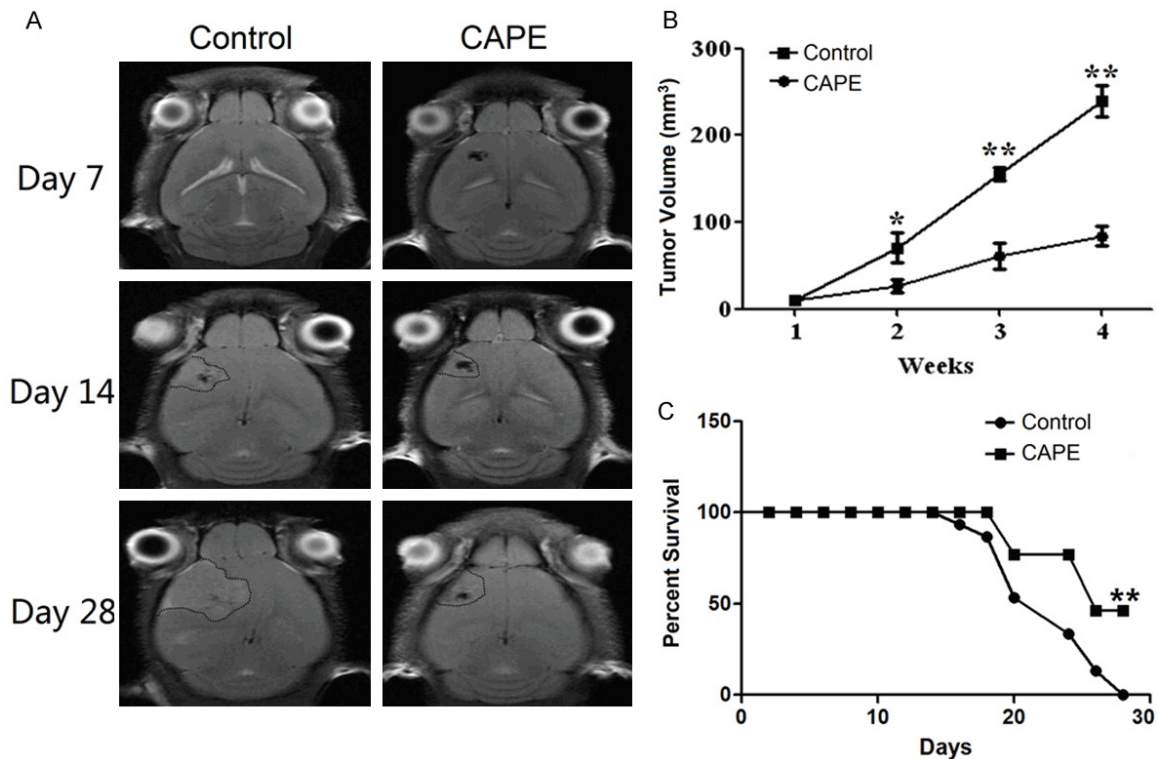


Figure 4. Anti-tumor activity of NF- κ B inhibitors in vivo. A. MRI image. MRI of tumors in nude mice at 7, 14, and 28 days after the injection of U87 cells. At 14 and 28 days, the tumor size in the CAPE-treated group was significantly reduced relative to that in the control group. B. The cumulative increase in tumor volume. The growth curves are plotted as the mean tumor volume \pm SD (standard deviation) (* $P < 0.05$, ** $P < 0.01$). C. Kaplan-Meier survival curve showing that CAPE administration significantly protects nude mice from death in a brain glioma orthotopic transplantation model (** $P < 0.01$).

activity in an in situ tumor formation mouse model.

Discussion

NF- κ B collectively describes a family of highly regulated dimeric transcription factors that control DNA transcription, cytokine production and cell survival [13, 14]. The role of NF- κ B in cancer has been studied extensively, and improper regulation of NF- κ B has been reported in numerous tumors and implicated in various stages of tumorigenesis [15, 16]. Moreover, defects in NF- κ B signaling result in increased susceptibility to cell death because NF- κ B regulates anti-apoptotic genes such as TRAF1 and TRAF2 and therefore abrogates the activity of enzymes in the caspase family, which are central to most apoptotic processes [17]. In tumor cells, NF- κ B is constitutively active due to mutations either in genes encoding the NF- κ B transcription factors themselves or in genes controlling NF- κ B activity. Furthermore, some tu-

mor cells secrete factors that cause NF- κ B activation, and NF- κ B inhibition can cause tumor cell death or apoptosis. Thus, NF- κ B is being actively researched by pharmaceutical companies as a target in anticancer therapy [18]. Many natural products or synthetic drugs have been promoted as having anticancer activity, and anti-inflammatory activity has also been shown to inhibit NF- κ B. For example, QNZ (EVP4593) potently inhibits both NF- κ B activation and TNF- α production [19]. SC75741 is a selective NF- κ B inhibitor that exhibits immunosuppressive activity by suppressing human PBMC proliferation [20] and inhibits the replication of influenza A and B viruses by transcriptionally inhibiting NF- κ B-mediated signaling [21].

In this study, a kinase inhibitor library containing over 400 compounds in the U87 cell line was used and the results were validated in M059K, U251, and primary malignant glioma cells. Surprisingly, we found that three NF- κ B

NF- κ B inhibitors suppress proliferation of glioma cells

inhibitors (CAPE, JSH-23 and S4-A) significantly reduced the growth of the glioma cell lines and of primary malignant glioma cells. CAPE showed promising anti-proliferative activity in a series of tumor cell lines [22, 23] and attenuated the pro-inflammatory phenotype of LPS-stimulated hepatic stellate cells (HSCs) and the LPS-induced sensitization of HSCs to fibrogenic cytokines by inhibiting NF- κ B signaling [24]. JSH-23 is another NF- κ B inhibitor that decreases LPS-induced apoptotic chromatin condensation [25]. Moreover, JSH-23 targets NF- κ B and reverses various deficits in experimental diabetic neuropathy [26]. S4-A is an antibiotic used to treat tuberculosis via NF- κ B inhibition and free radical scavenging. All three drugs can significantly inhibit proliferation of glioma cells, indicating their potential for the clinical treatment of malignant brain glioma.

The mechanism underlying the inhibitory effects of these drugs on the growth of malignant brain glioma cells was further examined. The NF- κ B signaling pathway has been to promote genetic instability and aid in the proliferation and survival of malignant cells, promote angiogenesis and metastasis, subvert adaptive immunity, and alter responses to chemotherapeutic agents [27-29], and inhibition of NF- κ B activity or targeting of inducible NF- κ B genes is an attractive therapeutic approach for brain cancer [30]. In general, the NF- κ B response is a hallmark of inflammation and has been shown to result in a poor prognosis in patients diagnosed with glioma [31]. At the molecular level, NF- κ B has been shown to negatively control both the nuclear transcription factor NFAT in bone marrow-derived macrophages and the inflammatory response [32]. Here, NF- κ B inhibitors were found to reduce phosphorylation of the key NF- κ B signaling pathway protein p-p65 in glioma cells *in vitro*, thus leading to decreased TNF- α expression at both the transcriptional and translational levels. This effect may be the major means by which NF- κ B inhibitors suppress the growth of glioma cells. Furthermore, the effect of NF- κ B inhibitors was assessed *in vivo*. CAPE and JSH-23 have been reported to inhibit the proliferation of glioma cells *in vitro*. Therefore, we attempted to investigate the effect of CAPE *in vivo*. Using a U87 cell orthotopic transplantation nude mouse model, we show that CAPE administration significantly decreased tumor volume and protected nude

mice from death. These results indicate that this NF- κ B inhibitor has strong antitumor activity *in vivo*.

In conclusion, three NF- κ B inhibitors (CAPE, JSH-23 and S4-A) significantly decreased the growth of glioma cell lines and of primary malignant glioma cells. Therefore, our research identifies a potential new treatment for malignant brain glioma. Treatment of glioma cells with CAPE, JSH-23 or S4-A led to decreased expression of p-p65 and reduced expression of TNF- α at both the transcriptional and translational levels. Moreover, our *in vivo* study indicated that CAPE, a drug that can cross the blood-brain barrier, inhibited the growth of glioma in an orthotopic transplantation nude mouse model.

Disclosure of conflict of interest

None.

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