Berberine induces senescence of human glioblastoma cells by downregulating EGFR-MEK-ERK signaling pathway

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Abbreviations: GBM, Glioblastoma multiforme; EGFR, epidermal growth factor receptor; MEK, MAP kinase-ERK kinase; ERK, extracellular signal-regulated kinase

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Abstract

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumors and has a poor prognosis. We here report a potent antitumor effect of berberine, an isoquinoline alkaloid, on GBM. Berberine was found to have an IC\textsubscript{50} that is much lower than temozolomide in vitro in U87, U251 and U118 glioblastoma cells. While previous studies showed that berberine primarily exerts its anticancer effect by inducing cell cycle arrest, apoptosis and autophagy, we observed that the antitumor effect of berberine on glioblastoma cells was primarily achieved through induction of cellular senescence. In glioblastoma cells treated with berberine, the level of epidermal growth factor receptor (EGFR) was greatly reduced. Examination of the activities of the kinases downstream of EGFR revealed that the RAF-MEK-ERK signaling pathway was remarkably inhibited, while AKT phosphorylation was not altered. Pharmacological inhibition or RNA interference of EGFR similarly induced cellular senescence of glioblastoma cells. Furthermore, the cellular senescence induced by berberine could be rescued by introduction of a constitutive active MKK.

Berberine also potently inhibited the growth of tumor xenografts, which was accompanied by downregulation of EGFR and induction of senescence. Our findings thus revealed a new route by which berberine exerts its anticancer activity. Because EGFR is commonly upregulated in glioblastoma, the demonstration of effective inhibition of EGFR by berberine points to the possibility of employing berberine in the treatment of patients with glioblastoma.
Introduction

Malignant gliomas are the most common primary malignant brain tumors, with an annual incidence of 5.26 per 100,000 population (1). These tumors usually have a poor prognosis, with a 5-year survival rate of WHO grade IV tumors (glioblastoma and variants) less than 5% (2, 3). Radiotherapy combined with DNA alkylating agent such as temozolomide is considered the first-line of adjuvant treatment for all patients (1, 3, 4). However, while this regimen can increase the 2-year survival rate from 10% to 27%, its benefit is still limited. Moreover, it causes serious side effects such as lymphopenia and nausea, and nearly all glioblastoma patients experience disease progression (1). More efficacious measures against malignant glioma thus remain to be explored.

Extensive molecular studies, including the recent genome-wide characterizations of large samples (5, 6), have greatly advanced our understanding of the genetic alterations that underlie the pathogenesis of glioblastoma. Three core signaling pathways, RTK/RAS/PI(3)K, p53 and RB, were shown to be altered in majority of the glioblastomas (5, 6). Alterations of EGFR, in the forms of amplification and point mutation, were detected in over half of the samples (5). The activation of EGFR probably plays a critical role in driving the proliferation and survival of glioblastoma cells, via upregulating the AKT and MAPK pathways. Inhibition of EGFR and other receptor tyrosine kinases may prove to be valuable for the treatment of glioblastoma.
Berberine, an isoquinoline alkaloid present in many medicinal herbs including Huanglian (Coptis chinensis), is one of the most commonly used herbal medicines (7-9). It is shown to possess anticancer activities against various types of cancer cells. Berberine can induce apoptosis and cell cycle arrest of tumor cells (10-14). In this report, we studied the effect of berberine on glioblastoma cells in vitro and on tumor xenografts. We found that berberine possesses a more potent antitumor effect on glioblastoma cells than temozolomide in U87, U251 and U118. Interestingly, the antitumor effect of berberine was attributable to its ability to induce cellular senescence. We further demonstrated that berberine can greatly reduce the levels of EGFR, leading to a downregulation of ERK signaling pathway.

Materials and Methods

Cells

U-87 MG, U251 MG, U-118 MG and SHG-44 human glioma cell lines were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai). The authenticity of cell lines was characterized at the Cell Bank using DNA markers DXS52, Apo-B, MD17S5 and D2S44. All experiments were performed using cells within 10 passages after receipt. The cells were maintained in RPMI-1640, MEM or DMEM (Gibco, Invitrogen) supplemented with 10% FCS, 100U/mL penicillin, and 100μg/mL streptomycin in a humidified 5% CO₂/95% air atmosphere at 37°C.

Chemicals

Berberine chloride, hereinafter as berberine, and temozolomide (TMZ) were from
Sigma-Aldrich (St Louis, MO). The structure of berberine is as shown in a review (9).

Berberine was dissolved in DMSO for use in cell culture and in carboxymethylcellulose sodium (7g/L) for use in oral gavage. MTT (3-[4, 5-dimethyl-2-yl]-2, 5-diphenyl tetrazolium bromide) and all other chemicals were of analytical grade and were also purchased from Sigma Chemical.

**Cell Viability Assay**

Cells were trypsinized and plated in 96-well cell culture plates at the concentration of 2-5×10³ cells/well. Twenty-four h later, the medium was removed and replaced with fresh medium with or without berberine or TMZ. Cell density was measured on day 1, 2 and 3 by using the MTT following the manufacturer’s instructions. The absorbance of converted dye is measured at the wavelength of 490 nm and the absorbance is directly proportional to cell viability. All experiments were repeated at least three times.

**Analysis of Cell Cycle and Apoptosis by Flow Cytometry**

Control and treated cells were harvested using 0.25% Trypsin-EDTA, centrifuged (300 g), and washed once with cold PBS. The pellet was resuspended in ice cold 70% ethanol and stored at -20 °C. Samples were incubated with 20 μg/mL propidium iodide/0.1% Triton X-100 staining solution with 0.1mg/mL RNase A. Cell cycle distribution was determined using the BD Biosciences FACSCanto II Analyzer. At least 20,000 cells were collected. For analysis of apoptosis, both adherent and floating cells were harvested, washed twice in PBS, and resuspended in 1× binding buffer at a density of 1×10⁶ cells/mL. Cells were assayed for apoptosis using an Alexa Fluor 488
annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer’s instructions. For these studies, all experiments were repeated three or more times.

*EdU incorporation*

EdU (Cell-Light™ EdU Cell Proliferation Detection kit, Guangzhou RiboBio, China) was added at 50 µM and the cells were cultured for an additional 2 h. After the removal of EdU-containing medium, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min, washed with glycine (2 mg/ml) for 5 min in a shaker, treated with 0.2% Trion X-100 for 10 min, washed with PBS twice. Click reaction buffer (Tris–HCl, pH 8.5, 100 mM; CuSO4, 1 mM; Apollo 550 fluorescent azide, 100 µM; ascorbic acid, 100 mM) was then added. After 10–30 min, the cells were washed with 0.5% Triton X-100 for three times, stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature, washed with 0.5% Triton X-100 for five times, and, finally, immersed in 150 µl PBS and examined under a fluorescence microscope.

*Senescence-associated acidic β-galactosidase staining*

A senescence β-Galactosidase Staining Kit purchased from Cell Signaling Technology (Beverly, MA) was employed. Four high-power fields per sample were counted in three-independent samples to score the number of senescent cells.

*Western Blot Analysis*

Cells were harvested after treatment with berberine, rinsed in ice-cold PBS, and lysed in lysis buffer containing 50 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 2 mg/mL leupeptin, 2 mg/mL aprotinin, 5 mg/mL benzamidine, 0.5 mmol/L
phenylmethylsulfonylfluoride, and 1% NP40. Equal amounts of protein were separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore, Billerica, MA), and blocked with 5% nonfat dry milk in TBS-Tween 20 (0.1%, v/v) for 1 hour at room temperature. The membrane was incubated with primary antibody overnight. Antibodies to p-b-Raf, p-c-Raf, p-MEK, MEK, p-ERK1/2, ERK1/2, p-AKT, p-p38 and p-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA); Anti-EGFR was from Abcam (Cambridge, UK); HA-tag was from proteintech (Wuhan, China) and GAPDH was from Chemicon (Temecula, CA). After washing, the membrane was incubated with the appropriate horseradish peroxidase secondary antibody (diluted 1:5,000; Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 hour. Following several washes, the blots were developed by enhanced chemiluminescence (Millipore, Billerica, MA).

Plasmids and transfection
MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) was as previously described [15]. The plasmids were transiently transfected into U87 and U251 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China) following the manufacturer’s protocol. The cells were stably selected with hygromycin B at a final concentration of 50μg/mL. Cells stably expressing MKK2-CA were treated by berberine to determine the effect of MKK1/2-ERK1/2 signaling on cell senescence.

Small interfering RNA
EGFR siRNA duplex 1, 2 and 3 (Cat. No.1523827,1523828,1523829) and UNIV
NEGATIVE CONTROL small interfering RNA (#2) were synthesized (Sigma, St. Louis, MO, USA) and transfected into U87 and U251 cells using lipofectamine2000 for 24 h. EGFR protein levels were determined by Western blotting analysis and cell senescence was examined by SA β-gal activity as described above.

*cDNA synthesis and real-time PCR*

RNA was isolated using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s protocol. cDNA was synthesized by reverse transcription of 1 μg of total RNA with random hexamers. The total volume of reverse transcription reaction was 20 μL. Real-time quantitative reverse transcription-PCR was performed using the LightCycler® 480 sequence Detection System (Roche Applied Science, Upper Bavaria, Germany). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The levels of EGFR and GAPDH mRNA were measured by SYBR Green I assay. EGFR was amplified by using the primers with the sequence 5’-AGGCACGAGTAACAAGCTCAC-3’ (forward) and 5’-ATGAGGACATAACCAGCCACC-3’ (reverse). The GAPDH primer was 5’-CAGAACATCATCCCTGCCTCTAC-3’ (forward) and 5’-TTGAAGTCAGAGGAGACCACCTG-3’ (reverse). The samples were loaded in quadruple, and the results of each sample were normalized to GAPDH.

*In vivo tumor xenograft study*

Six to eight-week-old BALB/c nude mice (Vital River Laboratories, Beijing, China) were anesthetized with phenobarbital sodium (60 mg/kg, i.p.). Approximately 1.5 × 10^5 U87 cells (>95% viability) in a volume of 3 μL were stereotactically
injected into the right caudate nucleus: bregma (anatomical point on the mouse skull at which the coronal suture is intersected perpendicularly by the sagittal suture) 0.5 mm; lateral, 1.75 mm. The needle was initially advanced to a depth of 3.5 mm and then withdrawn to a depth of 3 mm to limit reflux up the needle tract during injection of cells. Mice were gavaged with the vehicle alone (control), or berberine (50 and 100 mg/kg body weight) daily, and euthanized after five weeks (n=5-6 per group). Brains were fixed with 4% paraformaldehyde in phosphate buffered saline at 4°C overnight, dehydrated in 20% sucrose until the tissue sinks, embedded in OCT (Fisher) and then sectioned for hematoxylin and eosin (H&E) staining, immunofluorescence, senescence and TUNEL assay. Tumor volume was measured as L × W²/2, where L is the length and W is the width. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University.

*Fluorescence immunohistochemistry.*

Frozen sections (7 µm) were washed with PBS twice, and then blocked with 10% goat serum in PBS at 37°C for 1 hour, following which rabbit anti-Ki67 antibody (CST, Beverly, MA) or rabbit anti-EGFR antibody (Abcam, Cambridge, UK) was added at a dilution of 1:400 and 1:300 in 5% bovine serum albumin in PBS respectively and incubated overnight at 4°C. Sections were then washed thrice in PBS before incubating in the dark with a Rhodamine-labeled or FITC-labeled secondary antibody at a dilution of 1:300 in 5% bovine serum albumin in PBS for 60 minutes. The secondary antibody solution was then aspirated and the sections were washed four times in PBS. Sections then were incubated in the dark with DAPI (1 µg/mL) in PBS.
for 5 minutes and coverslips were mounted with an antifade solution (Molecular Probes, Eugene, OR). Slides were then examined on a fluorescence microscope. Negative controls were performed by omitting the primary antibodies.

**TUNEL assay**

TUNEL assay was performed using the *In Situ* Cell Death Detection Kit, TMR red (Roche) following the manufacturer's recommendations. After labeling, the slides were counterstained with DAPI and visualized under a fluorescence microscopy. As positive controls, frozen sections were treated with DNase (Life Technologies, Gaithersburg, Md.) at a concentration of 1.0 μg/mL and incubated for 10 min at room temperature to induce DNA strand breaks.

**Statistical Analysis**

For each measurement, three or four independent experiments were performed. Results were expressed as mean ± SD. Statistical calculations were performed using the SigmaPlot 2000 software (Systat Software, San Jose, CA). Differences in measured variables between experimental and control groups were assessed using t-test. *P*<0.05 was considered statistically significant. For statistical significance was also taken as *P* < 0.05 and **P* < 0.01.

**Results**

*Berberine potently inhibits the proliferation of glioblastoma cells*
The cytotoxic effects of berberine on four glioblastoma cell lines were first evaluated by MTT cell viability assay. As shown in Figure 1A, the cell viability was reduced in a dose-dependent and time-dependent manner in three of the four cell lines. Importantly, berberine possesses a more potent effect than temozolomide (TMZ) in the three cell lines. The IC_{50} values of berberine were at least 6-fold lower than those of TMZ (Figure 1B). SHG-44 cells appeared to be resistant to both berberine and TMZ. Due to its fluorescent nature, the intracellular accumulation of berberine can be visualized under fluorescence microscopy or detected by flow cytometry, and the cytotoxic effect of berberine depends on its intracellular accumulation (16). We observed that while the berberine-sensitive U87, U251 and U118 cells all exhibited remarkable intracellular accumulation of berberine, SHG-44 cells were devoid of berberine staining (data not shown), which may explain why the SHG-44 cells were more resistant to berberine than the other three cell lines.

Because MTT assay is based on MTT reduction by the mitochondrial succinate dehydrogenase (complex II), and TMZ-resistant glioma cells tend to have increased activities of complexes II-III and IV in the electron transport chain (17), there is the possibility that the MTT assay may have underestimated the cytotoxicity of TMZ. We therefore next used the EdU incorporation assay to compare the effects of berberine and TMZ on glioma cells. As shown in Figure 1C, berberine again showed a much more potent effect than TMZ. Furthermore, flow cytometric analysis of cell cycle distribution indicated that the berberine-treated glioblastoma cells were primarily
arrested at G1/G0 phase (Figure 1D). Taken together, our data suggest that berberine can potently arrest the proliferation of glioblastoma cells.

**Berberine induces cellular senescence of glioblastoma cells**

Berberine can induce apoptosis in a variety of cancer cells (12, 13, 18-20). We therefore determined whether berberine could also induce apoptosis of glioblastoma cells. We observed that the basal level of early apoptotic cells was approximately 1%. When treated berberine, there was hardly any increase in the percentage of apoptotic cells (Figure 2A), suggesting that the antitumor effect of berberine on the glioblastoma cells tested in this study was not mediated by induction of apoptosis.

Glioblastoma cells were recently shown to undergo either cellular senescence or apoptosis in response to ionizing radiation (IR) and the switch between senescence and apoptosis is determined by PTEN (21). Lack of PTEN, as in U87 and U251 cells, renders glioblastoma cells resistant to IR-induced apoptosis, but predisposes them to cellular senescence. We therefore tracked the fates of U87 and U251 cells treated with berberine using SA-β-gal staining assay. As shown in Figure 2B and 2C, berberine treatment led to a significant increase in the percentage of senescent cells. After being treated with berberine (15 μM) for seven days, over 70% U87 and 40% U251 cells became senescent. Consistent with the emergence of a high percentage of SA-β-gal positive cells, EdU incorporation assay showed that the percentages of EdU-positive cells were greatly reduced seven days after treatment with berberine (Figure 2D), suggesting that very few of the berberine-treated cells were in S phase. Taken together,
these results suggest that induction of cellular senescence is the major mechanism by which berberine exerts its antitumor effect.

We previously reported that berberine may exert its antitumor effect by functioning as a genotoxicant, especially as an inducer of DNA double-strand breaks (DSBs) (12, 16). Because genotoxic stress can induce premature cellular senescence, we speculated that the induction of senescence by berberine might be mediated by DNA damage response. We tested this notion by measuring the level of γ-H2AX, a marker of DSBs, in berberine-treated glioblastoma cells. However, while γ-H2AX positive cells were common in U251 cells in response to berberine treatment, they were rare in berberine-treated U87 cells (Fig S1). This result suggests that DNA damage response may not be required during berberine-induced cellular senescence. This notion is substantiated by results shown below.

**EGFR- RAF-MEK-ERK signaling pathway was inhibited by berberine**

Amplification and/or point mutation of EGFR are one of the most common genetic alterations in glioblastoma. Gain of function of EGFR can lead to the upregulation of RAS and PI3K-AKT signaling pathways, and thereby drives cell survival and proliferation (22-24). We therefore tested whether EGFR-initiated pathways are altered in glioblastoma cells treated with berberine. Interestingly, in both U87 and U251 cells treated with berberine, we observed a significant reduction in the level of EGFR (Figure 3). Furthermore, the levels of phosphorylated (active) form of RAF, MEK and ERK were all decreased (Figure 3). This result indicated that accompanying
the declined level of EGFR, the RAF-MEK-ERK pathways was significantly downregulated in berberine-treated glioma cells. However, PI3K-AKT pathway, the other pathway downstream EGFR, was not affected (Figure 3). It should be noted that while the amount of EGFR was reduced by berberine at protein level, the mRNA level instead showed an increase (Fig. S2), suggesting that the downregulation of EGFR by berberine was not due to decreased EGFR transcription.

**Downregulation of EGFR-RAF-MEK-ERK signaling pathway mediates berberine-induced senescence**

We next determined whether the downregulation of EGFR is responsible for the induction of cellular senescence in berberine-treated cells. We first tested whether erlotinib, an inhibitor of EGFR, can also induce senescence in glioma cells. We noted that over 60% of the U87 cells became senescent after they were treated with 10 μM erlotinib for 7 d (Fig. 4A). Like berberine, erlotinib also failed to induce apoptosis in U87 cells (Fig. S3). We also examined the fate of EGFR knockdown U87 cells by SA-β-gal staining. As shown in Figure 4B, EGFR knockdown in U87 cells also led to a great increase in the percentage of senescent cells. These results indicate that inhibition or downregulation of EGFR alone is sufficient to induce cellular senescence in U87 cells. They also substantiate that notion that the berberine can induce cellular senescence independent of its genotoxic effect. Furthermore, U0126, an MEK inhibitor, also induced senescence in U87 and U251 cells (Figure 4C). We next obtained U87 and U251 cells that stably express MKK2-CA and tested whether
those cells could resist berberine-induced senescence. As shown in Figure 4D, berberine-induced senescence was greatly attenuated in MKK2-CA cells. Correspondingly, inhibition of ERK activation by berberine was remarkably relieved in the presence of MKK2-CA. Together, these results indicate that berberine-induced senescence in glioma cells was mediated by the downregulation of EGFR-MEK-ERK signaling pathway.

**Berberine induces cellular senescence of glioblastoma xenografts**

The *in vitro* data shown above clearly demonstrated a potent antitumor effect of berberine against glioblastoma cells. We next determined whether berberine could inhibit the growth of glioma *in vivo*. For this purpose, U87 cells were inoculated into the right striatum of mouse brains and the tumor-bearing mice were administered with berberine (50 and 100 mg/kg body weight) daily for five weeks. Examination of brain sections revealed the presence of tumor mass in all five mice in the control group, in four out of five in the 50mg/kg group, but only in two out of six in the 100 mg/kg group (Figure 5A). Furthermore, the tumor volume was greatly reduced in mice administered with berberine (Figure 5B). Importantly, when stained for SA-β-gal, tumor sections obtained from berberine-administered mice showed a widespread distribution of SA-β-gal positive cells (Figure 5C). Like the glioma cells in culture, apoptotic cells, marked by transferase dUTP nick end labeling (TUNEL), were rather rare in tumor sections (Figure 5D). Staining with Ki67, which marks proliferating cells, indicated that the proliferation of glioma cells was greatly inhibited in berberine-treated mice (Figure 5E). As in cultured glioma cells, the level of EGFR
was remarkably diminished by berberine treatment (Figure 5F). Together, these results demonstrated a potent antitumor effect of berberine against glioblastoma cells in vivo. It should be noted that inhibition of tumor growth by berberine was not accompanied by reduced body weight. Temozolomide administered at 60 mg/kg (p.o., daily), on the other hand, resulted in a great reduction in body weight and early death (data not shown), thus precluding us from comparing its efficacy relative to berberine. Overall, these results suggest that berberine may exert a potent antitumor effect without causing serious side effects.

Discussion

Our results showed that berberine possesses a potent antitumor effect against some glioblastoma cells. It can effectively induce glioblastoma cells to undergo cellular senescence. Interestingly, while berberine has been reported to exert its cytotoxic effect by inducing apoptosis in numerous types of cancer cells (13, 16, 18, 19), none of the glioblastoma cell lines we tested in this study showed signs of increased apoptosis in response to berberine treatment. Therefore, induction of cellular senescence is probably the major mechanism by which berberine exerts its antitumor effect against glioblastoma cells. Indeed, induction of senescence has recently been recognized to be an important therapeutic strategy for various types of cancer (25, 26). We further showed that the level of EGFR was greatly reduced in berberine-treated cells and that pharmacological inhibition or RNA interference of EGFR similarly induced cellular senescence of glioblastoma cells. Accompanying the downregulation of EGFR, the RAF-MEK-ERK signaling pathway downstream of EGFR was
remarkably inhibited in berberine-treated cells. Since the cellular senescence induced by berberine could be rescued by introduction of constitutive active MKK, we conclude that the induction of cellular senescence in berberine-treated glioblastoma cells is likely mediated by the downregulation of EGFR-RAF-MEK-ERK pathway. Because EGFR amplification is characteristic of classical subtype of GBM and sustains the proliferation of GBM (27-29), the effective downregulation of EGFR by berberine suggests that use of berberine could be considered in the treatment of GBM.

It was recently reported that glioblastoma cells may either undergo cellular senescence or apoptosis in response to ionizing radiation (IR) depending on the status of PTEN (21). Upon treatment with IR, the PTEN-deficient glioblastoma cells invariably underwent senescence while those with wild-type PTEN all took the fate of apoptosis. Interestingly, the PTEN-deficient glioblastoma cells were not intrinsically resistant to apoptosis, since they could become apoptotic when treated with doxorubicin (21). Thus, the effect of berberine on GBM cells resembles that of IR, but differs from that of doxorubicin. However, although berberine can inflict DNA double-strand breaks and thus mimic the effect of IR (12, 16), it should be pointed out that DNA damage response pathway, including p53 activation, that usually results in cellular senescence or apoptosis is not required for the induction of senescence in berberine-treated GBM cells. This notion is supported by the fact that an increase in the level of DSBs was not detected in U87 cells treated with berberine at the concentration that induced cellular senescence. While it can be argued that p53 activation in GBM cells bearing DSBs may accelerate senescence, it does not appear
to be required for berberine-induced senescence in GBM cells. Importantly, inhibition of EGFR alone, by RNAi or by erlotinib, could effectively induce senescence in GBM cells. Thus, our results indicated that the downregulation of EGFR alone might be sufficient for the induction of senescence in some GBM cells. Our findings are in contrast to the report that inhibition of EGFR could radiosensitize non-small cell lung cancer cells by inducing senescence in cells sustaining DSBs (30).

Multiple signaling pathways, including PI3K-AKT and MEK-ERK, can be initiated from EGFR. While we detected a downregulation of the MEK-ERK pathway in berberine-treated glioblastoma cells, the PI3K-AKT pathway, which is upregulated in U87 and U251 cells due to loss of PTEN, appeared to be unaffected by berberine. Interestingly, application of U0126, an MEK inhibitor, similarly induced senescence in GBM cells, suggesting that the downregulation of MEK-ERK pathway is responsible for the induction of cellular senescence. We confirmed this notion by using GBM cells that express constitutively active MKK2 (MKK2-CA). Berberine-induced senescence was greatly attenuated in MKK2-CA cells. Correspondingly, inhibition of ERK activation caused by berberine was remarkably relieved in those cells. These findings establish EGFR-RAF-MEK-ERK signaling pathway as the mediator of berberine-induced senescence in GBM cells.

While the protein level of EGFR was greatly reduced in berberine-treated GBM cells and in xenotransplants, the mRNA level of EGFR even exhibited a modest increase, suggesting that downregulation of EGFR by berberine was not due to decreased
transcription. It was recently reported that berberine could stimulate the activation of ubiquitin ligase Cbl and promote EGFR ubiquitinylation and degradation in colon tumor cells (31). Future studies may reveal whether berberine promotes EGFR degradation by a similar mechanism.
Acknowledgements

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Legends to figures

Fig.1. Berberine inhibits cell proliferation and induces cell cycle arrest in glioma cells. (A). Cell viability determined by MTT assay. Cells were treated with the indicated concentrations of berberine or TMZ for 24 h, 48 h and 72 h, and cell viability was determined by MTT. (B). IC50 values of berberine and TMZ in four glioma cell lines. (C). EdU incorporation in berberine- or TMZ-treated glioma cells. Cells were treated with berberine or TMZ for 72 h before they were assayed for EdU incorporation. (D). Berberine induces G0/G1 arrest of glioma cells. Cells were treated without or with 15μM berberine for the indicated time and then the cell cycle population was measured by FACS analysis. Data are presented as the mean ± SD of values from triplicate experiments. * P<0.05 versus control. **P < 0.01 versus control.
**Fig.2.** Berberine induces cellular senescence, but not apoptosis, of glioma cells. (A). Apoptosis in glioma cells determined by flow cytometry. Cells were treated without or with 15μM berberine for 48 h, and then apoptosis was measured by flow cytometric analysis of cells stained with annexin V and PI. The percentages of annexin V- or/and PI-positive cells are indicated. (B,C). Induction of cellular senescence by berberine. Cells were treated without or with 15μM berberine for 7d. Cellular senescence was examined by SA β-gal staining. (D). Inhibitory effect of the berberine on cell proliferation determined by EdU incorporation assay in U87 and U251 cells. EdU incorporation was measured by immunofluorescence staining of EdU (red) and DAPI (blue) under the same microscopic magnification (×200). Scale bar, 25 μm. Left panel, representative EdU incorporation; Right panel, quantitation of EdU incorporation. The number of EdU positive cells per 200 nucleated cells was determined. Data are presented as averages of triplicate measurements. *, p < 0.05 versus control, and **, p < 0.01 versus control.

**Fig.3.** Berberine inhibits EGFR-MEK-ERK signaling pathway. Cells were treated without or with 15μM berberine for the indicated times, and then equal amounts of cell lysates were subjected to immunoblot analysis using the indicated antibodies. The immunoblots are representative of at least two independent experiments with GAPDH serving as a protein loading control.

**Fig.4.** Downregulation of EGFR-MEK-ERK signaling pathway mediates the induction of senescence in berberine-treated glioma cells. (A). EGFR inhibitor (Erlotinib) induces senescence in U87 cells. Cells were treated without or with 10μM
Erlotinib for 7d. Cell senescence was examined by SA β-gal activity analysis. (B). EGFR siRNA induces senescence in U87 cells. Top, downregulation of EGFR by siRNA as measured by Western blot. U87 cells were transfected with siRNA duplexes (200 nM) specific to EGFR or negative oligo in serum-free medium for 4 h, then were incubated with complete medium for 24 h. Middle, representative image showing cellular senescence. Cellular senescence was examined 7 d after RNAi of EGFR. Bottom, quantitative summary of senescent cells. (C), U0126 (MEK inhibitor) induces senescence in U87 and U251 cells. Top, cells were treated without or with 20μM U0126 for 7d. Bottom, cells were treated without or with 20μM U0126 for 24h and whole cell extracts were collected for western blot analysis using p-ERK and ERK antibodies. (D). Overexpression of MKK2 attenuates berberine-induced senescence. MKK2-CA expression vectors were transfected into U87 or U251 cells using Lipofectamine 2000. After expression for 48 h, add media with Hygromycin B to get a good stably transfected cells. Then the cells were treated with 15μM berberine for 24 h. Cell extracts were examined by Western blotting for the determination of phospho-ERK1/2, ERK1/2, and HA-MKK2-CA protein levels. Stable MKK2-CA expression cells were treated with 15μM berberine for 7d. Cell senescence was examined by SA β-gal activity analysis.

Fig.5. Efficacy of berberine against human glioma cells in vivo. U87 cells were inoculated into the right striatum of mouse brain (n=5-6 per group). Mice were gavaged with the vehicle alone (Cont), berberine (50 and 100 mg/kg body weight) daily, and euthanized after 35d treatments. Brains were fixed, frozen and
systematically sectioned throughout the tumor injection site. Frozen sections (7 μm) were obtained and immunochemical detection was performed. (A), Representative pictures of hematoxylin-eosin staining of tumor sections from mice of three groups (×10). Arrows show the edge of tumor. Scale bar, 1 mm. (B). Berberine-administered mice exhibited decreased tumor volumes. (C). Representative images of IHC showing the positive SA-β-gal staining of tumor sections from berberine-administered mice (×400). Arrows show blood vessels. Scale bar, 20μm. (D). Staining of transferase dUTP nick end labelling (TUNEL) (×400). (E-F), Representative IHC images showing cell proliferation marker-Ki67 (E) (×400) and EGFR (F) (×1000). 4′,6-Diamidino-2-phenylindole (DAPI) was used for the nuclear staining. (G), Schematic model of signaling pathways in berberine-induced cell senescence.
Figure 1

A

![Graphs showing cell viability for different treatments](image)

B

<table>
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<tr>
<th>Drug</th>
<th>IC50 Values (μM) for 72h</th>
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<td>Berberine</td>
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C

![Graphs showing cell proliferation for different treatments](image)

D

![Graphs showing cell cycle distribution for different treatments](image)
Figure 3

U87
15μM Berberine

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<th>p-MEK</th>
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U251
15μM Berberine

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Berberine induces senescence of human glioblastoma cells by downregulating EGFR-MEK-ERK signaling pathway

Qiao Liu, Xiuhua Xu, Minnan Zhao, et al.

Mol Cancer Ther Published OnlineFirst December 12, 2014.

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