4-3-2023

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RPS Pharmacy and Pharmacology Reports, 2023, **2**, 1–10
https://doi.org/10.1093/rpsppr/rqad014
Advance access publication 3 April 2023
Research Paper

RPS Pharmacy and Pharmacology Reports

OXFORD

Anti-cancer effect of *Cissus quadrangularis* on human glioblastoma cells

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Abstract

Objectives Glioblastoma multiforme (GBM) is a common and fatal brain tumour in the central nervous system with a poor survival rate and a median survival time of 15 months only. The standard treatment is aggressive surgical resection followed by radiotherapy and chemotherapy. However, effective drugs available in chemotherapy are limited. This study was designed to evaluate, for the first time, the potential therapeutic effect of *Cissus quadrangularis* (CQ) in human glioblastoma cells and to investigate its possible mechanisms of action.

Methods In this study, we examined the anticancer activity of CQ in human glioblastoma U87 MG cells by cell viability assay, cell migration assay, immunofluorescence staining and Western blot.

Results Our results demonstrated that CQ treatment induced U87 cytotoxicity, cell cycle arrest and cell death. The cytotoxicity of CQ mediates ER stress, autophagy and mitochondrial apoptosis by suppressing pro-survival signalling pathways (extracellular signal-regulated kinase and signal transducer and activator of transcription 3 pathways).

Conclusions The findings of this study imply that CQ is a promising anti-cancer candidate for the treatment of GBM.

Keywords: glioblastoma; *Cissus quadrangularis*; herb; cancer; chemotherapy

Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain tumour in the central nervous system, accounting for 47.7% of all cases.[1, 2] It may occur at any age with a median age of diagnosis at 65 years old. Survival rate is low with approximately 40% survival in the first year and 17% in the second-year post-diagnosis. Current treatment options for GBM are surgical excision followed by radiation and chemotherapy for majority of patients.[3] GBM is an aggressive brain tumour that infiltrates surrounding tissues, which makes it almost impossible to remove completely. Normally, a combination of radiation and chemotherapy is used to target the tumour cells that have invaded the surrounding normal brain tissue. However, effective chemotherapy remains unmet, especially at recurrence where both systemic treatment and tumour-treating fields have not shown significant improvement over placebo.[4] Therefore, development of better treatments for glioblastoma to improve the prognosis of this type of tumour is necessary and required.

Cissus quadrangularis (CQ), commonly known as Hadjod, is a vining plant native to south Asia and Africa.[5] The CQ extract contains phytosterols, anabolic steroidal substance, ascorbic acid, triterpennoids, vitamin E, β-carotene and calcium.[6, 7] CQ has previously been used for the treatment of osteoporosis,[8] osteoarthritis,[9] helminthiasis, digestive diseases, diabetes, skin, eye and ear diseases.[10] Recently, it is reported that CQ showed a significant antioxidant and anticancer activity against the Ehrlich Ascites Carcinoma cell line.[11] CQ also inhibits the growth of tumoroid and leads to cell death of Hela cells, whereas the same dose of CQ and treatment time produce no such inhibition of normal keratinocytes skin cells.[12] Although CQ has been used to treat various diseases, studies exploring its anticancer properties especially the molecular mechanisms underlying the effects of CQ remain poorly understood.[11, 12]

The goal of this study was to evaluate the therapeutic potential of CQ in treating glioblastoma and to investigate its underlying mechanisms. In our previous study,[13] we found that treating glioblastoma cells with niclosamide triggered endoplasmic reticulum (ER) stress and activated autophagy, which led to cell toxicity by increasing the expression of CCAAT/enhancer-binding protein (CHOP) and microtubule-associated protein light chain 3II (LC3II), respectively. Furthermore, we observed a decrease in cell viability due to the suppression of the pro-survival signal transduction pathway, ERK and STAT3 (extracellular signal-regulated kinase/signal transducer and activator of transcription 3). In this study, we investigated whether CQ also induced cell toxicity through similar mechanisms as niclosamide in GBM cells. Our results showed that, like niclosamide, CQ inhibited the pro-survival signal transduction pathway, ER stress and autophagy in U87 cells.

Received: March 27, 2023. Editorial Acceptance: April 2, 2023

Highlights

- The anticancer effect of *Cissus quadrangularis* (CQ) was studied in human glioblastoma U87 MG cells.
- It was demonstrated that CQ treatment induced cytotoxicity, cell cycle arrest and cell death in U87 MG cells.
- CQ may become a potential chemotherapy component for the treatment of glioblastoma multiforme.

Materials and Methods

Reagents

Ethanol-extracted *Cissus quadrangularis* was purchased from Xi'an Le Sen Bio-technology Co., Ltd., P. R. China. CQ was dissolved in dimethyl sulfoxide (DMSO) to 0.1 g/ml and stored at –20°C. Cells were exposed to 0.1–0.8 mg/ml dose of CQ in most of the treatment, the DMSO concentration was equal or less than 0.8% in the treatment. Hoechst 3342 and 2′, 7′-dichlorodihydrofluorescein diacetate were purchased from Life Technologies (Eugene, OR, USA), and MitoTracker Red CMXRos was from Invitrogen (Carlsbad, CA, USA). MTS assay kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was purchased from Promega (Madison, WI). Crystal violet powder was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The glioblastoma cell line (U87 MG) was purchased from the American Type Culture Collection (cat. no. ATCC HTB-14). This is a human glioblastoma cell line with an unknown patient origin.[14] Cells were cultured at 5% CO_2 at 37°C in Dulbecco's modified eagle medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (foetal bovine serum, Corning, Inc.), penicillin (100 U/ml) and streptomycin (100 µg/ml).

Cell proliferation and viability assays

Crystal violet staining and MTS assay method were used to assess cell proliferation and viability. Human U87 MG cells were seeded in a six-well plate and allowed to grow for 24 h. The cells were then treated with 0.1, 0.2, 0.4, 0.8 and 1.6 mg/ml of CQ and cultured at 37°C for 2 days. At the end of the treatment, media was removed, and the cells were washed with PBS and fixed with 100% methanol. 0.1% crystal violet staining solution was then added to each well and incubated for 10 min at room temperature. Stained cells were rinsed with ddH_2O and plates were allowed to air dry before images were taken using digital camera.

Cell viability was then measured using the MTS assay with CellTiter 96 non-radioactive cell proliferation colorimetric assay kit (Promega, Madison, WI). Briefly, cells were plated into 96-well plates. The following day, the cells were treated with 0.1, 0.2, 0.4 and 0.8 mg/ml CQ for 48 h. At the end of the treatment period, 100 µl media were removed from all wells, which was then followed by the addition of 20 µl MTS solution into each well and incubated for 1.5 h at 37°C. Sample in each well was then measured using a microplate reader at a wavelength of 490 nm. At least six replicates were performed for each treatment.

Wound-healing assay

The migration of cells was assessed using an *in vitro* wound-healing assay. U87 MG cells were cultured to 70% confluence in six-well plates. A scratch wound was generated with a 200 µl sterile pipette tip and the wells were washed with PBS to remove nonadherent cells. The cells were then treated with 0.1, 0.2 and 0.4 mg/ml CQ in serum-free DMEM and incubated in the incubator for 48 h. The migrated cells were fixed in cold 75% methanol for 30 min and washed with PBS. Hematoxylin/Eosin staining was used to highlight cell morphology. The wounded areas were observed and imaged under an inverted phase-contrast light microscope.

Mitochondrial membrane potential (MMP) visualization

MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA, USA) with DAPI (4′,6-diamidino-2-phenylindole) staining was used to visualize MMP. The MitoTracker probe passively diffuses across the plasma membrane and accumulates in active mitochondria. This accumulation can be used to visualize changes in MMP and mitochondria distribution. For this assay, cells were plated on glass-bottom dishes and treated with or without CQ. Cells were then incubated for 30 min in a media solution containing the MitoTracker probe (50 nM), rinsed with cell culture media and PBS (phosphate buffered saline), fixed using 3.7% paraformaldehyde (PFA). Cells were mounted using Vectashield Mounting Medium for Fluorescence containing DAPI (Vector Laboratories, Burlingame, CA, USA) and imaged using an Olympus FV1000 confocal microscope and Olympus FV10-ASW3.1 software.

MitoTracker staining

U87 MG cells were grown on coverslips and allowed to adhere for 24 h, which was followed with CQ treatment at 0.8 mg/ml for 24 h. After the treatment, cells were incubated with 50 nM of MitoTracker Red CMXRos (Ex 579nm; Em 599nm) for 15–45 min. Following the incubation, cells were washed three times in pre-warmed growth medium and fixed in 4% PFA. After fixation, cells were rinsed in PBS and permeabilized in 0.2% Triton X-100 at room temperature for 10 min before being counterstained with DAPI and observed under fluorescence microscope.

Cell morphology and immunocytochemistry

To test for morphological changes, cells were cultured in glass-bottom dishes. After a given treatment, the cells were visualized using a confocal microscope. For α-tubulin immunostaining, cells were fixed with 4% PFA in PBS for 15 min at room temperature, washed with PBS, and permeabilized with 0.5% Triton-X 100 in PBS for 5 min. Fixed cells were probed with α-tubulin antibody (1:200) overnight at 4°C, followed by AlexaFluor 488-conjugated secondary antibody (1:1 000; Life Technologies). After incubating for 1 h, the cells were again washed three times with PBS and mounted with Vectashield Mounting Medium for Fluorescence. An Olympus FV1000 confocal microscope and Olympus FV10-ASW3.1 software was used to take differential interference

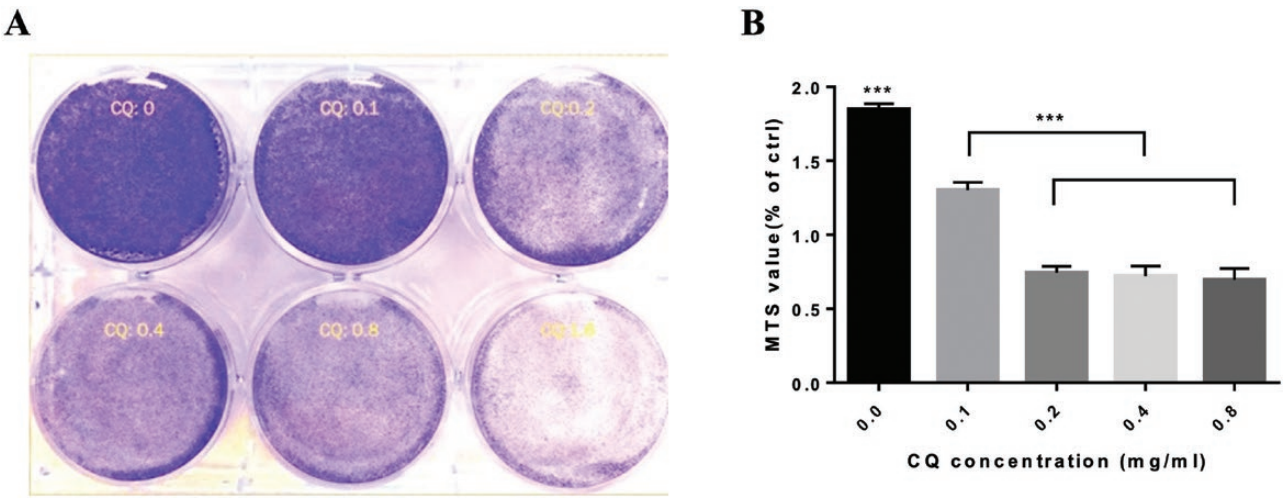

Figure 1 Cissus chloroquine (CQ) inhibits U-87 cell proliferation/viability. (A) Cells were treated with various concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6 mg/ml) of CQ as indicated for 48 h and then stained with Crystal Violet. (B) Cells were treated with various dose of CQ for 48 h. MTS assay was carried out using MTS assay following the manufacture's instruction. ***$P < 0.001$.

contrast images. For comparison purposes, microscope settings were unchanged across each treatment group.

DNA damage and cell apoptotic Determination

DNA damage and chromatin condensation were detected using Hoechst staining. Cells were cultured in glass bottom dishes. After treatment, cells were fixed with 3.7% PFA, washed with PBS and stained with Hoechst 33342 (5 μg/ml) solution (Sigma). Stained cells were mounted and imaged using a confocal microscope.

Western blot analysis

Protein homogenates were prepared as follows: U87 MG cells were treated with various concentrations of CQ for 24 h. Following treatment, cells were lysed in ice-cold radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Inc.) containing protease and phosphatase inhibitor cocktails (Santa Cruz Biotechnology, Inc.). Equal amounts of protein (20–30 μg) were subjected to 4–20% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad Laboratories, Inc.) and transferred onto nitrocellulose membranes. The following primary antibodies were used: β-actin (SC-47778), ERK (SC-1647), survivin (SC-17779), STAT3 (SC-8019), p-STAT3 (SC-8059) and cyclin D1 (SC-8396) were purchased from Santa Cruz Biotechnology, Inc., phospho-ERK (4370), CCAAT/enhancer-binding protein, CHOP (2895) and microtubule-associated protein light chain 3, LC3 (2775) were purchased from Cell Signaling Technology, Inc. After incubating with primary antibodies, the membranes were washed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (SC-2357 and SC-516102, Santa Cruz Biotechnology, Inc.). The protein bands were visualized using a chemiluminescent method, according to the manufacturer's protocol.

Statistical analysis

SPSS software Amos was used for statistical analysis and the measurement data were presented as the mean ± standard error of mean (≥three replicates). One-way ANOVA analysis was used, followed by Tukey's multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference. $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$***.

Results

Cissus quadrangularis inhibits glioblastoma cell viability

To evaluate the effect of CQ on cell viability and proliferation, we first conducted a crystal violet assay and screened the dosage effects of CQ on glioblastoma cells. U87 MG cells were treated with various concentrations of CQ and cultured for 5 days to allow cell growth and colonies to develop. Following the treatment, cells were stained with crystal staining. As shown in Figure 1A, in control group (CQ = 0 mg/ml), cells were treated with DMSO and showed dark purple staining. The staining intensity was decreased with the increased CQ concentrations, indicating the inhibitory effect of CQ on cell proliferation and viability. To quantify this inhibitory effect, a parallel MTS assay was used to find statistical significance. U87 MG cells were treated with increasing concentrations of CQ (0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml and 0.8 mg/ml) for 48 h, and MTS assay was carried out to measure cell proliferation and viability. As shown in Figure 1B, cell proliferation/viability was robustly decreased when compared to the control.

The effects of *Cissus quadrangularis* on glioblastoma cell migration

Migration is an important factor to target in potential cancer treatments, especially for containment of extremely invasive tumours like glioblastoma. The wound-healing assay is a comparatively simple method used to observe migration *in vitro*, approximating the healing process of cells *in vivo*.[15] To explore the effect of CQ on cellular migration, a wound-healing assay was performed. The wound-healing results revealed that CQ (0.4 mg/ml) treatment for 24 h significantly reduced U87 MG cell migration compared with the control group. As shown in Figure 2, CQ treatment effectively suppressed migration of U87 cells in a dose-dependent manner.

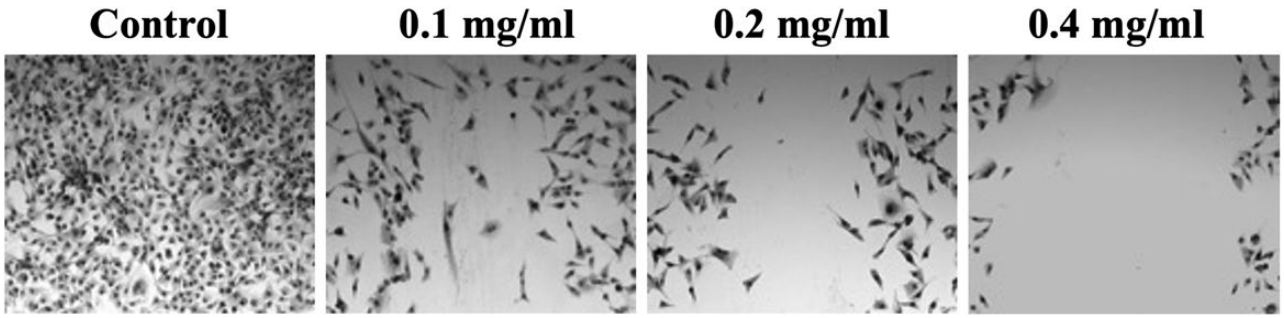

Figure 2 CQ inhibits U-87 cell migration in a dose-dependent manner. Lines of equal width were scratched into similar dishes of cells which were then treated with various concentrations of CQ for 24 h and then stained with Haematoxylin/Eosin for clarity.

Cissus quadrangularis causes mitochondrial fragmentation in U87 cells

Mitochondrial functional markers, including mitochondrial distribution, conformation and membrane potential (MMP), are often used to detect apoptosis.[16, 17] Disruption of MMP is a crucial event for initiating cellular apoptosis, making it an important target to be explored with potential treatment.[18] In the current study, MitoTracker Red CMXRos staining was used to visualize mitochondrial distribution and compare the MMP of CQ-treated versus untreated U87 cells. Figure 3A shows the red fluorescent aggregates of stained mitochondria with high MMP in healthy and untreated U87 cells. In contrast, CQ-treated cells exhibit diffused mitochondria. Many small red dots were observed in cells treated with 0.8 mg/ml of CQ for 24 h. These small red dots presented in the cytoplasm indicated the mitochondrial fragmentation.

Cissus quadrangularis induces cytoskeletal deformation in U87 cells

Microtubules are key components of the cytoskeleton. Thus, tubulin has emerged as a target for cancer therapy drugs.[19, 20] The effect of CQ on cytoskeleton and cell morphology was therefore investigated in U87 cells using alpha-tubulin fluorescent immunostaining. As shown in Figure 3A, CQ was found to induce microtubule disruption characterized by the presence of short, contorted fibres as opposed to the long fibres found in the untreated controls. Furthermore, most CQ-treated cells displayed spreading of cytoplasm (enlarged cell bodies) as well as a reduction in cell number. By contrast, the control cells maintain normal tubulin distribution and cell morphology.

Cissus quadrangularis causes nuclear condensation and fragmentation

A typical morphological change associated with apoptosis is the formation of apoptotic bodies with condensed chromatin and DNA fragmentation. To visualize nuclear morphology, we stained cells with the DNA dye Hoechst 33342. As shown in Figure 3B, most of the nuclei in the control cells were stained homogenously indicating these nuclei are normal, however, when cells were exposed to various concentrations of CQ for 24 h, a number of cell nuclei exhibited intense fluorescence corresponding to pyknotic nuclei and/or chromatin condensation and DNA fragmentation indicating these cells were undergoing apoptosis after insulting with CQ.

Cissus quadrangularis represses the expression of cell cycle regulator

Cyclin D and survivin are proteins that are associated with proliferation and cell survival. The overexpression of cyclin D1 is associated with tumorigenesis of many types of cancer.[21–23] Similarly, as an apoptosis inhibitor survivin is also highly expressed in cancer cells.[24] As shown in Figure 4, CQ treatment for 24 h suppressed both cyclin D1 and survivin expression in U87 MG cells. These findings indicate that CQ may suppress cell cycle progression and reduce cell viability by limiting the expression of cyclin D1 and survivin in U87 cells.

Cissus quadrangularis induces ER stress and stimulates autophagy

ER is a multifunctional organelle that guides protein folding to generate functional proteins. Accumulation of misfolded proteins caused by DNA damage or oxidative stress can induce ER stress and if prolonged, can activate apoptosis signalling.[25] One of the components of the ER stress mediated apoptosis pathway is C/EBP homologous protein (CHOP).[26] In order to determine if CQ treatment induces ER stress, U87 MG cells were treated with 0 mg/ml, 0.2 mg/ml, 0.4 mg/ml and 0.8 mg/ml CQ for 24 h. Following the treatment, cells were collected and lysed. Cell lysates were applied for Western blot analysis and probed with CHOP antibody. As shown in Figure 5A, CQ treatment enhanced CHOP protein expression in a dose-dependent manner, indicating the induction of ER stress by CQ in U87 MG cells. These results suggest that CQ causes apoptosis in glioma cells at least partially via ER stress.

Recent reports have shown autophagy can be activated in response to ER stress.[27] Microtubule-associated protein light chain 3 (LC3) is widely used to measure autophagy,[28] especially the conversion of LC3I to LC3II. To determine whether CQ increases autophagic activity in U87 cells, we compared levels of LC3I and LC3II using Western blot analysis. As shown in Figure 5, LC3 conversion in U87 cells is upregulated with CQ treatment in a dose-dependent manner. The increasing trend is correlated with increasing levels of CHOP expression (Figure 5), confirming the correlation between ER stress and autophagy.

Cissus quadrangularis downregulates pro-survival signalling pathways in glioblastoma cells

STAT3/interleukin-8 and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) are two

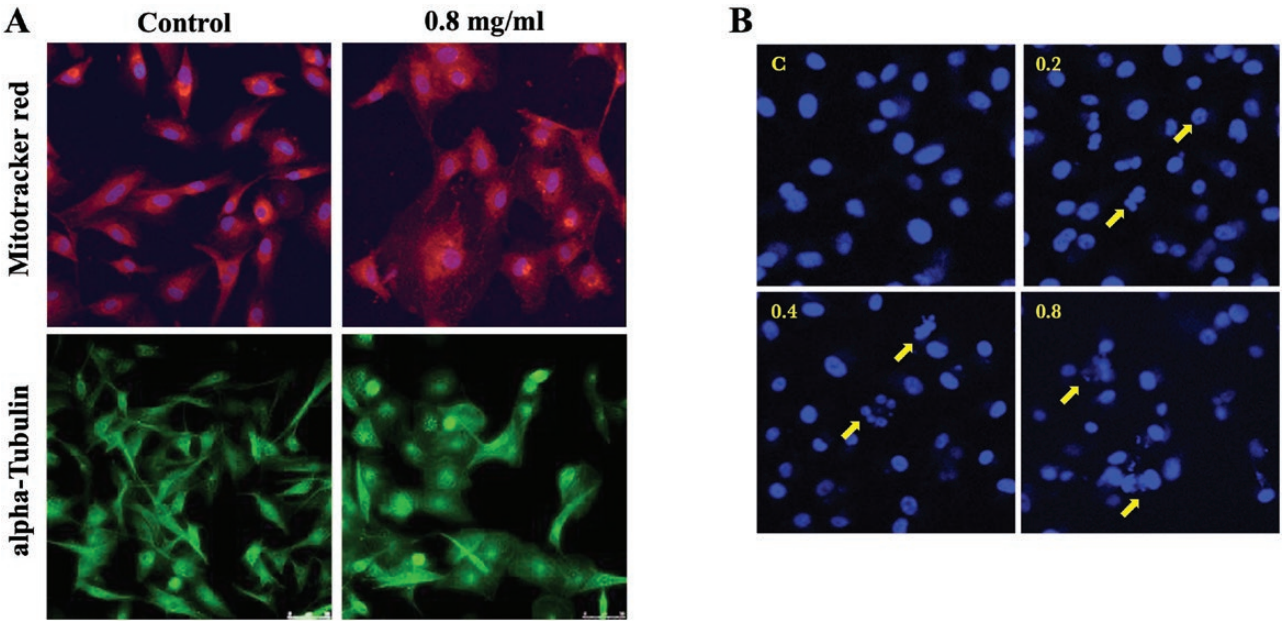

Figure 3 CQ causes mitochondrial fragmentation, cytoskeletal deformation and nuclear condensation in U-87 cells. (A) Control and 0.8 mg/ml of CQ. U-87 cells were treated for 24 h, then stained with Mitotracker red and alpha-Tubulin according to the protocol. The nucleus appears in blue. (B) Cells were treated with various concentrations of CQ (mg/ml) for 24 h. Cells were then stained using Hoechst staining and photographed under fluorescence.

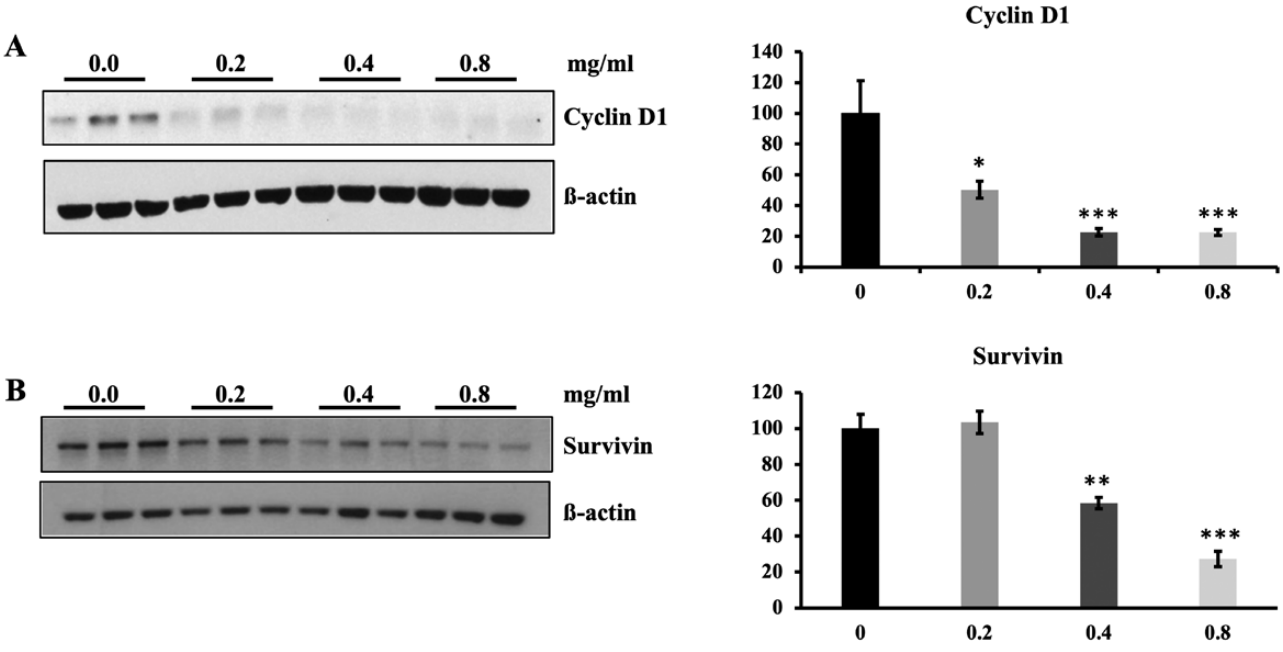

Figure 4 CQ treatment represses Cyclin D and Survivin protein expression in U-87 cells in a dose-dependent manner. U87 cells were treated with various concentrations of CQ for 24 h. Lysates were prepared for Western blotting, then probed with the anti-cyclin D1 (A) and anti-survivin (B) antibodies indicated in the figure. β-actin was used as a loading control. Expression levels of cyclin D1 and survivin were quantified by densitometry. Data represent the mean ± SEM of three replicates. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

pro-survival signalling pathways typically associated with cancer development.[29, 30] So far, no studies have shown CQ is able to affect cell signalling pathways in cancer cells especially in U87 cells. To explore the effect of CQ on signalling pathways, cells were treated with various concentrations of CQ for 24 h. Since CQ has been shown to inhibit glioblastoma cell viability and migration (Figures 1 and 2), we next studied if CQ played any roles in the regulation of MAPK/ERK pathway. Our data demonstrated that following 24 h treatment with various CQ concentrations, ERK phosphorylation was significantly inhibited by CQ (Figure 6). This indicated CQ might reduce glioblastoma cell viability and migration by inhibiting MAPK/ERK pathway.

Signal transducer and activator of transcription (STAT) includes a family of transcription factors. One of the STAT family members STAT3 has been reported to involve in tumour progression and the constitutively activated/phosphorylated STAT3 plays a crucial role in the carcinogenesis of

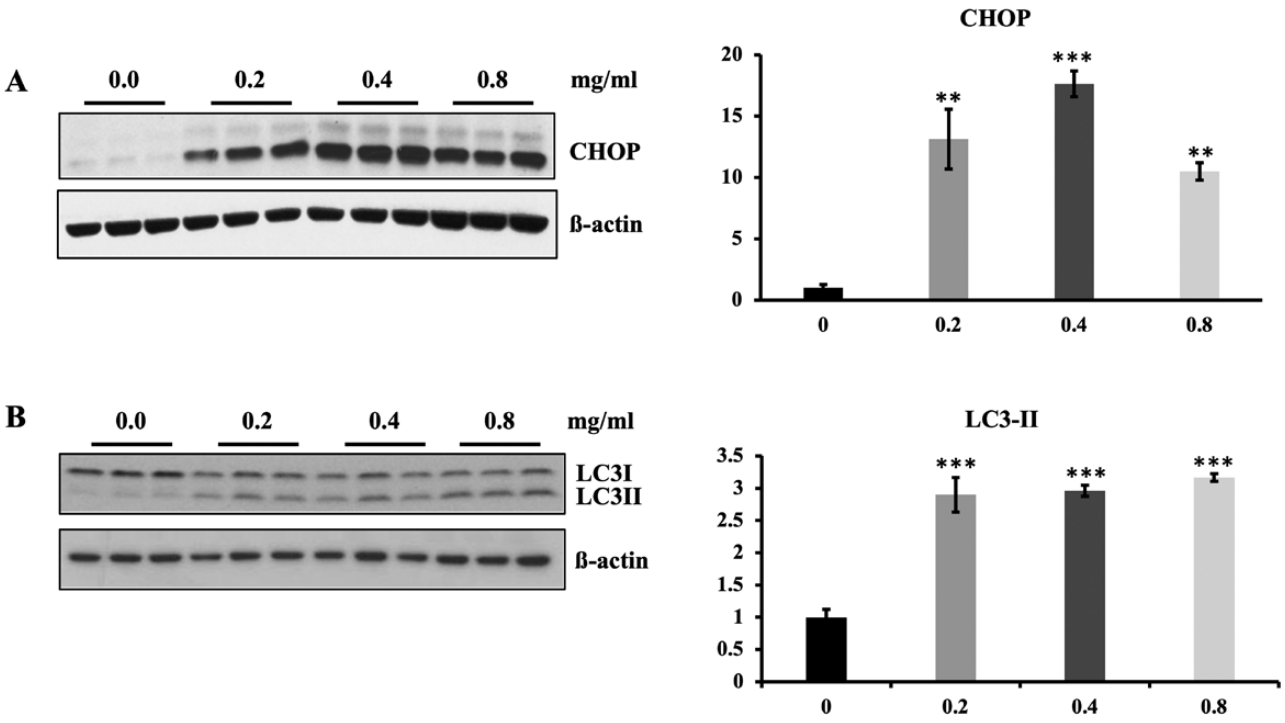

Figure 5 CQ treatment enhances CHOP (A) and LC3II (B) protein expression in U-87 cells in a dose-dependent manner. U87 cells were treated with various concentrations (mg/ml) of CQ for 24 h. Lysates were prepared for Western blotting, then probed with the anti-CHOP (A) and anti-LC3 (B) antibodies indicated in the figure. β-actin was used as a loading control. Expression levels of CHOP and LC3II were quantified by densitometry. Data represent the mean ± SEM of three replicates. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

head and neck cancer and myeloma cells.[31] In our study, CQ treatment also led to a significant decrease in both STAT3 and p-STAT3 expression in concentration-dependent fashion (Figure 6). A similar pattern was also observed in MAPK/ERK pathway following CQ treatment.

Discussion

CQ, also known as 'Hadjora', is a perennial herb from the grape family. It has been used for its healing properties in the repair of bone fractures and injured tendons. Additionally, CQ has been found to have antioxidant properties[32] and can help reduce inflammation.[33] Recent studies have also suggested that CQ may be beneficial in reducing obesity.[34, 35] Recently, CQ was also reported to have potential anticancer activity. Sheikh and his colleagues' study showed CQ contains several bioactive compounds that cause cancer cell morphological changes, ROS release, cell cycle arrest, MMP decrease, p53 up-regulation and Bcl-2 down-regulation in oral epidermoid carcinoma cell line (KB).[36] In addition, CQ is observed to cause DNA fragmentation and cell death in Hela cell line[37] and demonstrates strong antioxidant effects against leukemic cells.[38]

To further understand the anti-cancer mechanism of CQ, we investigated the effects and underlying mechanisms of CQ on human glioblastoma U87 MG cells. Glioblastoma multiforme is the most common primary malignant brain tumour in adults. At the beginning of this study, we examined the effects of CQ on glioblastoma cell viability. Previously, it is reported that CQ was able to promote osteoblast differentiation in a dosage-dependent manner. However, higher concentrations of CQ (≥0.1 mg/ml) adversely affect the cell growth, while lower concentrations of CQ (≤50 μg/ml) are non-toxic and significantly increase cell proliferation.[7] In our study, higher concentrations of CQ (0.1-1 mg/ml) were used to treat U87 MG cells. Crystal violet staining and MTS assay showed that CQ significantly inhibited U87 MG cell growth and proliferation. Additionally, the wound-healing assay indicated that CQ not only inhibited cell growth but also suppressed migration of glioma cells.

CQ has displayed the anti-cancer effects on different cancer cell lines.[39] For instance, CQ could induce A431 cell death via mitochondria-mediated apoptosis by increasing mitochondrial membrane's permeability.[40] Studies have also shown that mitochondrial fragmentation is involved in mitochondrial injury and is associated with cell death.[41] In our study, the diffused mitochondria in the cytoplasm and the mitochondrial fragmentation were observed after CQ treatment monitored by Mitotracker red. The abnormal distribution and fragmentation of the mitochondria implied low MMP and dysfunction of mitochondria. These results suggest that CQ is able to induce the disruption of the MMP in U87 cells. Moreover, nuclear condensation and chromatin fragmentation detected by Hoechst staining further confirmed the cytotoxic effects of CQ in U87 MG cells. This result is consistent with other studies that CQ elicits DNA fragmentation in various cancer cells.[18]

Microtubules are key components of the cytoskeleton and play crucial roles in the development and maintenance of cell shape, cell mitosis, cell migration and it is an important target for anticancer medications.[42] In our study, it was observed that cell size in the treatment group increased, and tubulin ectopically distributed all over the cytoplasm as compared to the control groups. Many studies have reported that

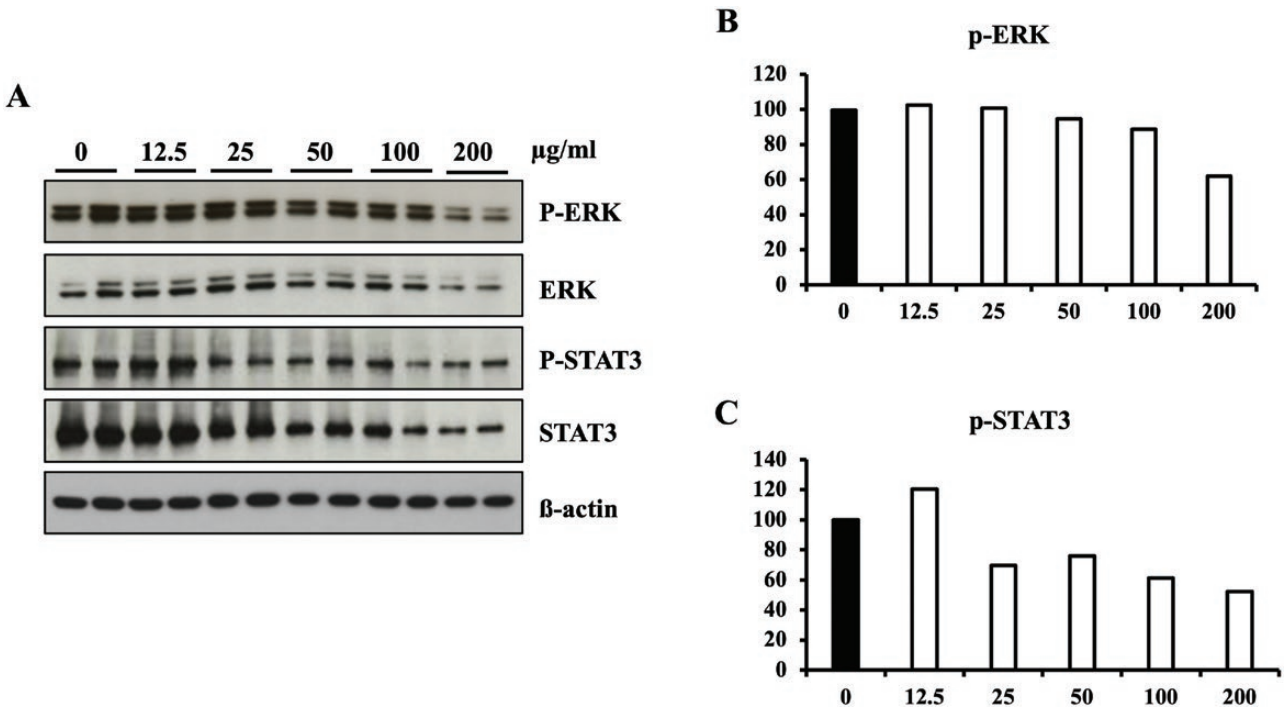

Figure 6 CQ inhibits both MAPK/ERK and STAT3 expression and phosphorylation in a dose-dependent manner. U87 cells were treated with various concentrations of CQ for 24 h. Lysates were prepared for Western blotting, then probed with the antibodies indicated in the figure. β-actin was used as a loading control. Expression levels of p-ERK and p-STAT3 were quantified by densitometry. Data represent the mean of two replicates.

anticancer medications inhibit microtubule depolymerization, enhance tubulin ectopic nucleation and lead to apoptosis.[43, 44] The observed potential apoptotic effect of CQ on tubulin proteins suggest that cell viability reduction might be caused by tubulin stability changes induced by CQ. Additionally, carbon nanotubes (CNT) are a potential new class of microtubule-stabilizers in cancer treatment by incorporating into microtubules and forming mixed hybrid tubulin polymers. It was shown that CNT could reduce cell migration speed in different cell types, including U87 MG cell, leading to cell apoptosis.[45] Our data showed CQ treatment significantly reduced U87 MG cell migration compared with the control group. It is possible that the effect of CQ on U87 MG cell migration is mediated by α-tubulin post-translational modification change, which further inhibited cell migration.

D-type cyclins regulate cell cycle progression and are tightly controlled in normal cells. However, overexpression of cyclin D has been linked to tumorigenesis in many types of cancer, including breast cancer and squamous cell carcinoma of the head and neck.[46–48] High levels of cyclin D1 activity have been shown to lead to uncontrolled tumour cell proliferation.[49] To limit the proliferation of cancer cells in advanced breast cancer, CDK4/6 inhibitors, such as ribociclib, abemaciclib and palbociclib are used.[50]

In our study, we detected cyclin D1 expression in U87 MG cells, in agreement with previous reports of CCND1 (cyclin D1 gene) amplification in malignant gliomas.[51] However, treatment with CQ for 24 h dramatically suppressed cyclin D1 expression, indicating that CQ may target the cell cycle and regulate cell proliferation via cyclin D1. Additionally, cyclin D1 has been found to control cellular invasiveness and aggressiveness by targeting substrates involved in cytoskeletal modelling, cell adhesion and motility.[52] Therefore, it is possible that CQ could inhibit cell migration by suppressing cyclin D1 levels.

Survivin is an inhibitor of apoptosis proteins that is highly expressed in most cancers and associated with chemotherapy resistance. It can be activated by the transcription factor STAT3,[53] and its overexpression could help cancer cells avoid apoptosis, increase cell proliferation, promote tumour aggressiveness and cause cancer relapse. Since its discovery, various methods have been tried to reduce survivin expression, including RNA interfering, antisense oligonucleotides, ribozyme approach and survivin inhibitors.[54, 55] In malignant gliomas, survivin levels increase with malignancy.[56] In our study, survivin levels gradually decreased in response to increasing doses of CQ, suggesting that CQ could induce apoptosis of glioma cells by inhibiting survivin expression.

In addition to regulating cell cycle and apoptosis inhibitors, CQ was also found to induce ER stress in our study. The ER plays a key role in protein synthesis, modification and folding. Protein-folding could be disrupted, and the misfolded proteins accumulate in ER which provokes a situation called ER stress. If unresolved, extreme ER stress can lead to cell death.[57] CHOP is considered an ER stress marker and serves an essential role to induce apoptosis.[58] Emerging evidence has shown the tumour suppression mechanism is mediated by autophagy-dependent cell death.[59, 60] In mammalian cells, a key step in the induction of autophagy is the conversion of microtubule-associated protein light chain 3-I (LC3I) to LC3II.[61, 62] In our study, CQ was observed to upregulate CHOP expression in U87 MG cells, indicating that CQ causes U87 cell apoptosis by inducing unresolved, lethal ER stress. Furthermore, following various concentrations of CQ treatment for 24 h, we detected the level of LC3II increased gradually in response

to the increased concentration of CQ. This implied that CQ could induce autophagy in U87 MG cells. Glioma cells are more likely to respond to therapy through autophagy than through apoptosis. Autophagy induction in gliomas by CQ thus represents a promising mechanism that may lead to new glioma therapies.

Mitogen-activated protein kinase (MAPK) cascades are important signalling pathways involved in regulating various cellular processes, including proliferation, differentiation, apoptosis and stress responses.[63–65] Among these pathways, the Ras/Raf/MAPK (MEK)/ERK pathway plays a critical role in tumour cell survival and development, and has been shown to be involved in glioma tumorigenesis.[66, 67] The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is associated with haematopoiesis, immune function, inflammation and apoptosis.[68] Recent studies have reported that the JAK/STAT pathway is also involved in malignancies and autoimmune diseases.[69–72] Therefore, inhibiting the JAK/STAT pathway holds promise for the treatment of various diseases, including cancer.

In our study, we observed a marked decrease in the expression of ERK, p-ERK, STAT3 and p-STAT3 in U87 MG cells treated with various concentrations of CQ for 24 h. These results indicate that CQ-induced cell death is mediated by suppressing the ERK and STAT3 pathways. Furthermore, studies have shown that inhibiting p-STAT3 can promote the expression of CHOP and activate ER stress in chronic stress-induced splenocyte apoptosis.[73] In addition, p-STAT3 inhibition could also play a role in activating autophagy. For example, biguanide metformin downregulates STAT3 activity and induces autophagy,[74] and sorafenib activates autophagy in multiple cell lines by downregulating p-STAT3.[75]

In our study, we observed enhanced expression of CHOP and LC3II in U87 MG cells following CQ exposure, suggesting that CQ-induced ER stress and autophagy activation might be mediated through downregulation of the STAT3 signalling pathway.

CQ is available as an aqueous extract, ethanolic extract, methanolic extract or chloroform water extract, each containing various phytochemical components.[76] This herbal medicine has been traditionally used safely and offers a promising alternative to harmful chemotherapy treatments. However, further experimentation is necessary to identify the potential anticancer components in CQ extracts and to develop efficient methods for delivering CQ or its potent anticancer components through the blood–brain barrier.

Conclusion

Our study demonstrated the cytotoxic and anticancer effects of CQ in human glioblastoma U87 MG cells. Treatment with CQ inhibited pre-survival signalling pathways, induced apoptosis and cell cycle arrest, reduced cell viability, inhibited cell migration, and caused changes in MMP and cell/nuclear morphology. To the best of our knowledge, this is the first study to reveal the mechanisms by which CQ may be useful in treating various types of cancers, including glioblastoma. Considering these aforementioned findings, it can be speculated that CQ may serve as a potential lead compound for the development of future anticancer therapies. However, further preclinical and clinical trials are needed to fully elucidate the spectrum of anticancer effects of CQ, either alone or in combination with other standard drugs, to validate its usefulness as a potent anticancer agent.

Author Contributions

B.C. and X.F. designed the experiment. B.C., Y.W., L.G., R.S., S.B. and L.V. performed the experiments. X.F. analysed the data and performed the statistical analyses. B.C. and X.F. drafted the manuscript, and all authors discussed the results and commented on the manuscript. All authors approved the final manuscript.

Funding

This research was supported by funds from Innovative Research and Development Program (Texas State), and UTRGV/SOM.

Ethical Statement

There were no animals or human volunteers included in this study.

Conflict of Interest

All authors declare that they have no conflicts of interest in this manuscript.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

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