Original Article

Quinacrine enhances temozolomide cytotoxicity in temozolomide-sensitive and -resistant glioblastoma cells

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Abstract

Background: The alkylating agent temozolomide (TMZ) is widely used in glioblastoma multiforme (GBM) therapy. Unfortunately, TMZ-resistance frequently occurs in recurrent GBM and is the major cause of treatment failure. The anti-malarial drug quinacrine (QC) harbors antitumor and chemosensitivity properties, but its interactions with TMZ in GBM remain unclear. This study aimed to investigate whether QC would sensitize TMZ in TMZ-sensitive and TMZ-resistant GBM cells as well as the underlying mechanisms. **Materials and Methods:** The cytotoxicity of QC and TMZ in TMZ-sensitive and TMZ-resistant GBM cells was evaluated using *in vitro* cell viability assay and colony formation assay. Cellular apoptosis and protein expression levels were determined using TUNEL assay and immunoblotting, respectively. **Results:** QC substantially enhanced TMZ cytotoxicity in both TMZ-sensitive and TMZ-resistant cells. Such cytotoxic effect was accompanied by changes in the expression levels of LC3II, p62 and cleaved caspase 3, and increased cellular apoptosis. The results suggested that QC could sensitize GBM cells to TMZ at least partially through apoptosis induction, in which autophagy inhibition might be involved. **Conclusion:** The antimalarial drug QC may hold promise as a potentiation of TMZ treatment in GBM, especially in cases of TMZ-resistance.

Keywords: Apoptosis, autophagy, glioblastoma multiforme, quinacrine, temozolomide

Introduction

Glioblastoma multiforme (GBM) is one of the most lethal primary brain tumors in adults. Despite standard therapy consisting of surgery, chemotherapy, and radiotherapy, patient prognosis remains poor. The median survival is around 15 months after diagnosis.[1] Resistance to the commonly used chemotherapeutic drug temozolomide (TMZ) in primary and recurrent GBM patients is a major cause of treatment failure. The development of new strategies to overcome TMZ-resistance is urgently needed to improve the prognosis of GBM patient.

The alkylating agent TMZ acts by methylating DNA at different positions to form DNA adducts. In particular, the formation of O6-methylguanine lesion is the most important effect that accounts for TMZ's therapeutic benefit. TMZ-induced DNA adducts interfere with DNA replication and consequently trigger apoptosis, autophagy, senescence and possibly other cellular responses.[2,3] However, TMZ-induced autophagy may prevent cell death by suppressing apoptosis and activating senescence,[2] while the inhibition of autophagy has been shown to enhance TMZ-induced apoptosis in GBM.[4,5] The previous study revealed that autophagy inhibition by a natural compound would sensitize GBM cells to TMZ.[6] These findings suggest that agents and/or factors targeting autophagy may be an effective strategy for promoting the therapeutic efficacy of TMZ.

Recently, the autophagy inhibitor quinacrine (QC) has attracted attention as a potential agent to overcome TMZ-resistance. QC is widely used as an antimalarial drug with proven records of patient tolerance and clinical safety. Furthermore, available evidence also suggests that QC may have anticancer and chemosensitizing properties.[7-9] In glioma cells, it has been reported that QC significantly inhibited cell proliferation,[10]

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and increased the anticancer effect of cediranib, an inhibitor of the tyrosine kinase receptor.[11] However, the effect of QC when used in combination with TMZ remains unexplored. This study aimed to investigate whether and how QC could enhance TMZ cytotoxicity in both TMZ-sensitive and TMZ-resistant GBM cells. Our findings demonstrated that QC might be a potential chemosensitizer of TMZ in GBM cells though at least partially apoptosis induction.

Materials and Methods

Cell culture

Human GBM cell lines U87 and U251 (American Type Culture Collection, Manassas, VA, USA) and TMZ-resistant U87-R and U251-R cell lines were maintained in MEMα medium (Gibco, USA) supplemented with 10% fetal bovine serum and 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco, USA). All cells were cultured at 37°C in a 5% CO_2 humidity incubator.

TMZ-resistant cell lines U87-R and U251-R were established by treating the parental U87 and U251 cells with a very low dose of TMZ at the beginning, then gradually increasing the doses very 2 weeks with >10 months treatment. The IC50 of TMZ-resistant cell lines were assessed using Thiazolyl blue tetrazolium bromide (MTT, Sigma, USA) assay.

Reagents and antibodies

TMZ was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) at a concentration of 10 mM. QC was obtained from Sigma (St. Louis, MO, USA) and dissolved in Milli-Q water at a concentration of 5 mM. Antibodies against LC3, p62, caspase 3, cleaved caspase 3 and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell viability assay

Cell viability was evaluated by MTT assay. Briefly, cells were seeded in triplicates on 96-well plates at 5×10^3 cells per well and treated with different concentrations of TMZ and/or QC on the next day. At the end of 72 h of treatment, MTT reagent was added to each well for 3 h of incubation. The supernatants were gently aspirated, and the formazan crystals were dissolved in DMSO. Cell viability was measured at 590 nm absorbance with Thermo Varioskan Flash Reader.

Clonogenic assay

Cells were seeded on 6-well plates in triplicates at a concentration of 500 cells per well. Next day, the cells were treated with QC and/or TMZ. After 8–12 days of incubation, cells were fixed by 75% ethanol for 30 min and then stained by 0.5% crystal violet for 30 min. The number of colonies was determined under a microscopy. Only colonies consisting of at least 50 cells were counted.

TdT-mediated dUTP-X nick end labeling assay

Cells were seeded on coverslips on 6-well plates 1 day before treatment with QC and/or TMZ. Apoptotic cells were assessed using *in situ* cell death detection kit (Roche, USA). Briefly, cells were fixed with 4% paraformaldehyde at room temperature for 1 h, and then incubated in freshly prepared permeabilization solution (0.1% Triton X-100 diluted in 0.1% sodium citrate) on ice for 2–5 min. Then, 50 μL of the TdT-mediated dUTP-X nick end labeling (TUNEL) mixture (mixing enzyme solution with label solution [1:9]) was directly added to the coverslips and incubated in cell culture incubator for 1 h. Finally, DAPI (4',6-diamidino-2-phenylindole) (Thermo, USA) in mount solution was used to stain cell nuclei. Apoptotic cells were visualized and counted under fluorescence microscopy.

Immunoblotting

Cells were lysed in radio immunoprecipitation assay lysis buffer (Cell Signaling Technology, USA) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Total cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membrane was blocked in nonfat milk and then incubated with a primary antibody (for LC3II, p62, or cleaved caspase 3) at 4°C overnight. After washing thrice, the membrane was incubated with HRP-linked secondary antibody at room temperature for 1 h. Protein bands were detected with chemiluminescent reagents (GE Healthcare, Buckinghamshire, UK) and then exposed to X-ray film.

Statistical analysis

The software SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. Student's *t*-test was used to evaluate the difference between groups. A value of $P < 0.05$ was considered statistically significant.

Results

Quinacrine is cytotoxic in both temozolomide-sensitive and temozolomide-resistant glioblastoma multiforme cells

Before investigating the effect of QC on GBM cells, we examined the resistance of U87-R and U251-R cells to TMZ using MTT assay. As shown in Figure 1, on exposure to different concentrations of TMZ (from 62.5 to 2000 μM), the number of viable cells in the U87-R and U251-R lines was significantly increased as compared to that in the paired TMZ-sensitive U87-S and U251-S cells, respectively. The IC50 values of U87-R and U251-R cells were 1314 μM and 707.9 μM, compared to 361.6 μM and 156.8 μM in U87-S and U251-S cells, which represented a 3.6-fold and 4.5-fold increase, respectively. These results suggest that U87-R and U251-R cells were TMZ-resistant GBM cells.

Furthermore, to determine the cytotoxic effect of QC on both TMZ-sensitive and TMZ-resistant cells, MTT assay was used. As shown in Figure 2, on QC treatment, the number of viable cells was obviously decreased in both TMZ-sensitive cells U87-S, U251-S and TMZ-resistant cells U87-R, U251-R in a dose-dependent manner. The rate of decrease in U87-R and

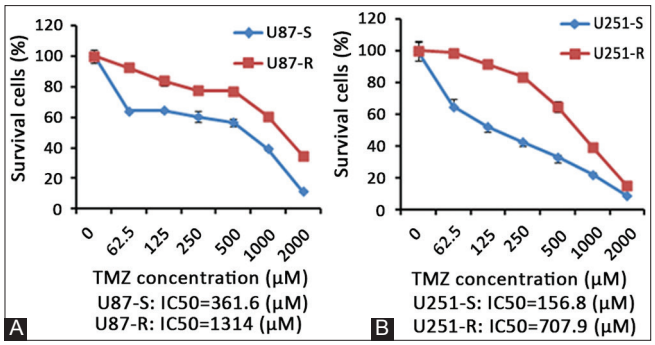

Figure 1: Temozolomide-resistant U87-R and U251-R glioblastoma multiforme cells. U87-S, U87-R (A) and U251-S, U251-R (B) were treated with temozolomide at concentrations of 62.5, 125, 250, 500, 1000, and 2000 μM for 72 h. Cell viability and the IC50 value were assessed using MTT assay

U251-R cells was similar to their parental cells. Less than half of viable cells were observed after treatment with 10 μM QC in all these cells. These results indicated that QC had the same cytotoxic effect on TMZ-resistant as on TMZ-sensitive GBM cells.

Quinacrine enhances temozolomide cytotoxicity in both temozolomide-sensitive and temozolomide-resistant glioblastoma multiforme cells

Next, we evaluated the cytotoxic effect of QC in combination with TMZ using MTT assay with short-term culture time, and clonogenic assay with long-term culture time. We found that QC significantly enhanced the cytotoxic effect of TMZ in both TMZ-sensitive cells (U87-S, U251-S) and TMZ-resistant cells (U87-R, U251-R). On MTT assay, 250 μM TMZ only reduced the survival of U87-S and U87-R cells to 60% and 80%. The addition of 4 μM QC further decreased the number of viable cells to 40% and 60%, respectively [Figure 3A and B]; Similarly, 250 μM TMZ treatment led to 40% U251-S and 80% U251-R cell survival as compared to the control, whereas 4 μM QC in combination with 250 μM TMZ further decreased the number of viable cells to 30% and 40%, respectively [Figure 3C and D]. On clonogenic assay, the clonogenic survival was significantly decreased by combined QC and TMZ treatment when compared with QC or TMZ treatment alone in the two paired cell lines [Figure 4]. For example, 50 μM TMZ reduced the survival clones of U87-S and U87-R cells to 60% and 90%. The addition of 0.5 μM QC further decreased the survival clones to 40% and 65%, respectively. These results suggested that QC could significantly enhance the efficacy of TMZ treatment in both TMZ-sensitive and in TMZ-resistant GBM cells.

Quinacrine increases temozolomide-induced apoptosis in both temozolomide-sensitive and temozolomide-resistant glioblastoma multiforme cells

We next explored the potential mechanisms of QC's action. QC is a late-stage autophagy inhibitor.[10,12-14] During autophagy, LC3I is conjugated to phosphatidylethanolamine to form LC3II. LC3II is recruited to autophagosomal membranes and degraded after the fusion of autophagosomes with

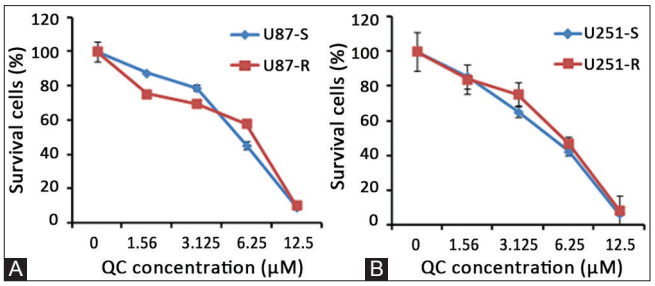

Figure 2: Quinacrine has cytotoxic effect on temozolomide-sensitive and temozolomide-resistant cells. U87-S, U87-R (A) and U251-S, U251-R (B) were treated with quinacrine at concentrations of 1.56, 3.125, 6.25, and 12.5 μM for 72 h. Cell viability was assessed using MTT assay

lysosomes. P62, an LC3-binding protein, is downregulated by activating autophagy flux. In general, when the degradation of autophagosome is blocked, an accumulation of LC3II and p62 expressions would occur.[15-17] To confirm whether QC could interfere with cellular autophagy in our model, the autophagy markers of LC3I/II and p62 were assessed. Cells were exposed to QC and/or TMZ, and immunoblotting was performed to detect LC3II and p62 expression levels. As expected, QC upregulated both LC3II and p62 expressions [Figure 5E and F], indicating autophagosome accumulation and the inhibition of autophagy on QC treatment.

Apoptosis is an important mechanism of cell death in GBM. We investigated whether the elevated cytotoxicity of TMZ when used in combination with QC in the two paired cell lines (U87-S and U87-R; U251-S, and U251-R) was due to apoptosis induction. Our results showed that QC plus TMZ upregulated the Bax and cleaved caspase 3 expression levels compared to treatment with QC or TMZ alone in all four cell lines [Figure 5E and F], suggesting that cellular apoptosis could be increased by the combinational treatment. To further confirm the effect of apoptosis induction, TUNEL assay was performed. As shown in Figure 5A-D, QC plus TMZ significantly elevated cellular apoptosis in both TMZ-sensitive and TMZ-resistant cells as compared to QC or TMZ alone. For example, more than 25% of U87-R [Figure 5B] and U251-R [Figure 5D] cells were TUNEL-positive with the combinatorial treatment which was significantly higher than that treated with QC or TMZ alone (<10% TUNEL-positivity). Taking together, our results suggested that QC could enhance TMZ-induced apoptosis in GBM cells.

Discussion

Acquired TMZ-resistance is an important factor accounting for the poor prognosis of GBM patients. TMZ-induced autophagy has been shown, in addition to other known mechanisms such as DNA repair system[18,19] and the mismatch-repair response, to play an important role in the development of TMZ-resistance.[20,21] QC acts as an inhibitor of autophagy and has antitumor activities.[7-9] In this study, we investigated the effect of QC in combination with TMZ on TMZ-sensitive and TMZ-resistant GBM cells.

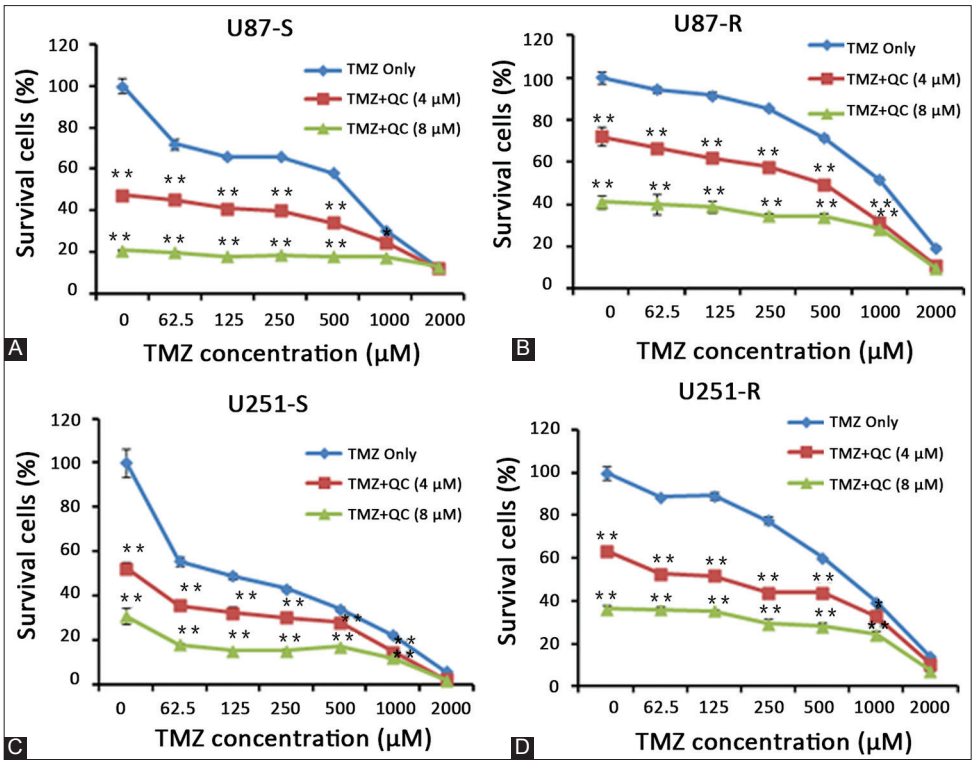

Figure 3: Quinacrine is an effective chemosensitizer of temozolomide in both temozolomide-sensitive and temozolomide-resistant cells. U87-S (A) and U87-R (B), U251-S (C), and U251-R (D) cells were treated with quinacrine at concentrations of 4 or 8 μM and/or in combination with different concentrations (62.5, 125, 250, 500, 1000, and 2000 μM) of temozolomide for 72 h. Cell viability were detected by MTT assay. Data were analyzed using Student's *t*-test and represented the mean ± standard deviation; $*P < 0.05$; $**P < 0.01$

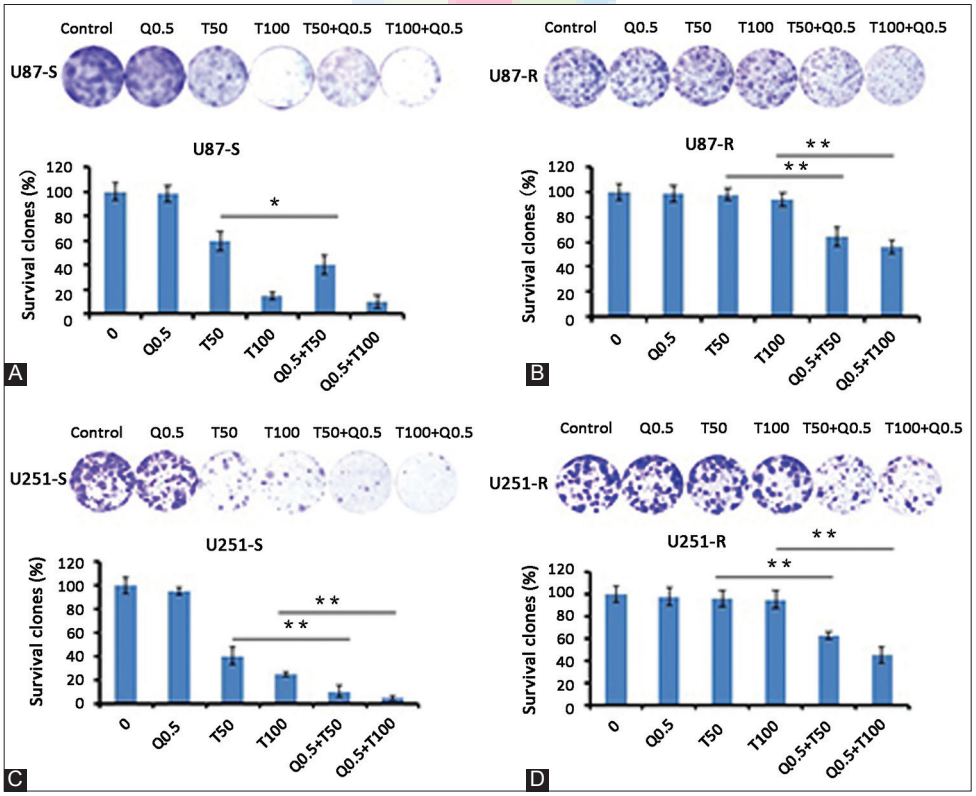

Figure 4: Quinacrine increases temozolomide efficacy in both temozolomide-sensitive and temozolomide-resistant cells. U87-S (A) and U87-R (B), U251-S (C), and U251-R (D) cells were treated with 0.5 μM QC and/or 50 or 100 μM temozolomide for 10–14 days. Survival clones were determined under a microscope. Data were analyzed by Student's *t*-test and represented the mean ± standard deviation; $*P < 0.05$; $**P < 0.01$

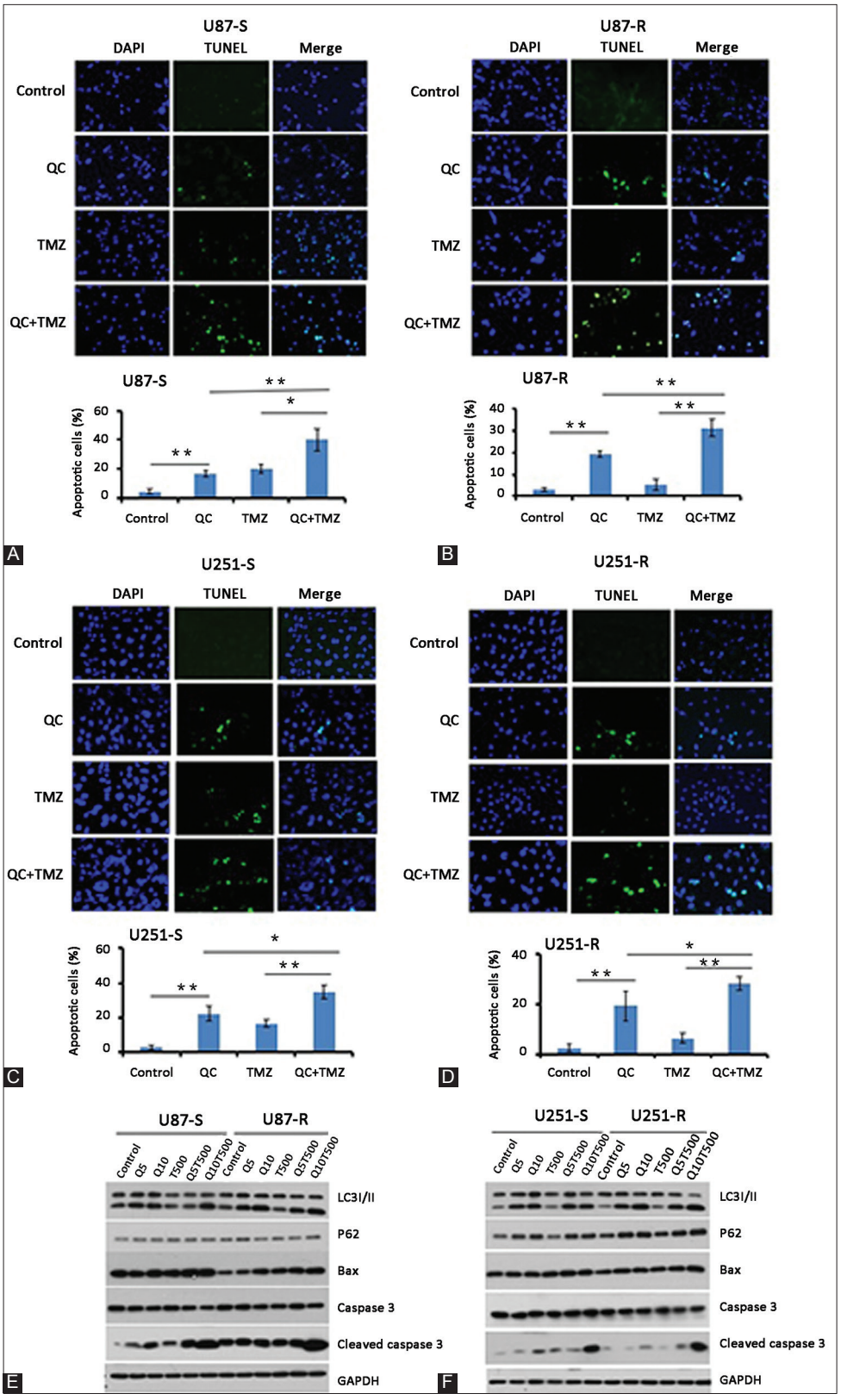

Figure 5: Quinacrine significantly enhances temozolomide chemosensitivity through apoptosis induction. U87-S (A), U87-R (B), and U251-S (C), U251-R (D) cells were treated with 10 μM QC with or without 500 μM temozolomide for 48 h. Apoptotic cells were detected by TUNEL assay. Data were analyzed by Student's *t*-test and all graphs show the mean ± standard deviation; **P* < 0.05; ***P* < 0.01. U87-S and U87-R (E), U251-S and U251-R (F) cells were treated with 5 (Q5) or 10 μM (Q10) quinacrine with or without 500 μM temozolomide (T500) for 48 h. Protein expressions were determined by immunoblotting

The antitumor activities of QC have already been identified in several cancers.[7,22,23] QC could enhance chemosensitivity in both chemosensitive and chemoresistant ovarian cancer cells.[7] In several colorectal cancer cells, QC could act synergistically with a variety of chemotherapeutic agents.[8,24] QC also enhanced the sensitivity of hepatocellular carcinoma to several chemotherapeutic agents, except for TRAIL (tumor necrosis factor-related apoptosis-inducing ligand).[9] Golden *et al*.[10] found that several antimalarial drugs including QC may exert cytotoxic effects on glioma cells including those that exhibited high degrees of chemoresistance. In addition, QC could significantly enhance cediranib efficacy in glioma both *in vitro*[25] and *in vivo*.[11] Our results demonstrated that QC could substantially promote TMZ cytotoxicity, even in cells that are highly resistant to TMZ, indicating that this time-honored antimalarial drug, when used in combination with TMZ, could provide a promising therapeutic strategy for GBM including treatment-naïve lesions with intrinsic TMZ-resistance and recurrent cases with *de novo* TMZ-resistance.

In terms of mechanism of action, we found that QC may act, at least partially, through the induction of apoptosis. Previous studies showed that QC-induced cancer cell death was mostly due to cellular apoptosis in, for example, colorectal cancer,[24,26] ovarian cancer,[7] breast cancer,[23,27] and gastric cancer.[28] In addition, QC could enhance the efficacy of factors such as suberoylanilide hydroxamic acid (SAHA),[29] TRAIL,[9,30] and cediranib[25] through apoptosis induction. In agreement with these findings, our results further demonstrated that QC could enhance TMZ efficacy through apoptosis induction in GBM cells.

Although QC functions as an autophagy inhibitor, whether QC-induced apoptosis is indeed related to autophagy inhibition remains controversial. In colonic cancer cells, QC-induced apoptosis was found to be positively associated with the accumulation of autophagic vacuoles and increased LC3II expression.[26] Moreover, QC also sensitized ovarian cancer cells to cisplatin in an autophagy-dependent manner.[7] However, in nonsmall-cell lung cancer, QC-induced apoptosis was probably independent of autophagy inhibition.[14] Our results suggested that in glioma cells, QC could block autophagy and that may account for the increase in apoptosis following combinatorial treatment with TMZ, but further investigations are needed.

In conclusion, our preliminary study demonstrated that QC could sensitize GBM cells to TMZ, possibly through apoptosis induction and the inhibition of autophagy. Given that QC is a clinically accessible, safe, and well-tolerated drug, its potential role in GBM treatment warrants further translational and clinical investigations. Future studies may focus on the appropriate treatment window and dosing regimen as well as QC's potential interactions with radiotherapy and TMZ rechallenge.

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Conflicts of interest

There are no conflicts of interest.

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