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Temozolomide and AZD7762 Induce Synergistic Cytotoxicity Effects on Human Glioma Cells

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Abstract. *Background/Aim: This study investigated the effects of temozolomide (TMZ) and/or checkpoint kinase inhibitor AZD7762 in human glioma cells. Materials and Methods: Glioma cells were treated with TMZ and/or AZD7762 for 24 or 48 h, then the cellular survival was studied and the expression of various proteins was investigated. Results: Both TMZ and AZD7762 induced concentration- and time-dependent cytotoxic effects, and combined TMZ and AZD7762 (TMZ+AZD) caused synergistic cytotoxic effects in glioma cells ($p<0.05$). AZD7762 suppressed the O^6-methylguanine-DNA-methyltransferase (MGMT) expression. TMZ+AZD increased the expression of phospho-p53 (p-p53), p-p38 mitogen-activated protein kinase, and phosphatase and tensin homolog; and decreased the expression of p-extracellular signal-regulated kinase 1/2 and p-signal transducer and activator of transcription 3 in glioma cells. Conclusion: TMZ and AZD7762 combined induced synergistic cytotoxic effects on human glioma cells and such effects may be related to the AZD7762-induced suppression of MGMT expression and the modulation of multiple signaling pathways.*

Malignant glioma is the most common primary brain tumor, and is histologically heterogeneous and invasive (1-4). The prognosis of patients with malignant gliomas is poor, even under treatment with surgery, radiotherapy, and chemotherapy (1, 2, 4). In recent years, concomitant temozolomide (TMZ) and radiotherapy have been demonstrated to prolong patients' survival (1, 5). However, not all patients with glioblastoma multiforme (GBM) respond to TMZ and drug resistance may develop after treatment (2, 5-7). In addition, these treatments only modestly enhance patients' survival (8). Therefore, it is crucial to develop better therapies to eliminate the drug resistance and improve patients' prognosis.

TMZ, a DNA methylating imidazole tetrazinone, acts as a cytotoxic prodrug that, when hydrolyzed, suppresses DNA replication by methylation of guanines at the O^6 position to produce O^6-methylguanine in DNA, which then mispairs with thymine (9). O^6-methylguanine-DNA-methyltransferase (MGMT) is involved in the repair of DNA damage induced by TMZ, and the effect of TMZ and the development of drug resistance after TMZ treatment are closely related to the expression and the methylation status of the *MGMT* gene (1, 5, 7, 10, 11). TMZ can also induce the phosphorylation/activation of checkpoint kinase 1 (Chk1) and Chk2, which are required for TMZ-induced G_2/M arrest (12-14). In contrast, Chk inhibitors can abolish cell-cycle arrest in transformed cells treated with DNA-damaging agents such as TMZ, causing inappropriate G_2/M progression and death (13, 14). Therefore, to overcome the drug resistance or enhance the antitumor effect, combination therapy with TMZ and a Chk inhibitor is a reasonable approach for the treatment of malignant gliomas (15). However, the clinical benefits of the combination of TMZ with various Chk inhibitors in cancer treatment are still unsatisfactory (16). AZD7762, an adenosine triphosphate-competitive Chk1/2 inhibitor, has been found to induce antitumor effects in various cancer types (16-21). It can increase DNA damage and abrogate cell-cycle arrest in cells treated with genotoxic agents, and has been shown to have a synergistic effect with DNA-damaging agents and radiation to cause cancer cell apoptosis (16-21). In the literature, limited studies about the effects of AZD7762 on brain tumor cells showed that AZD7762 can trigger apoptosis and necrosis of medulloblastoma cells (22) and induce cell death of glioma cells (8, 23). However, there is no report investigating the effects of combined TMZ and AZD7762 (TMZ+AZD) on cancer cells including glioma cells. Therefore, in this study, we investigated the effects of TMZ+AZD on the viability of glioma cells and explored the possible underlying mechanisms of their synergistic cytotoxicity effects.

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Key Words: Temozolomide, AZD7762, cytotoxicity, glioma.

Materials and Methods

Cell lines and cell culture. Three human glioma cell lines T98G, U138MG and U87MG (American Type Culture Collection, Manassas, VA, USA) were used in this study. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum plus 2 mmol/l L-glutamine, 1 IU/ml penicillin G, and 1 μg/ml streptomycin.

Reagents. Temozolomide was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and AZD7762 (3-(carbamoylamino)-5-(3-fluorophenyl)-N-[(3S)-3-piperidyl] thiophene-2 carboxamide) was from AstraZaneca (Wilmington, DE, USA). Both drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Cell viability assay. To analyze the cytotoxicity effects of TMZ and/or AZD7762 on glioma cells, cells were exposed to various concentrations of TMZ and/or AZD7762 for 24 or 48 h, and then the cellular viability was determined *in vitro* by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide)-based colorimetric assay (MTT assay). Briefly, 5×10^3 cells were seeded in triplicate wells of a flat-bottomed 24-well microtiter plate and cultured overnight before treatment. Then, cells were treated with various regimens for 24 or 48 h. After drug removal, cells were incubated for five days in total. Cell proliferation and viability were measured by the MTT assay. The drug concentrations used in the experiments were chosen according to the literature (8, 9, 18, 22, 24, 25). The drug concentration at which 50% of cells were killed was defined as the LC_{50}. The dose enhancement ratio of TMZ was calculated by dividing the TMZ concentration required to kill 50% of the cells during TMZ treatment alone by the TMZ concentration required to kill 50% of the cells during TMZ+AZD treatment. The dose enhancement ratio of AZD7762 was calculated by dividing the AZD7762 concentration required to kill 50% of the cells during AZD7762 treatment alone by the AZD7762 concentration required to kill 50% of the cells during TMZ+AZD treatment. The difference in the cytotoxicity induced by various treatments was analyzed.

Whole cell extract preparation and western blot analysis. To explore the mechanisms of the cytotoxicity of the glioma cells treated with TMZ and/or AZD7762, glioma cells were exposed to various concentrations of TMZ, AZD7762, or TMZ+AZD for 24 or 48 h, and then analysed by western blot. The primary antibodies against MGMT (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (Santa Cruz Biotechnology), phospho-p53 (p-p53, Ser 46) (Santa Cruz Biotechnology), BAX (Santa Cruz Biotechnology), extracellular signal-regulated kinase 1/2 (ERK 1/2) (Santa Cruz Biotechnology), p-ERK 1/2 (Santa Cruz Biotechnology), p38 mitogen-activated protein kinase (p38 MAPK) (Santa Cruz Biotechnology), p-p38 MAPK (Santa Cruz Biotechnology), protein kinase B (AKT) (Abcam, Cambridge, UK), p-AKT (Cell Signaling Technology, Danvers, MA, USA), phosphatase and tensin homolog (PTEN) (R&D Systems, Minneapolis, MN, USA), signal transducer and activator of transcription 3 (STAT3) (Bethyl Lab Inc., Montgomery, TX, USA), p-STAT3 (Bethyl Lab Inc.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EMD Millipore, Billerica, MA, USA) were used.

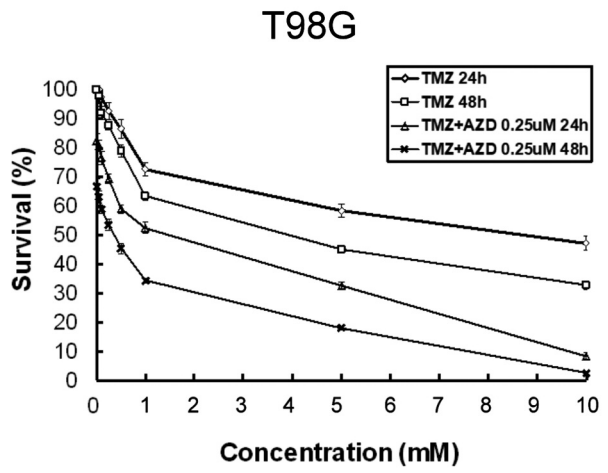
Statistical analysis. We used one-way analysis of variance (ANOVA) by Scheffe's multiple comparison for statistical analyses of the extent of cytotoxicity of the glioma cells induced by various treatments. Statistical significance was defined as $p<0.05$.

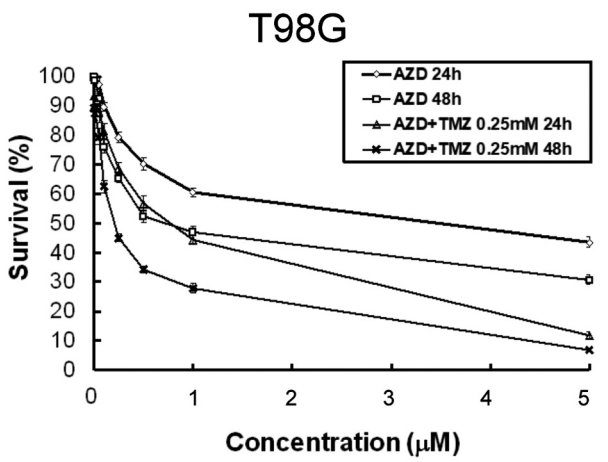
Results

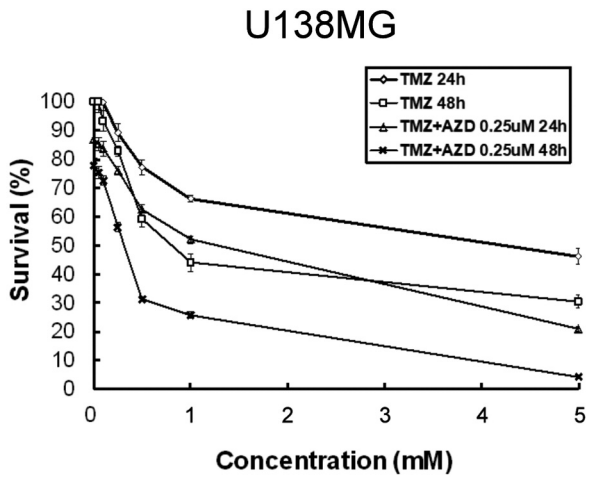
Temozolomide and AZD7762 induced cytotoxicity in glioma cells. The glioma cells were treated with various concentrations of TMZ or AZD7762 for 24 or 48 h, and then the viability of the glioma cells was analyzed by MTT assay (Figures 1 and 2). Both TMZ and AZD7762 induced concentration- and time-dependent cytotoxicity in glioma cells ($p<0.05$). The LC_{50}s for TMZ at 24 and 48 h of treatment were 8.75 mM and 3.93 mM for T98G cells, 4.24 mM and 0.81 mM for U138MG cells, and 4.69 mM or 0.93 mM for U87MG cells, respectively (Table I). The T98G cells had the highest LC_{50} for TMZ among these three cell lines. The LC_{50}s for AZD7762 at 24 and 48 h of treatment were 3.48 μM and 0.71 μM for T98G cells, 7.86 μM and 5.32 μM for U138MG cells, and 2.34 μM or 0.81 μM for U87MG cells, respectively (Table II). The U138MG cells had the highest LC_{50} for AZD7762 among these three cell lines.

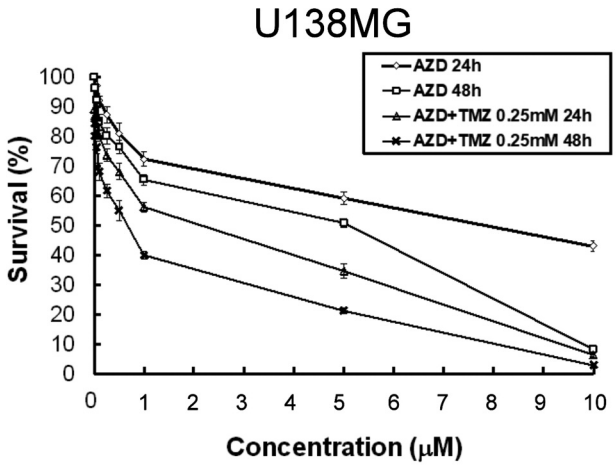
Combined temozolomide and AZD7762 treatment induced synergistic cytotoxic effects on glioma cells. The effects of TMZ+AZD on the viability of the glioma cells were studied. Glioma cells were treated with various concentrations of TMZ and 0.25 μM AZD7762 for 24 or 48 h, the viability of the glioma cells was analyzed by MTT assay (Figure 1), and the dose enhancement ratios were calculated (Table I). The viability of the cells treated with various concentrations of TMZ and 0.25 μM AZD7762 was significantly less than that of cells treated with TMZ only ($p<0.05$). In addition, the TMZ LC_{50}s in the cells treated with TMZ+AZD were lower than those in the cells treated with TMZ alone (Table I). The dose enhancement ratios of TMZ for T98G, U138MG and U87MG cells, respectively, were 4.4, 4.2, and 4.3 folds for 24 h treatment, and 8.7, 2.6 and 3.2 folds for 48 h treatment (Table I).

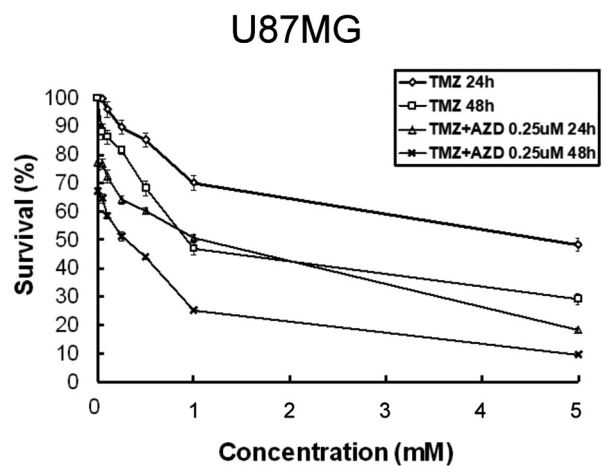
The cells were also treated with various concentrations of AZD7762 and 0.25 mM TMZ for 24 or 48 h. Then, the viability of the glioma cells was analyzed by MTT assay (Figure 2) and the dose enhancement ratios were calculated (Table II). The viability of the cells treated with various concentrations of AZD7762 and 0.25 mM TMZ was significantly less than that of the cells treated with AZD7762 only ($p<0.05$). In addition, the AZD7762 LC_{50}s in the cells treated with TMZ+AZD were lower than those in the cells treated with AZD7762 alone (Table II). The dose enhancement ratios of AZD7762 for T98G, U138MG and U87MG cells, respectively, were 4.5, 3.7, and 3.8 folds for 24 h treatment, and 3.4, 7.8 and 4.8 folds for 48 h treatment (Table II). Collectively, these data indicate that TMZ and AZD induced synergistic cytotoxic effects on the glioma cells.











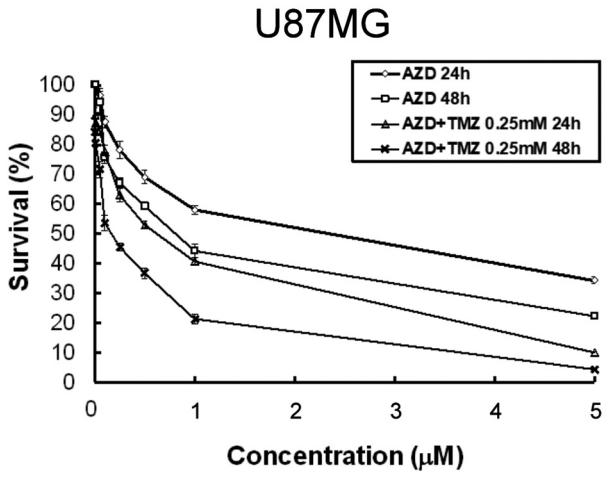


Figure 1. *Effects of temozolomide alone or temozolomide plus AZD7762 on the viability of glioma cells. The T98G, U138MG, and U87MG glioma cells were treated with various concentrations of temozolomide (TMZ) or TMZ plus 0.25 μM AZD7762 (AZD) for 24 or 48 h. Then, cell viability was measured using MTT assay. Each point is the average of three independent trials (nine determinations for each concentration); data are presented as mean±standard deviation.*

Figure 2. *Effects of AZD7762 alone or AZD7762 plus temozolomide on the viability of glioma cells. The T98G, U138MG, and U87MG glioma cells were treated with various concentrations of AZD7762 (AZD) or AZD7762 plus 0.25 mM temozolomide (TMZ) for 24 or 48 h. Then, cell viability was measured using MTT assay. Each point is the average of three independent trials (nine determinations for each concentration); data are presented as mean±standard deviation.*

Table I. *Dose enhancement ratio* of temozolomide (TMZ) in the glioma cells treated with various concentrations of TMZ or TMZ plus 0.25 μM AZD7762.*

Treatment duration	24 h	48 h
T98G		
TMZ	8.75 mM	3.93 mM
TMZ+AZD7762	2.01 mM	0.45mM
Dose enhancement ratio	4.4 folds	8.7 folds
U138MG		
TMZ	4.24 mM	0.81 mM
TMZ+AZD7762	1.02 mM	0.31 mM
Dose enhancement ratio	4.2 folds	2.6 folds
U87MG		
TMZ	4.69 mM	0.93 mM
TMZ+AZD7762	1.08 mM	0.21 mM
Dose enhancement ratio	4.3 folds	4.4 folds

*The dose enhancement ratio was calculated by dividing the TMZ concentration required to kill 50% of the cells during TMZ treatment alone by the TMZ concentration required to kill 50% of the cells during combined TMZ and 0.25 μM AZD7762 treatment.

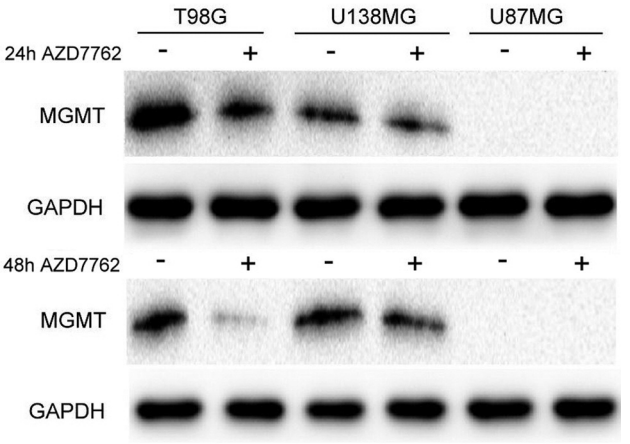


Figure 3. *Effects of AZD7762 on the O^6-methylguanine-DNA-methyltransferase (MGMT) expression in the glioma cells. The T98G, U138MG, and U87MG glioma cells were treated with 0.25 μM AZD7762 for 24 for 48 h. Then, the protein levels of MGMT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed by western blotting using specific antibodies.*

Table II. *Dose enhancement ratio* of AZD7762 in the glioma cells treated with various concentrations of AZD7762 or AZD7762 plus 0.25 mM temozolomide (TMZ).*

Treatment duration	24 h	48 h
T98G		
AZD7762	3.48 μM	0.71 μM
AZD7762+TMZ	0.77 μM	0.21 μM
Dose enhancement ratio	4.5 folds	3.4 folds
U138MG		
AZD7762	7.86 μM	5.32 μM
AZD7762+TMZ	2.15 μM	0.68 μM
Dose enhancement ratio	3.7 folds	7.8 folds
U87MG		
AZD7762	2.34 μM	0.81 μM
AZD7762+TMZ	0.62 μM	0.17 μM
Dose enhancement ratio	3.8 folds	4.8 folds

*The dose enhancement ratio was calculated by dividing the AZD7762 concentration required to kill 50% of the cells during AZD7762 treatment alone by the AZD7762 concentration required to kill 50% of the cells during combined AZD7762 and 0.25 mM TMZ treatment.

The effect of AZD7762 on MGMT expression in glioma cells. Figure 3 shows the expression of MGMT in the human glioma cells treated with 0.25 μM AZD7762 for 24 or 48 h. Among these three glioma cell lines, T98G cells had the highest constitutive MGMT expression level, U138MG cells had relatively low expression, and the U87MG cells had almost no expression. AZD7762 treatment suppressed the MGMT expression in both T98G and U138MG cells. The U87MG cells had almost no MGMT expression after AZD7762 treatment.

Effects of temozolomide and/or AZD7762 on the expression of proteins related to the proliferation and survival of glioma cells. The expression of various proteins related to the proliferation and survival of glioma cells after TMZ and/or AZD7762 treatment for 48 h was studied by western blot analysis (Figure 4). AZD7762 and TMZ+AZD significantly increased p-p53 expression in the investigated glioma cells; however, TMZ only mildly enhanced its expression. Furthermore, TMZ, AZD7762 and TMZ+AZD increased BAX expression in T98G and U138MG cells, while failed to affect its expression in U87MG cells. TMZ enhanced p-ERK 1/2 expression in T98G and U138MG cells, but did not affect its expression in U87MG cells. In contrast, AZD7762 and TMZ+AZD suppressed p-ERK 1/2 expression in the investigated glioma cells. Additionally, TMZ did not affect p-p38 MAPK expression in T98G and U138MG cells, while enhanced its expression in U87MG cells. AZD7762 increased p-p38 MAPK expression in T98G and U87MG cells, but showed no effect on its expression in U138MG cells. In contrast, TMZ+AZD enhanced the p-p38 MAPK expression in all investigated glioma cells. AZD7762 did not affect PTEN expression in T98G cells, while enhanced its expression in U138MG and U87MG cells. In contrast, TMZ and TMZ+AZD increased PTEN expression in the investigated glioma cells. TMZ showed no effect on the p-AKT expression in the investigated glioma cells; in contrast, AZD7762 and TMZ+AZD suppressed its expression in

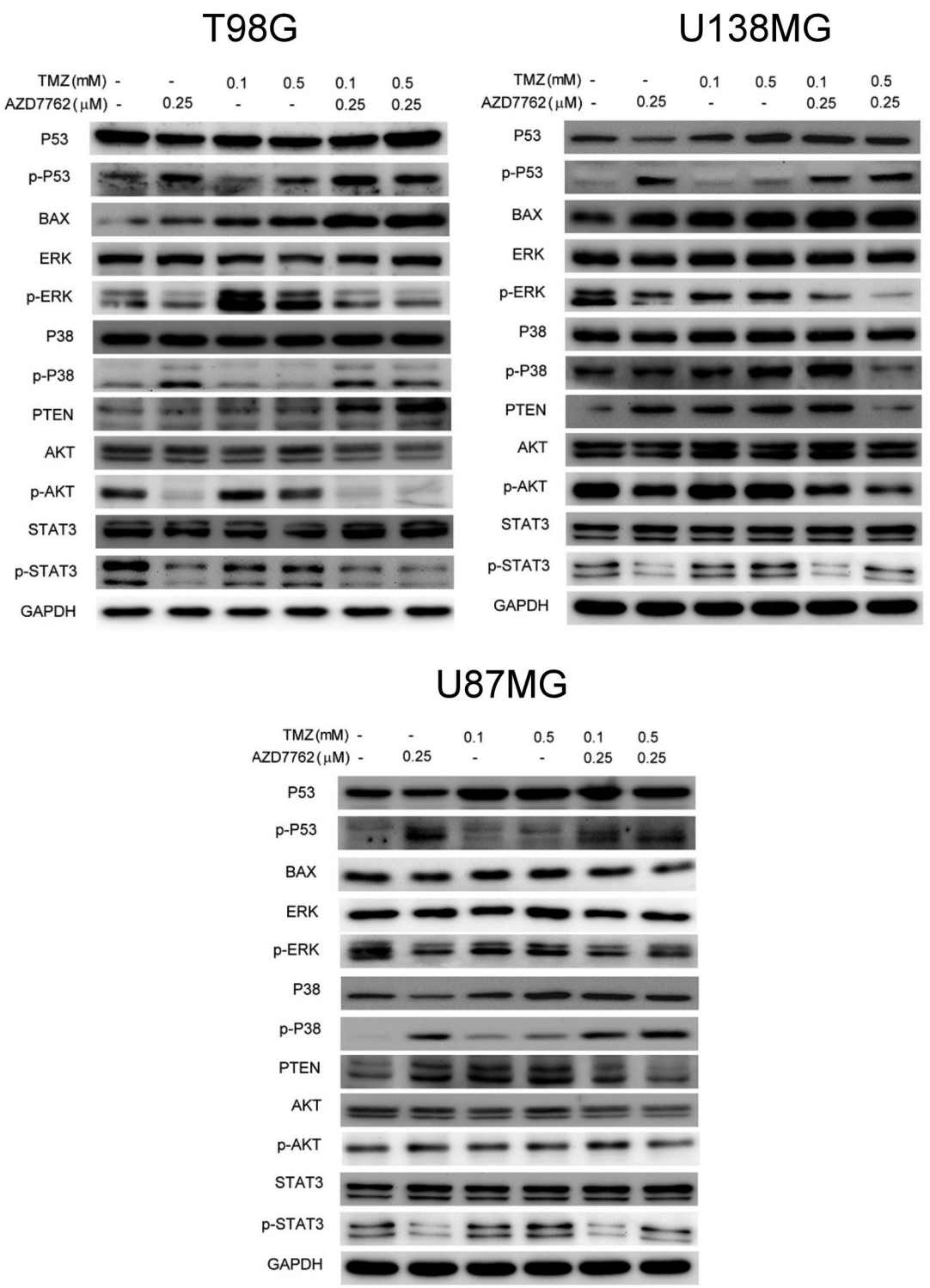


Figure 4. *Effects of temozolomide, AZD7762, or combined TMZ and AZD7762 on various proteins in the glioma cells. The T98G, U138MG, and U87MG glioma cells were treated with temozolomide (TMZ, 0.1 or 0.5 mM) and/or AZD7762 (0.25 μM) for 48 h. Then, the protein levels of p53, phospho-p53 (p-p53), BAX, extracellular signal-regulated kinase 1/2 (ERK 1/2), p-ERK 1/2, p38 mitogen-activated protein kinase (p38), p-p38 MAPK (p-p38), phosphatase and tensin homolog (PTEN), protein kinase B (AKT), p-AKT, signal transducer and activator of transcription 3 (STAT3), p-STAT3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed by western blotting using specific antibodies.*

T98G and U138MG cells, while failed to affect expression in U87MG cells. TMZ did not affect p-STAT3 expression, in contrast, AZD7762 and TMZ+AZD inhibited expression in the investigated glioma cells. Taken together, TMZ+AZD enhanced the expression of p-p53, p-p38 MAPK, PTEN; and suppressed the expression of p-ERK 1/2, and p-STAT3 in glioma cells, in contrast to the variable effects on the expression of these proteins caused by TMZ or AZD7762 treatment alone. Additionally, TMZ+AZD increased BAX expression and decreased p-AKT expression in T98G and U138MG cells, but showed no effects in U87MG cells.

Discussion

In this study, TMZ exerted concentration- and time-dependent cytotoxicity in glioma cells, with the LC_{50}s being 4.24-8.75 mM for 24 h treatment and 0.81-3.93 mM for 48 h treatment, which were relatively higher than those for glioma cells (about 0.01 ~1.5 mM) reported in the literature (26). AZD7762 also induced concentration- and time-dependent cytotoxicity in glioma cells, with the LC_{50}s being 2.34-7.86 μM for 24 h treatment and 0.71-5.32 μM for 48 h treatment. Treatment with 0.1-0.3 μM AZD7762 for 24 or 48 h has been found to cause only about 8-12% cell death in GBM5 and GBM6 glioma cells; and treatment with 0.1 μM AZD7762 for 48 h reduces about 40% colony formation at 14 days after treatment in GBM6 cells (8). Another report has shown that treatment with 0.1-1 μM AZD7762 for 24 h reduces the colony forming ability of D54MG, U251, SKMG3 and U87 GBM cells to 50% at 14 days after treatment (23). The LC_{50} levels of AZD7762 for glioma cells in our study are consistent with or slightly higher than those in the literature (8, 23). We further found that TMZ+AZD induce synergistic cytotoxicity in glioma cells, shown as lower LC_{50} levels as compared with TMZ or AZD7762 treatment only; and 2.6 to 8.7 folds of dose enhancement ratio after 24 or 48 h treatment in different glioma cells.

The mechanisms of the synergistic cytotoxic effects induced by the combination of TMZ and AZD7762 are unclear and may be multifactorial. As mentioned above, TMZ can phosphorylate Chk1 and Chk2, which are important for TMZ-induced G_2/M arrest (12-14). Chk inhibitors can abrogate TMZ-induced G2/M arrest, and trigger cell cycle progression and cell death (13, 14). In addition to the interaction in cell cycle, the influence of AZD7762 on the MGMT expression may also be important in the synergistic cytotoxic effects of TMZ+AZD. MGMT is an important factor involved in the repair of DNA damage induced by TMZ (1, 5, 7, 10, 11, 27). The development of drug resistance after TMZ treatment is closely related to the expression and the methylation status of the *MGMT* gene, and the equilibrium between the rate of O^6-methylguanine formation and the rate of repair by MGMT (1, 5, 7, 10, 11, 27). Therefore, the constitutive MGMT expression in the glioma cells and the effect of AZD7762 on the MGMT expression might play a role in the response of cells to TMZ+AZD treatment. According to the literature and our study, T98G cells have high constitutive MGMT expression (28, 29), which is consistent with the relatively high TMZ LC_{50}; whereas U138MG cells have low and U87MG cells have no MGMT expression (28, 29), which are compatible with their relatively low TMZ LC_{50}s. Furthermore, AZD7762 was noted to suppress the MGMT expression in T98G and U138MG cells, and not modify the negative MGMT expression in U87MG cells. The suppression of MGMT expression by AZD7762 may reduce the DNA repair after TMZ treatment, and thus contribute to the synergistic cytotoxicity effects of TMZ+AZD. STAT3, an oncogenic protein, is important for glioma cell survival, growth, and invasion; and increased STAT3 expression predicts a poor clinical outcome of GBM patients (30-32). A previous study has also noted that inhibition of STAT3 can reduce TMZ resistance in GBM by suppressing MGMT expression (30). We also found that AZD7762 suppresses p-STAT3 expression, in addition to the MGMT expression. The suppression of the p-STAT3 and MGMT expression by AZD7762 may thus contribute to the synergistic cytotoxic effect of TMZ+AZD in the glioma cells.

In addition to MGMT, apoptosis may also play a role in the synergistic cytotoxic effects induced by TMZ+AZD, as both TMZ and AZD7762 have been found to induce cellular apoptosis in glioma or medulloblastoma cells (1, 22, 33). Many factors including p53 and Bcl-2 family members are related to cellular apoptosis (14, 34, 35). We found that TMZ+AZD significantly increased p-p53 expression in the investigated glioma cells and enhanced BAX expression in T98G and U138MG cells. p53 is a tumor suppressor protein (35), and BAX, belonging to the Bcl-2 family, is a pro-apoptotic factor (36). The increased expression of p-p53 and BAX may enhance cellular apoptosis and thus, contribute to the synergistic cytotoxic effects of TMZ+AZD in glioma cells. Further, the MAPK pathway including p38 MAPK and ERK1/2 is also related to the survival and apoptosis of cancer cells (14, 34). p38 MAPK is a pro-apoptotic MAPK and activation of p38 MAPK is correlated with tumor cell death treated with chemotherapeutic agents (8). In addition, p38 MAPK plays an important role in the G_2/M arrest in glioma cells treated with TMZ (13). In contrast, activated ERK1/2 may protect tumor cells from toxic actions of chemotherapeutic drugs and ionizing radiation (37), and high p-ERK 1/2 expression is associated with poor survival in patients with GBM (38). In this report, we found that TMZ+AZD significantly increased p-p38 MAPK expression and suppressed p-ERK 1/2 expression in glioma cells. These data indicate that the combination of TMZ and AZD7762 may modulate the MAPK pathway to enhance the pro-apoptotic p38 MAPK and suppress the oncogenic ERK activity, which

then increase the cytotoxic effects of TMZ+AZD in glioma cells. Such features are compatible with the enhanced tumor growth inhibition observed by combining selumetinib (MAPK kinase 1/2 inhibitor, MEK1/2 inhibitor) with TMZ, as compared with monotherapies (34). However, a previous report mentioned contradictory results, that treatment with 25 or 50 nM AZD7762 for 24 h increases p-ERK expression in GBM5, GBM6, GBM14 and DAOY cells (8). Such difference might be due to differences in the concentrations and treatment duration of AZD7762, and the different cell lines.

Glioma cells often have loss of PTEN and/or activation of the phosphatidylinositol-3-kinase (PI3K)/AKT/mTOR pathway, and these features correlate with increased tumorigenicity, invasiveness and stemness of glioma cells (6, 8, 39, 40). In contrast, suppression of the PI3K/AKT/mTOR pathway can induce apoptosis in malignant cells including glioma cells (36, 40). TMZ has been found to activate AKT activity, which may protect glioma cells from TMZ-induced cytotoxicity (6, 8, 40). In addition, AZD7762 does not affect p-AKT expression in GBM6 cells and DAOY glioma cells (8). However, in this report, we found that TMZ increases PTEN expression, while fails to affect p-AKT expression in glioma cells. In contrast, AZD7762 increases PTEN and suppresses p-AKT expression in two of the three investigated glioma cells. Furthermore, TMZ+AZD appear to increase PTEN and decrease p-AKT expression, which may thus contribute to the synergistic cytotoxic effects of the combination therapy in the glioma cells.

In summary, this study revealed that both TMZ and AZD7762 exerted cytotoxicity effects on glioma cells, and combined TMZ and AZD7762 induced synergistic cytotoxic effects. The synergistic cytotoxic effects of TMZ+AZD in glioma cells may be related to the AZD7762-induced suppression of MGMT expression, and the modulation of the p53, BAX, PTEN/AKT, ERK 1/2, p38 MAPK, and STAT3 pathways. As Chk1 inhibitors have low neurotoxicity and a broad therapeutic window, and non-toxic concentrations of AZD7762 have been found to sensitize medulloblastoma cells to cisplatin (22), therefore, AZD7762 may be used to boost the effect of temozolomide in the treatment of gliomas (22). Certainly, more studies are necessary before any clinical application.

Conflicts of Interest

The Authors have no conflicts of interest to report regarding this study.

Authors' Contributions

All Authors assisted in the experiments and writing of the manuscript. Yun Chen reviewed the literature, designed the experiments and prepared the first draft of the manuscript. Bor-Jiun Tseng reviewed the literature and performed the experiments. Ya-Hui Tsai performed the experiments and statistical analysis. Sheng-Hong Tseng designed the experiments and the basic structures of this paper, and wrote/edited the paper.

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