ORIGINAL ARTICLE

Enhancement of temozolomide-induced apoptosis by valproic acid in human glioma cell lines through redox regulation

Ching-Hsein Chen · Yu-Jia Chang · Maurice S. B. Ku · King-Thom Chung · Jen-Tsung Yang

Abstract Temozolomide (TMZ) is an oral alkylating agent that has been widely used in the treatment of refractory glioma, although inherent and acquired resistance to this drug is common. The clinical use of valproic acid (VPA) as an anticonvulsant and mood-stabilizing drug has been reported primarily for the treatment of epilepsy and bipolar disorder and less commonly for major depression. VPA is also used in the treatment of glioma-associated seizures with or without intracranial operation. In this study, we evaluated the potential synergistic effect of TMZ and VPA in human glioma cell lines. Compared with the use of TMZ or VPA alone, concurrent treatment with both drugs synergistically induced apoptosis in U87MG cells as evidenced by p53 and Bax expression, mitochondrial transmembrane potential loss, reactive oxygen species production, and glutathione depletion. This synergistic effect correlated with a decrease in nuclear translocation of the nuclear factor-erythroid 2 p45-related factor and corresponded with reduced heme oxygenase-1 and γ-glutamylcysteine synthetase expression. Pretreatment with *N*-acetylcysteine partially recovered the apoptotic effect of the TMZ/VPA combination treatment. The same degree of synergism is also seen in p53-mutant Hs683 cells, which indicates that p53 may not play a major role in the increased proapoptotic effect of the TMZ/VPA combination. In conclusion, VPA enhanced the apoptotic effect of TMZ, possibly through a redox regulation mechanism. The TMZ/VPA combination may be effective for treating glioma cancer and may be a powerful agent against malignant glioma. This drug combination should be further explored in the clinical setting.

Keywords Temozolomide · Valproic acid · Glioma · Glutathione depletion · Heme oxygenase-1 · Bax

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Introduction

Temozolomide (TMZ) is an oral alkylating agent that has been widely used in the treatment of high-grade glioma, a type of cancerous brain tumor. TMZ efficacy in glioma treatment may involve a first event characterized by changes in heterochromatin organization and its silencing,
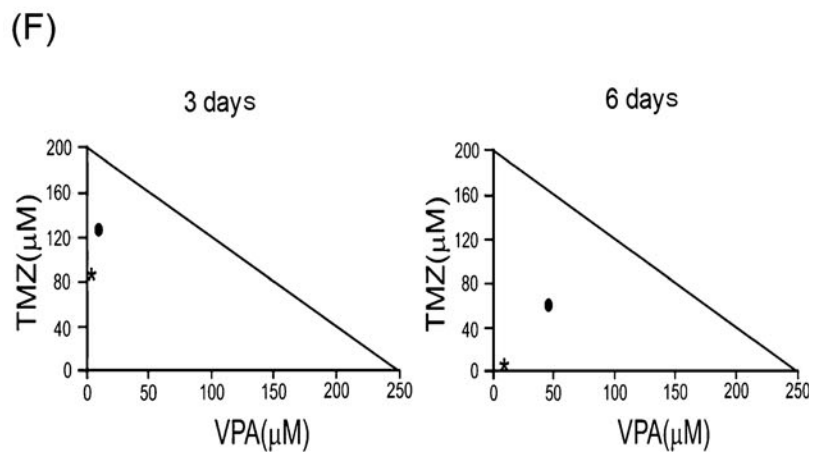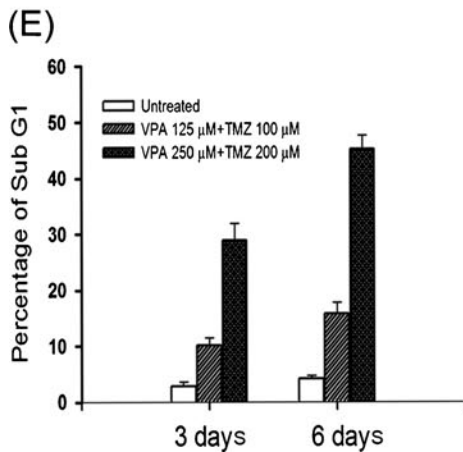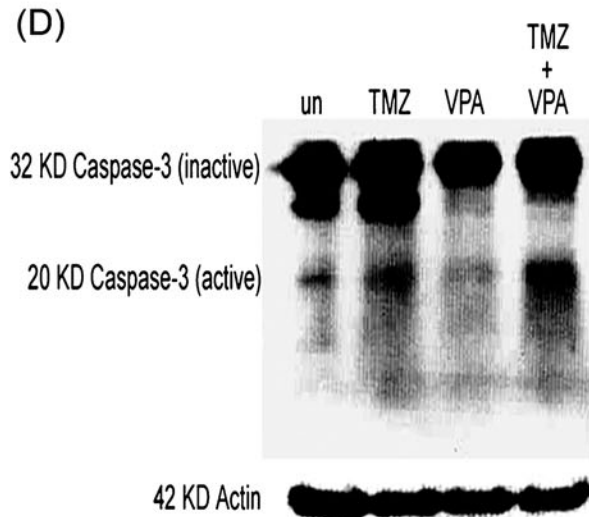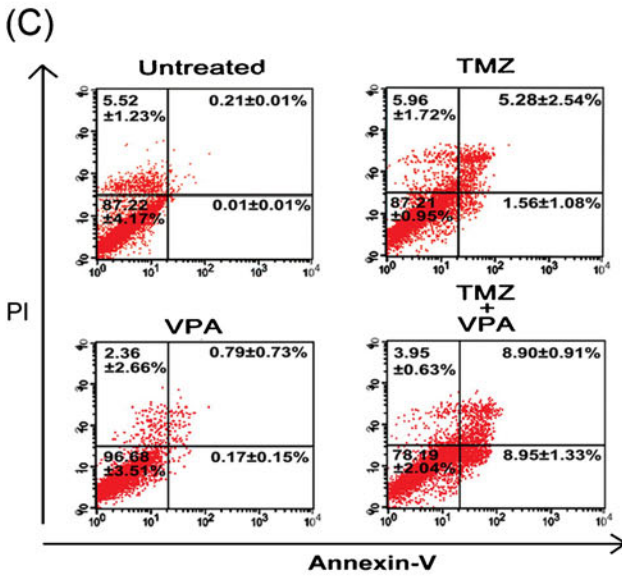
which is then followed by apoptosis and senescence [1]. TMZ-induced apoptosis in human malignant melanoma cells is related to p53 response. The p53 protein is a key regulator of cell responses to DNA damage and has been shown to sensitize glioma cells to TMZ by upregulating the extrinsic apoptotic pathway of cells [2].

Several studies have shown that combining TMZ with other agents enhances its effectiveness on glioma cells. Drugs such as betulinic acid, lonidamine, and CD437 target the mitochondrial pore and function as cytotoxic and cytostatic agents in TMZ-resistant glioma cells [3]. Combining PI-103, an isoform-selective class I phosphatidylinositol-3 kinase and mammalian target of rapamycin inhibitor, with TMZ significantly improved the response in U87MG human glioma xenografts [4]. Dihydroartemisinin potentiates the cytotoxic effect of TMZ in rat C6 glioma cells by generating intracellular reactive oxygen species (ROS) [5]. In a previous study, combination therapy involving TMZ and tamoxifen or hypericin potently inhibited tumor growth by inducing apoptosis and provided an effective means of treating malignant glioma [6].

Valproic acid (VPA), which is well established as a first line and widely used antiepileptic agent, is well tolerated in most patients and has an impressive safety profile [7]. The gene expression induced by VPA is related to the activation of redox-sensitive transcription factors, such as nuclear factor-erythroid 2 p45-related factor (Nrf2), for interaction with the antioxidant response element that is responsible for producing ROS [8]. It has been demonstrated that VPA upregulates ROS to enhance cisplatin-mediated cytotoxicity in ovarian carcinoma cells; VPA not only exhibited synergistic cytotoxicity with cisplatin in all of the ovarian carcinoma cells tested but also resensitized those cells that had developed resistance to cisplatin [9].

Despite the effectiveness of TMZ in treating brain tumors, many patients fail to benefit from this drug because of
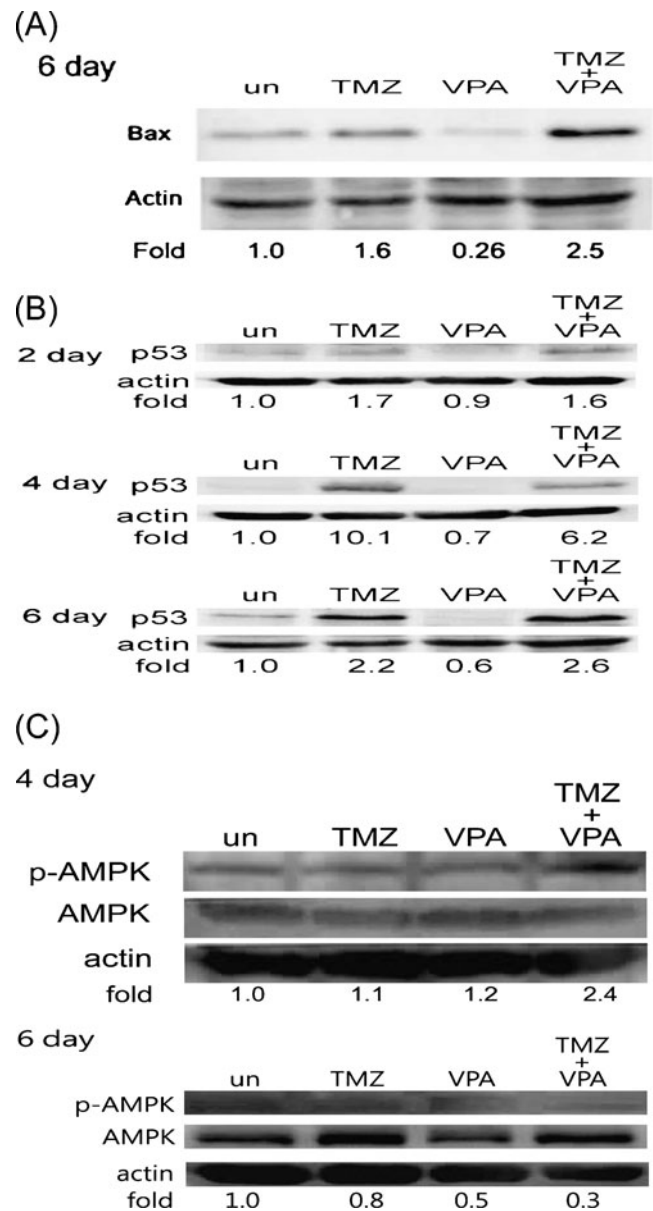
Fig. 2 Effect of the temozolomide/valproic acid (TMZ/VPA) combination on **a** Bax expression, **b** p53 expression, and **c** AMPK phosphorylation (pAMPK). U87MG cells were untreated (*un*) or treated with TMZ, VPA, or their combination. After treatment, the cells were washed with PBS and extracted with protein extraction buffer. Fifty micrograms of each protein was loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel. The expression of p53, Bax and, pAMPK at the indicated time points was evaluated by western blotting as outlined in the "Materials and methods" section. These experiments were performed at least three times, and a representative experiment is presented

acquired resistance. High-grade gliomas are one of the most difficult cancers to treat, and despite surgery, radiotherapy, and TMZ-based chemotherapy, the prognosis of glioma patients remains poor. Resistance to TMZ is the major barrier to effective therapy [3]. Therefore, it is necessary

to seek alternative strategies to enhance the anticancer effect of TMZ in refractory brain tumors. In this study, we evaluated the possible synergistic anticancer efficacy of TMZ/VPA combination treatment. Specifically, we examined the molecular mechanisms underlying synergistic

apoptosis, induction of oxidative stress, glutathione (GSH) depletion, inhibition of Nrf2-related antioxidant drug-metabolizing enzymes (phase II—conjugating enzymes), and disturbance of the mitochondrial transmembrane potential in the U87MG cell line.
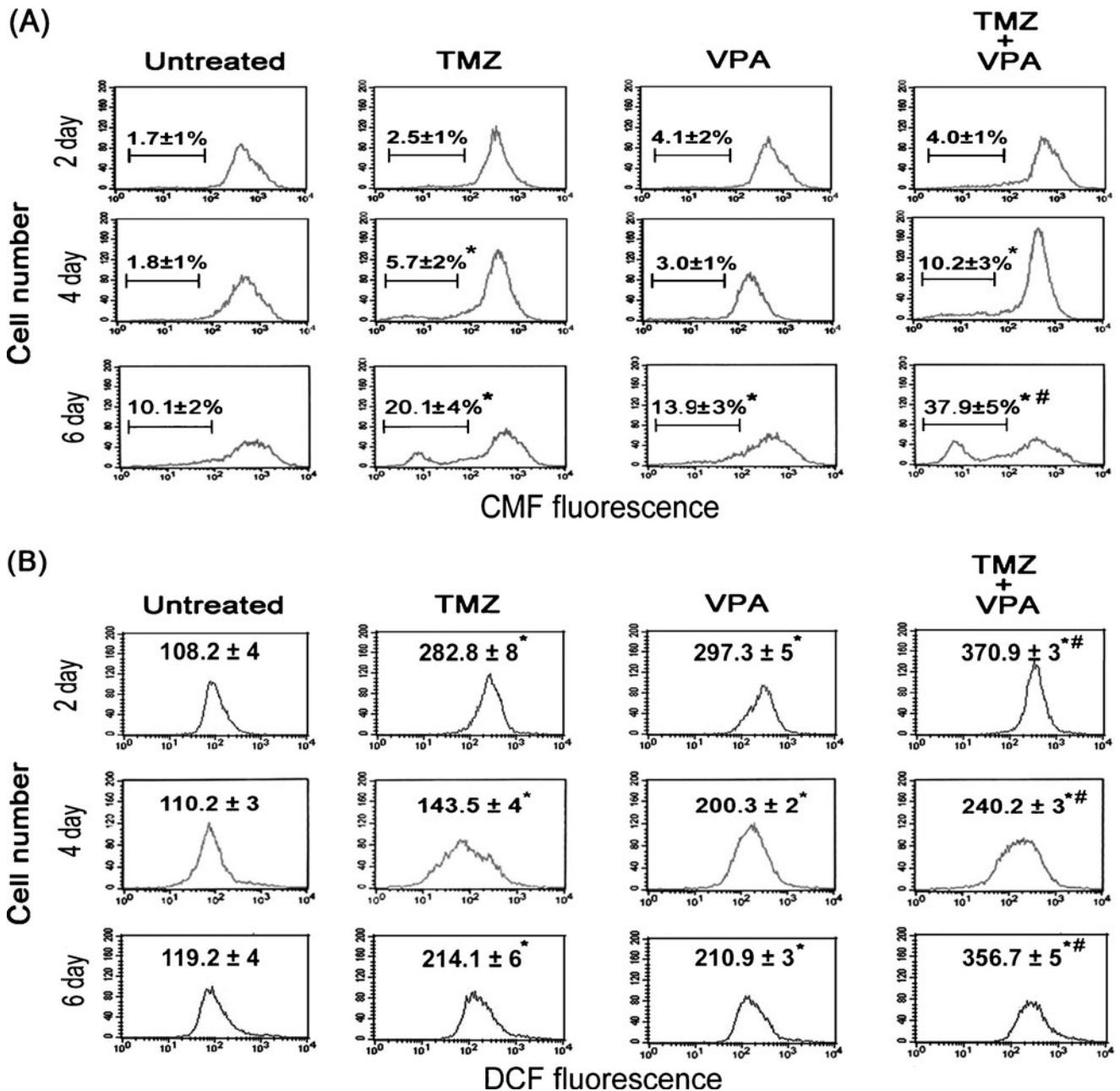
Fig. 3 Effect of the temozolomide/valproic acid (TMZ/VPA) combination on **a** glutathione (GSH) depletion, **b** reactive oxygen species (ROS) production, and **c** mitochondrial transmembrane potential disruption. U87MG cells were treated with TMZ, VPA, or their combination for 2, 4, and 6 days. After treatment, the cells were stained with chloromethylflourescein diacetate (CMF-DA), 2′,7′-dichlorflouorescein-diacetate (DCFH-DA), and rhodamine 123 for GSH, ROS, and mitochondrial transmembrane potential analyses, respectively, and were then evaluated by flow cytometry as outlined in the "Materials and methods" section. **a** Data in each panel represent the percentages of GSH-depleted cells. **b**, **c** Data in each panel represent the fluorescence intensity within the cells. The values are represented as mean ± standard deviation ($n=5–8$) of individual experiments. Significant differences for the untreated group and TMZ group are $*P<0.05$ and $\#P<0.05$, respectively

(C)

Rhodamine 123

Fig. 3 (continued)

Materials and methods

Cell lines and reagents

The human glioma cell lines, U87MG, Hs683, and DBTRG-05MG, were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan, Republic of China). TMZ was supplied by Schering-Plough Corporation (Kenilworth, NJ). The Annexin V–FLUOS staining kit was purchased from Roche Appli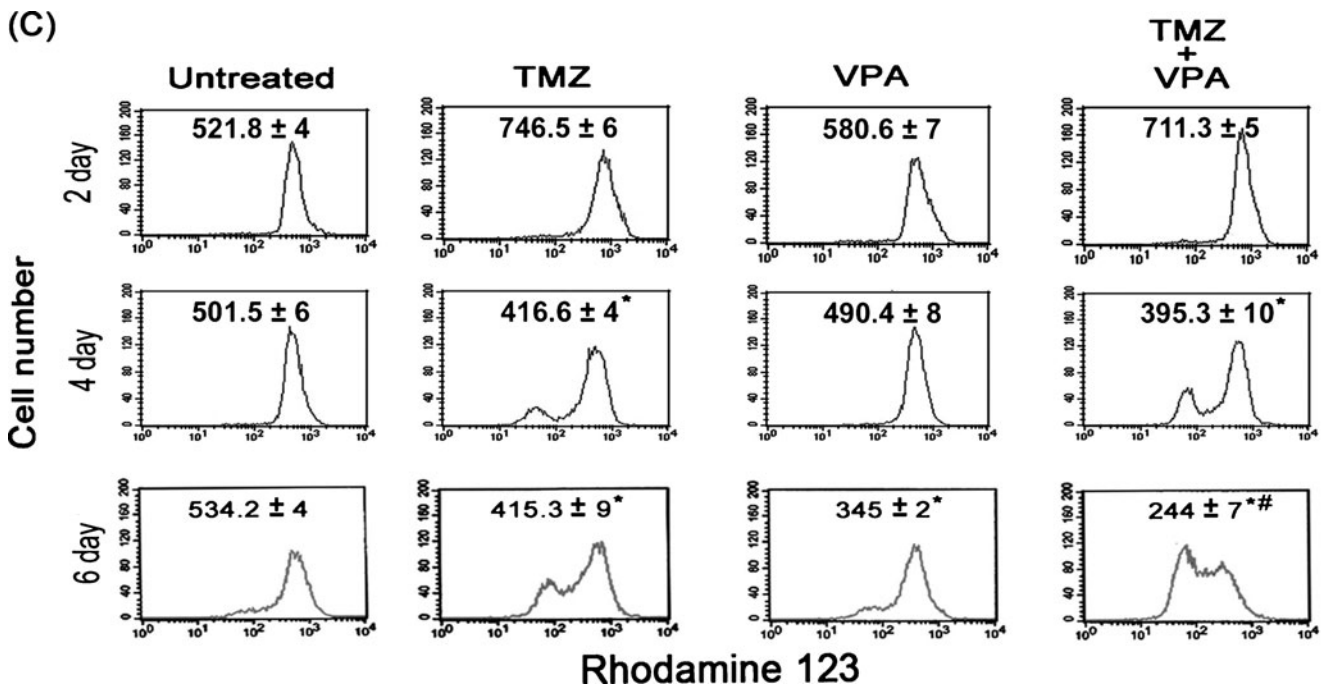ed Science Company (Penzberg, Germany). Chloromethylflourescein diacetate (CMF-DA) was acquired from Invitrogen Corporation (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (South Logan, UT). Anti-Bax, anti-p53, and other antibodies were purchased from Santa Cruz Biotechnology (Lab Vision, Santa Cruz, CA). Arrest-In transfection reagent was acquired from Thermo Fisher Scientific Inc. (Pittsburgh, PA). VPA, propidium iodide (PI), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture and treatment

U87MG and Hs683 cells were cultured in DMEM containing 10% FBS. DBTRG-05MG cells were cultured in RPMI 1640 medium with 10 mg/L adenine, 1 mg/L adenosine triphosphate, 100 mg/l L-cytine, 5,950 mg/l HEPES, 15 mg/l hypoxanthine, 50 mg/l L-isoleucine, 50 mg/l L-proline, 100 mg/l sodium pyruvate, and 1 mg/l thymidine, 90%; FBS, 10%. Glioma cells (1×10^6) were cultured in 60-mm tissue-culture dishes for 24 h. The culture medium was changed, and the cells were exposed to a medium with or without various concentrations of drugs for the indicated times. TMZ or VPA stock solution was dissolved in DMSO, and the final concentration was prepared in cultured medium. The concentrations of TMZ and VPA used in this study were 200 and 250 μM, respectively, which are equivalent to the clinical concentrations.

Cell cycle and DNA damage analysis of glioma cells

Cell cycle and DNA damage were assessed by PI staining and flow cytometry. After treatment, adherent and floating cells were fixed in a phosphate buffered saline (PBS)–methanol solution, and then maintained at 4°C for at least 18 h. After one wash with PBS, cell pellets were stained with a fluorescent probe solution containing PBS, 40 μg/mL PI, and 40 μg/mL DNase-free RNase A for 30 min at room temperature in the dark. DNA fluorescence of PI-stained cells was analyzed by a Becton-Dickinson FACScan flow cytometer (Franklin Lakes, NJ). The DNA histograms were evaluated further using Modfit software on a PC workstation to calculate the percentage of cells in various phases of the cell cycle.
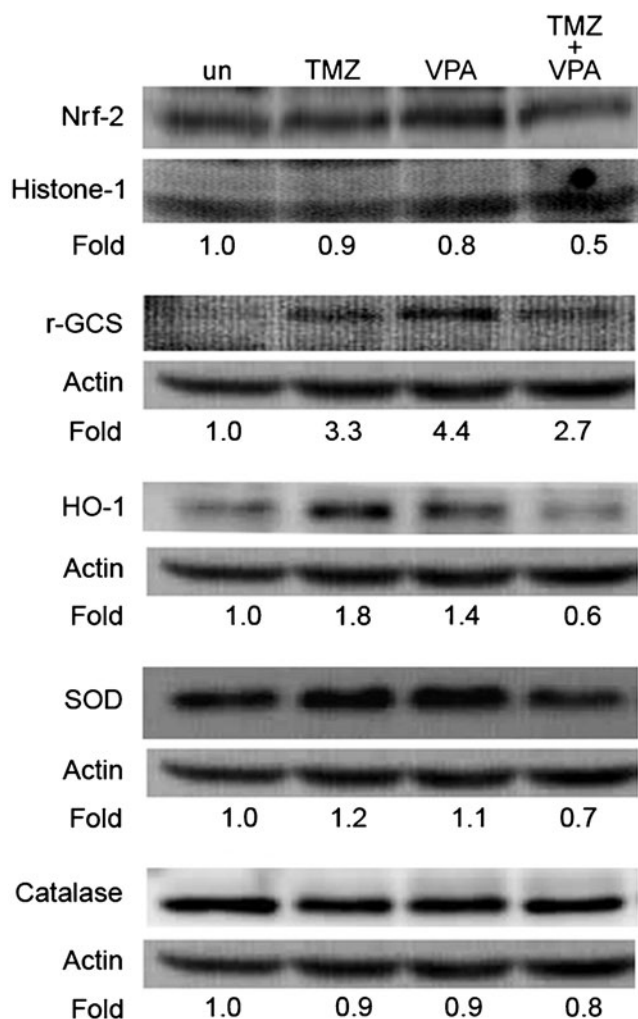
Fig. 4 Effect of the temozolomide/valproic acid (TMZ/VPA) combination on both nuclear factor-erythroid 2 p45-related factor (Nrf2) nuclear translocation and phase II enzyme expression. U87MG cells were untreated (*un*) or treated with TMZ, VPA, or their combination for 6 days. After treatment, 50 μg of nuclear proteins for Nrf2 or 50 μg of total proteins for phase II enzymes was loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel. Nrf2 nuclear translocation and phase II enzyme and actin expression were evaluated by western blotting, as outlined in the "Materials and methods" section. These experiments were performed at least three times, and a representative experiment is presented

Flow cytometric analysis of annexin V binding

Apoptosis was measured with annexin V binding and flow cytometry. After treatment, U87MG cells were washed once with incubation buffer, collected by centrifugation, and suspended in an annexin V–FITC–PI reactive solution. Following 15 min of incubation in annexin V–FITC–PI solution at room temperature, the percentage of apoptotic cells was measured by flow cytometry.

Measurement of GSH depletion

After treatment, U87MG cells were incubated with 25 μM CMF-DA for 20 min at 37°C in a CO_2 incubator. Intracellular GSH, as indicated by chloromethylflourescein fluorescence, was measured with a Becton-Dickinson FACScan flow cytometer.

Measurement of intracellular ROS by flow cytometry

Treated U87MG cells were incubated with 10 μM 2′,7′-dichlorfluorescein-diacetate for 30 min in the dark, washed once with PBS, detached by trypsinization, collected by centrifugation, and resuspended in PBS. The intracellular ROS, as indicated by dichlorofluorescein fluorescence, was measured with a Becton-Dickinson FACScan flow cytometer.

Measurement of mitochondrial transmembrane potential by flow cytometry

After treatment, U87MG cells were trypsinized and then incubated with 5 μM rhodamine 123 for 30 min at 37°C in a CO_2 incubator. Following incubation, we measured mitochondrial membrane potential, as indicated by the changed fluorescence level of rhodamine 123, using a Becton-Dickinson FACScan flow cytometer.

Overexpression of Nrf2 in U87MG cells

Full-length Nrf2 cDNA was generated by polymerase chain reaction and subcloned into the pCMV6-XL5 expression vector (OriGene Technologies Inc., Rockville, MD). The full-length sequence was determined by automatic sequencing (ABI). The pCMV6-XL5-Nrf2 plasmid was transfected into cells by using the Arrest-In transfection reagent. Cells were harvested, and their nuclear proteins were extracted to determine the expression level of Nrf2 at specific time intervals by means of western blotting.

Preparation of nuclear protein extracts from U87MG cells for Nrf2 analysis

After treatment, U87MG cells were washed with cold PBS and suspended in 0.1 ml of hypotonic lysis buffer containing protease inhibitors for 30 min. Cells were then lysed with 3.2 μl of 10% Nonidet P-40. The homogenate was centrifuged, and the supernatant containing the cytoplasmic extracts was stored frozen at −80°C. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the

extract was centrifuged and the supernatants containing the nuclear extracts were secured. Nuclear Nrf2 expression was evaluated by western blotting.

Measurement of protein expression by western blotting

After treatment, U87MG cells were washed with PBS, resuspended in a protein extraction buffer for 10 min, and then centrifuged at $12,000 \times g$ for 10 min at 4°C to obtain total extracted proteins (supernatant). Protein concentrations were measured using a Bio-Rad protein assay reagent. Protein expression was evaluated by western blotting.

Apoptotic chromatin condensation examination

The treated U87MG cells were washed with PBS and resuspended in the same buffer. One hundred microliters of each cell suspension (1×10^6/ml) was incubated with 1 μl of Hoechst 33258 (1 mg/ml in ddH$_2$O) for 10 min. Apoptotic chromatin condensation was evaluated by fluorescence microscopy.

Statistical analysis

Data are presented as the mean ± standard deviation from at least three independent experiments and analyzed using Student's t tests. A P value of <0.05 was considered statistically significant [10]. Isobologram analysis of the TMZ/VPA combination in glioma cells was calculated using the CalcuSyn software and combination index values were plotted [11].

Results

Combination of VPA and TMZ increased apoptosis-related DNA damage in U87MG cells

We first used PI staining and flow cytometry to evaluate whether VPA could enhance the apoptosis-related DNA damage of TMZ and to assess the percentage of cells in the subG$_1$ phase upon treatment with VPA, TMZ, or their combination. We found no significant difference in the percentage of cells in the subG$_1$ phase (<2.0%) between the VPA-treated and untreated groups after 6 days (Fig. 1a). However, we noticed a large increase in the percentage of subG$_1$ cells after the combination treatment (46.8%) for 6 days, whereas the percentage of cells in the subG$_1$ phase after treatment with TMZ alone was much less (13.3%). We further used Hoechst 33258 staining to examine apoptotic chromatin condensation. TMZ or VPA alone had little effect

on chromatin condensation, whereas concurrent administration of both agents resulted in markedly increased chromatin condensation (Fig. 1b). In addition, the percentage of annexin V positive cells, an indicator of apoptosis, was greater in the TMZ/VPA combination group than in the groups treated with either TMZ or VPA (Fig. 1c). Caspase-3 activation in cells treated with the TMZ/VPA combination was greater than that for either TMZ or VPA alone (Fig. 1d). We also treated U87MG cells with lower concentrations of TMZ and VPA (100 and 125 μM, respectively) to evaluate the proapoptotic synergism at 3 and 6 days of treatment. The formal assessment of synergism was conducted using isobologram analysis. As shown in Fig. 1e and f, treatment of U87MG cells with the TMZ/VPA combination exhibited both time- and dose-dependent apoptotic effects.

Effects of TMZ/VPA combination on the expression of Bax and p53 and the phosphorylation of AMPK

Bax is a proapoptotic protein that is overexpressed during the apoptotic process [12]. We monitored Bax expression in U87MG cells that were treated either with TMZ, VPA, or their combination. Our results showed that VPA alone failed to induce Bax expression in cells, whereas TMZ alone slightly increased the level of Bax (1.6-fold; Fig. 2a). Interestingly, Bax was greatly overexpressed (2.5-fold) in cells treated with the TMZ/VPA combination.

Figure 2b clearly shows that the extent of p53 induction is essentially the same (if not greater, at certain time points) in the TMZ-alone group than in the combination group. We further evaluated the activation of AMPK (an upstream regulator of p53), which is indicated by its phosphorylation, in U87MG cells. AMPK phosphorylation in cells treated with the TMZ/VPA combination was obviously increased compared with phosphorylation in cells treated with TMZ or VPA alone after 4 days, but declined after 6 days of treatment (Fig. 2c). We also demonstrated that MAPK and Akt signaling were obviously inhibited in samples treated with the TMZ/VPA combination after 6 days but not after 2 or 4 days (data not shown), which suggests that signaling through the MAPK and Akt cascades does not seem to play a major role in combination treatment, rather they are modulated only at late time points, likely as a consequence of the already advanced cell death process.

TMZ/VPA combination treatment enhanced intracellular GSH depletion, ROS production, and mitochondrial transmembrane potential disruption

The major function of GSH is to prevent ROS-induced cellular damage and to detoxify exotoxins [13]. It is understood that either intracellular GSH depletion or
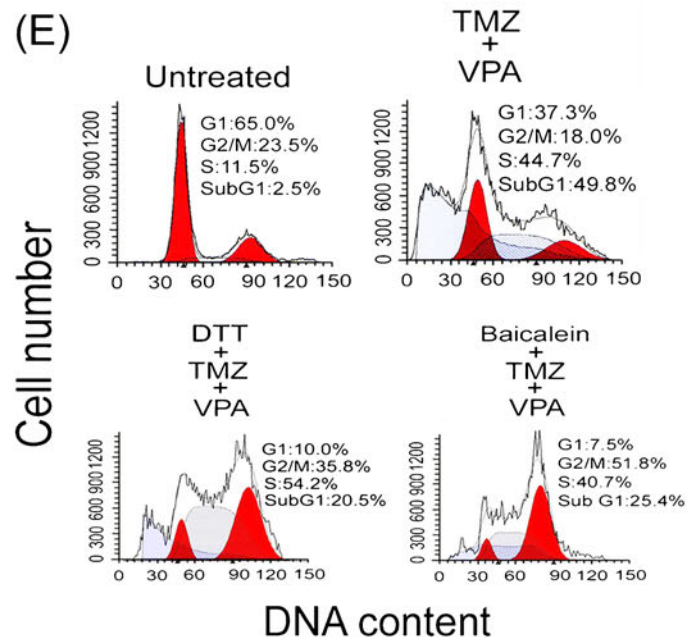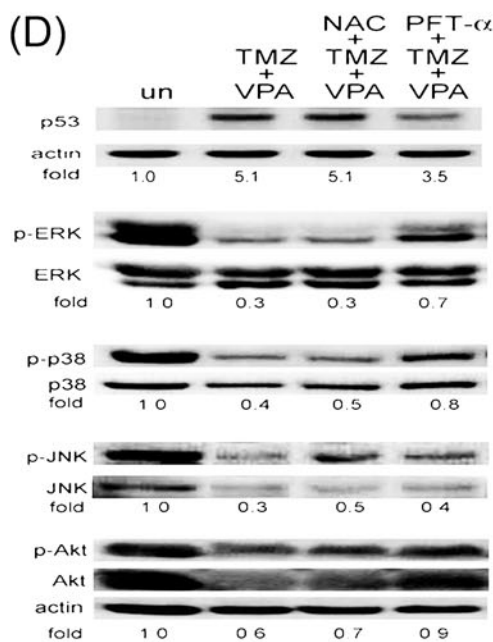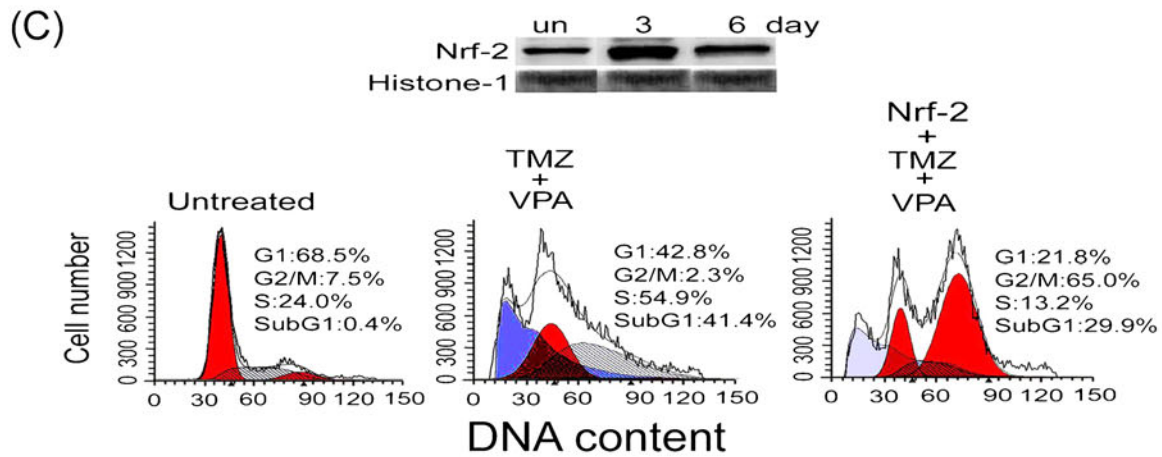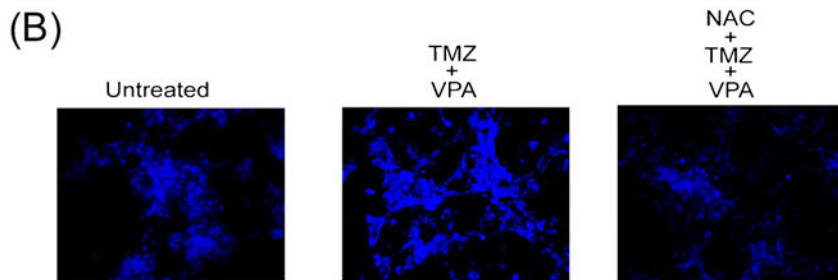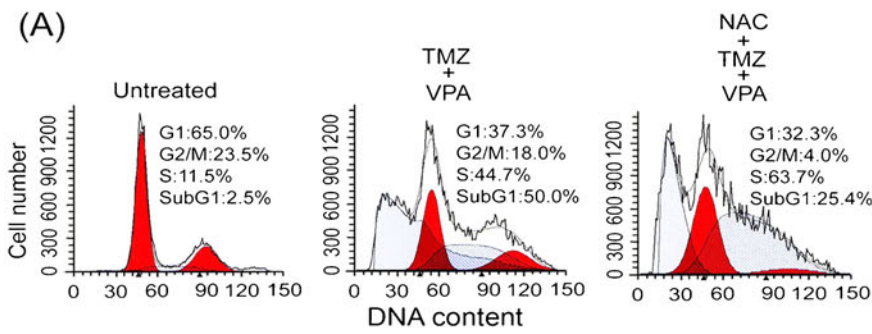
◀ **Fig. 5** Evaluation of a critical event in the temozolomide/valproic acid (TMZ/VPA) combination-induced apoptosis. **a** U87MG cells were untreated (*un*) or pretreated with 10 mM *N*-acetylcysteine (NAC) for 2 h, followed by TMZ/VPA combination treatment for 6 days and were then included in a DNA damage assay as outlined in the "Materials and methods". **b** After treatment, the apoptotic chromatin condensation of U87MG cells was determined with Hoechst 33258 staining to identify apoptotic cells and was evaluated under a fluorescent microscope (original magnification ×200). Apoptotic chromatin condensation showed concentrated dense granular fluorescence. **c** U87MG cells were transfected with pCMV6-XL5-nuclear factor-erythroid 2 p45-related factor (Nrf2) plasmid, and the over-expression of nuclear Nrf2 was assessed by western blotting at 3 and 6 days. Cells were untreated (*un*) or treated with the TMZ/VPA combination for 6 days and then included in a DNA damage assay as outlined in the "Materials and methods". **d** U87MG cells were untreated (*un*) or pretreated with 10 μM PFT-α or 10 mM NAC for 2 h, followed by TMZ/VPA combination treatment for 6 days and were then assessed for ERK, p38, JNK, and Akt phosphorylation by western blotting as outlined in the "Materials and methods". **e** U87MG cells were untreated (*un*) or pretreated with 1 mM dithiothreitol (DTT) or 25 μM baicalein for 2 h, followed by TMZ/VPA combination treatment for 6 days and were then included in a DNA damage assay as outlined in the "Materials and methods". These experiments were performed at least three times, and a representative experiment is presented

intracellular GSH excretion outside the cell leads to apoptosis [14]. To fully evaluate the possible involvement of ROS and GSH balance and redox status of the cells in the proapoptotic effects of combined VPA and TMZ treatment, we detected the GSH depletion, ROS accumulation, and mitochondrial potential loss after 2, 4, and 6 days. As shown in Fig. 3, GSH depletion and ROS accumulation occurred in a time-dependent manner in the combined VPA and TMZ treatment group. VPA or TMZ alone had little effect on GSH depletion and ROS accumulation compared with their combination. The loss of mitochondrial potential is also enhanced by VPA and TMZ combination at 4 and 6 days. These results indicate that GSH depletion, ROS accumulation, and mitochondrial transmembrane potential loss are involved in the proapoptotic effects of combined VPA and TMZ treatment.

Role of Nrf2 and Nrf2-related proteins in mediating VPA-induced sensitization to TMZ

Nrf2 is a critical transcription factor that enhances the resistance of cancer cells to chemotherapeutic drugs [15]. To determine whether enhanced GSH depletion in cells treated with the TMZ/VPA combination was mediated specifically by Nrf2, we repeated the experiment shown in Fig. 3 to evaluate the nuclear translocation of Nrf2 and its related antioxidant drug-metabolizing enzymes in U87MG cells by western blotting. As shown in Fig. 4, there was no marked inhibition on nuclear translocation of Nrf2 in the treatments with TMZ or VPA alone compared with the untreated group. Although the

TMZ/VPA combination treatment caused a dramatic decrease in Nrf2 nuclear translocation (by 0.5-fold), the expression of heme oxygenase-1 (HO-1) and γ-glutamylcysteine synthetase (γ-GCS) obviously increased in samples treated with either TMZ or VPA alone; however, TMZ/VPA combination treatment resulted in marked loss in γ-GCS and HO-1 expression. TMZ or VPA alone treatment induced a slight increase in superoxide dismutase (SOD) expression (by 1.2- and 1.1-fold, respectively), whereas the combination drug treatment inhibited SOD expression to 0.7-fold of that of the basal level (untreated group). All drug-treated groups showed a slight decrease in catalase expression.

Critical apoptosis pathway in cells concurrently treated with TMZ and VPA was partially attributable to GSH depletion

To determine whether the apoptosis induced by concurrent treatment with TMZ and VPA was specifically attributable to a GSH-depletion–dependent pathway, we used *N*-acetylcysteine (NAC), an intracellular GSH synthetic agent, in this study. We found 50.0% apoptotic cells after cotreatment with TMZ and VPA for 6 days, whereas only 2.5% of untreated cells were apoptotic (Fig. 5a). NAC pretreatment moderately inhibited TMZ/VPA-induced apoptosis, as the percentage of apoptotic cells decreased to 25.4%. The untreated cells displayed nonapoptotic nuclei, whereas the cells treated with the combination exhibited an increase in apoptotic chromatin condensation (Fig. 5b). In NAC-pretreated cells, the amount of apoptotic chromatin condensation was partially decreased (Fig. 5b), suggesting that enhanced GSH depletion mediated the apoptotic events observed in the TMZ/VPA combination group.

We carried out additional studies to establish the cause–effect relationship between various markers and TMZ/VPA combination treatment. To do so, we first engineered U87MG cells that transiently overexpressed Nrf2. Our results showed increased Nrf2 expression in the cell nuclei at 3 and 6 days after pNrf2 cell transfection (Fig. 5c). Nrf2 overexpression partially abrogated the percentage of cells in subG$_1$ that were exposed to TMZ/VPA combination treatment (Fig. 5c), which confirmed that the reduction of Nrf2 nuclear translocation participated in the combination effect. We also found that pretreatment of U87MG cells with NAC or pifithrin-α (PFT-α) reversed the effect of the TMZ/VPA combination on ERK, Akt, JNK, and p38 phosphorylation in the cells (Fig. 5d). The reduced effects of the combination on MAPK and Akt signaling by cotreatment with PFT-α and NAC may simply be attributable to the decreased percentage of apoptosis in these cells. To enhance the GSH-depletion and ROS-dependent mechanisms on combination treatment with TMZ/VPA, we also used two antioxidants, dithiothreitol (DTT), and baicalein. DTT is an antioxidant used to stabilize enzymes and other
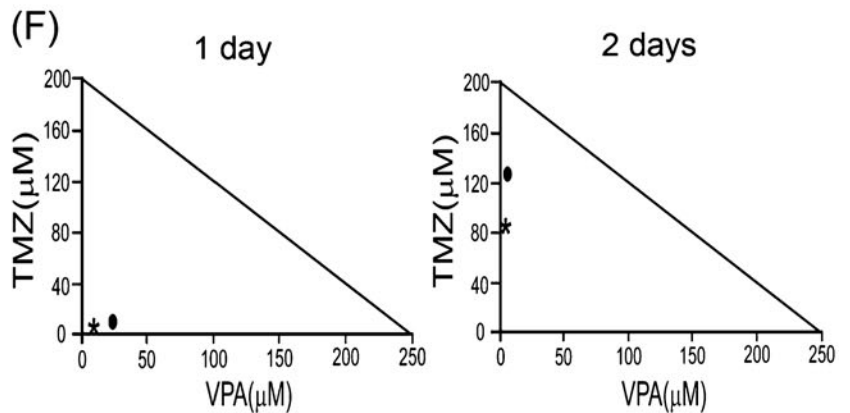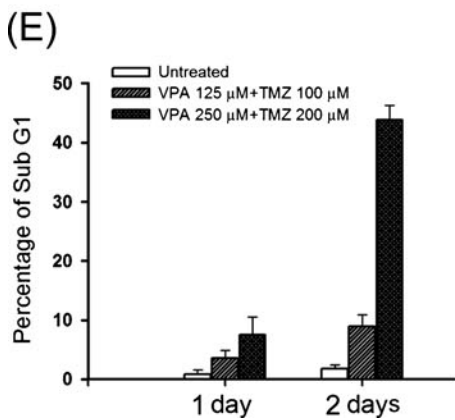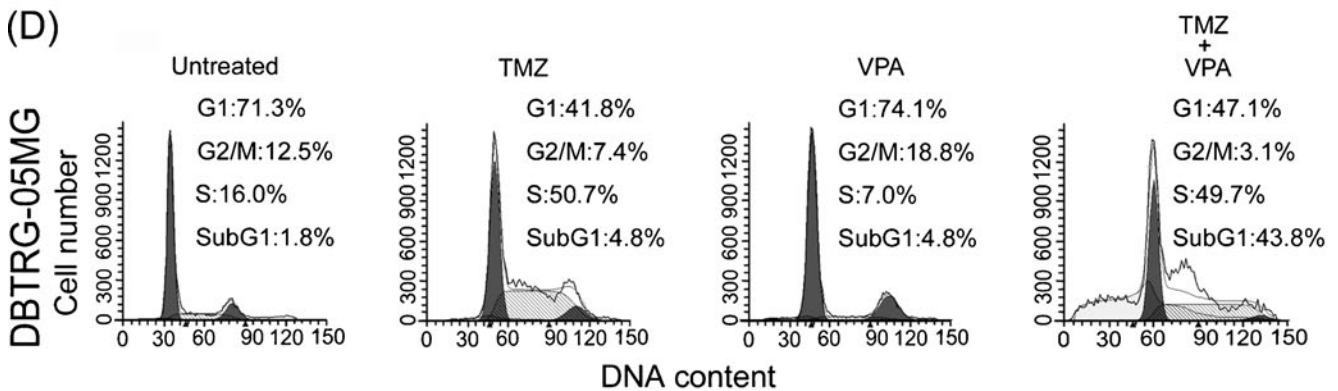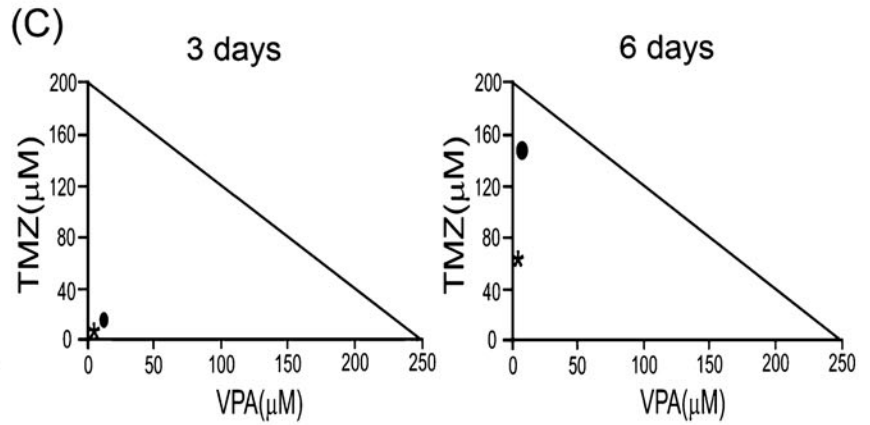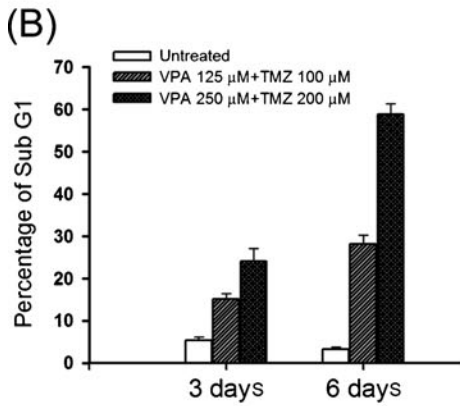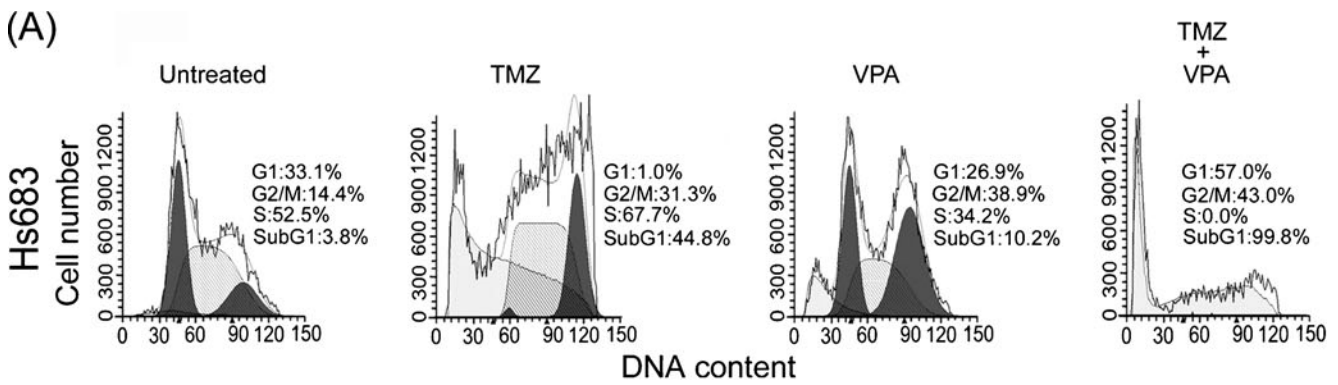
◀ **Fig. 6** Analysis of DNA damage in the Hs683 and DBTRG-05MG glioma cell lines. **a** Hs683 cells were treated with temozolomide (TMZ; 200 μM), valproic acid (VPA; 250 μM), or TMZ/VPA combination for 6 days. The percentages of subG$_1$ cells, as indicated by the DNA damage, were determined as outlined in the "Materials and methods". **b** Percentage of cells in the subG$_1$ phase after treatment with the TMZ/VPA combination for 3 and 6 days. Values are represented as mean ± standard deviation (n=5–8) of individual experiments. **c** Isobologram analysis of the TMZ/VPA combination in Hs683 cells. Combination index (CI) values were calculated using CalcuSyn software. *Points below the line* indicate synergy. **d** DBTRG-05MG cells were treated with TMZ (200 μM), VPA (250 μM), or TMZ/VPA combination for 2 days. The percentages of subG$_1$ cells, as indicated by DNA damage, were determined as outlined in "Materials and methods". **e** Percentage of cells in the subG$_1$ phase after treatment with the TMZ/VPA combination for 1 and 2 days. Values are represented as mean ± standard deviation (n=5–8) of individual experiments. **f** Isobologram analysis of the TMZ/VPA combination in DBTRG-05MG cells. CI values were calculated using CalcuSyn software. *Points below the line* indicate synergy. These experiments were performed at least three times, and a representative experiment is presented

Fig. 7 The proposed model of the temozolomide/valproic acid (TMZ/VPA) combination that enhanced apoptosis in glioma U87MG cells

proteins containing sulfhydryl groups, while baicalein is a flavonoid that has antioxidant and free radical scavenging effects [16, 17]. As expected, the percentage of subG$_1$ cells was partially decreased by pretreatment of cells with DTT or baicalein before TMZ/VPA combination treatment, suggesting that the GSH depletion and the enhanced ROS production are the major mechanisms of the combination treatment (Fig. 5e).

VPA and TMZ combination increased apoptosis-related DNA damage in other glioma cell lines

We also evaluated the effect of VPA/TMZ treatment on apoptosis in the p53 mutant Hs683 cell line. As shown in Fig. 6a, these cells were treated with VPA, TMZ, or TMZ/VPA combination for 6 days, which resulted in 10.2%, 44.8%, and 99.8% of cells in the subG$_1$ phase, respectively. These results indicate that the synergistic effect also existed in the p53 mutant glioma cell line and seem to suggest the clinical potential of this combination despite the occurrence of p53 mutations in gliomas. We also evaluated the combination effect of VPA and TMZ on apoptosis in another glioma cell line, DBTRG-05MG, by measuring the percentage of cells in the subG$_1$ phase by flow cytometry. As shown in Fig. 6d, VPA and TMZ cotreatment increased the percentage of cells in subG$_1$ (43.8%) compared to treatment with VPA (4.8%) or TMZ (4.8%) alone. We further treated Hs683 and DBTRG-05MG cells with lower concentrations of TMZ and VPA (100 and 125 μM, respectively) to evaluate proapoptotic synergism. As shown in Fig. 6b and e, treatment of Hs683 or DBTRG-05MG cells with the TMZ/VPA combination exhibited both time- and dose-dependent apoptotic effects. The synergism also appeared in treatment of Hs683 or DBTRG-05MG cells
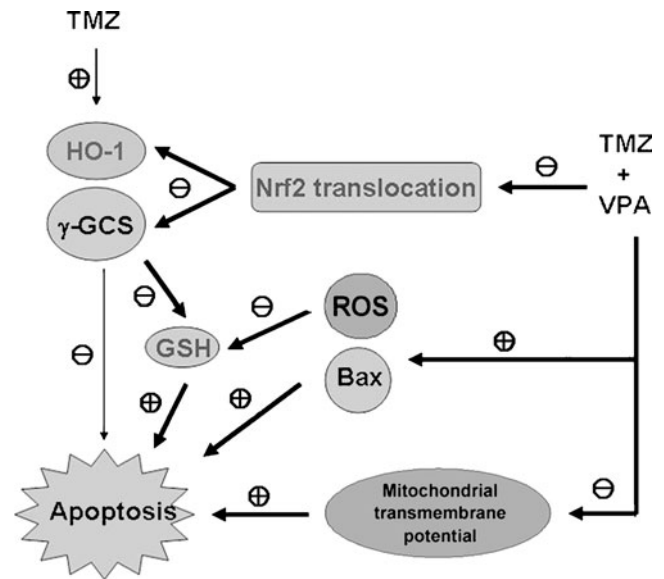
with TMZ/VPA combination (Fig. 6c and f). These results further substantiate the potential therapeutic relevance of this drug combination.

Discussion

In this study, we investigated the cellular and molecular effects of VPA on the apoptotic pathways of TMZ in the U87MG cell line to evaluate the therapeutic potential of combining TMZ and VPA. VPA is a potent inhibitor of the liver cytochrome P450 enzyme, which has important metabolic interactions with drugs used in glioma therapy. However, TMZ does not require hepatic metabolism involving the P450 system and shows poor drug interaction with VPA [18, 19].

AMPK phosphorylation is clearly induced only by combination treatment compared to p53, which is mainly modulated by TMZ alone. This finding is similar to a previous observation of the crucial role of AMPK phosphorylation in apoptosis induced by acadesine in B cell chronic lymphocytic leukemia cells [20]. Acadesine had no effect on p53 expression, indicating a p53-independent mechanism in apoptosis induction. Another anticancer compound, JKA97 (methoxy-1-styryl-9 H-pyrid-[3,4-b]-indole), also induced apoptosis via a Bax-dependent, p53-independent mechanism in human colon cancer [21]. In addition, the same degree of synergism is seen in p53-mutant glioma cells (Fig. 6a), which indicates that p53, may not play a major role in the increased proapoptotic effect of the TMZ/VPA combination. Accumulation of p53 may be a by-product of the ongoing DNA damage in cells treated

with the TMZ/VPA combination. In line with these observations, our data indicate that Bax expression also obviously increased by combination treatment compared with treatment with TMZ or VPA alone (Fig. 2a). These results suggest that the effects of the TMZ/VPA combination on pAMPK and Bax may be further developed independent of p53 regulation.

There is substantial evidence that phase II enzyme expression regulation is most likely mediated by Nrf2 and antioxidant response elements [22]. Here, we demonstrated for the first time that treating cells with the TMZ/VPA combination resulted in obvious abrogation of Nrf2 translocation to cell nuclei (Fig. 4). Some studies have focused on the fact that Nrf2 and its downstream genes are overexpressed in numerous cancer cell lines and human cancer tissues and concluded that this overexpression provides cancer cells growth and survival benefits [23]. Because ROS generation was more pronounced after TMZ/VPA combination treatment than after treatment with VPA alone, the apoptosis observed from the drug combination could be inhibited by nuclear Nrf2 overexpression and pretreatment with three antioxidants (NAC, dithiothreitol, or baicalein; Fig. 5c and e). The results suggest that ROS generation, inhibition of Nrf2 nuclear translocation, and GSH depletion are the three main mechanisms that induce apoptosis after cell treatment with the TMZ/VPA combination.

It has been shown previously that HO-1 induced by stress stimuli represents a prime cellular defense mechanism, but it may be associated with enhanced cell proliferation and chemoresistance in some cancer cells [24]. Numerous tumors express high levels of HO-1 that confer resistance of tumors to various anticancer drugs [25]. For example, tumor cells with altered HO-1 expression exhibit resistance to doxorubicin [26]. Constitutive overexpression of Nrf2-dependent HO-1 in A549 cells contributes to resistance to apoptosis induced by epigallocatechin 3-gallate [23]. Furthermore, an increase in the level of cellular HO-1 markedly reduces the sensitivity of papillary thyroid carcinoma cells to tumor necrosis factor-α and cycloheximide [27]. These reports are in agreement with our finding that treatment of cells with TMZ alone increased HO-1 expression (Fig. 4). HO-1 expression seems to provide a resistance mechanism for U87MG cells against TMZ treatment. One of the most noteworthy findings in this study is that VPA enhanced the downregulation of HO-1 in TMZ-treated glioma cells (Fig. 4), which suggests that HO-1 may provide a potential therapeutic target for TMZ-resistant glioma cells.

Multidrug resistance in cancer cells is often associated with an elevated concentration of GSH and expression of γ-GCS, a rate-limiting enzyme for GSH [28]. γ-GCS is thought to play a significant role in intracellular detoxification, especially of anticancer drugs. Increased levels of intracellular GSH are commonly found in drug-resistant

human cancer cells [29]. Our results demonstrate that VPA increased ROS but failed to induce obvious apoptosis in U87MG cells (Figs. 1 and 3), indicating that VPA is an intracellular ROS inducer that may trigger γ-GCS expression to maintain GSH level against ROS injury. To our knowledge, this is the first finding of TMZ-induced γ-GCS overexpression in human glioma cells, suggesting that there is a resistant system against TMZ in glioma cells that functions via γ-GCS expression. Importantly, the γ-GCS expression levels were lower in cells treated with the TMZ/VPA combination than in cells treated with TMZ or VPA alone. This observation suggests that γ-GCS inhibition is involved in the sensitivity of U87MG cells to TMZ and that it may act synergistically with VPA via an unknown mechanism. On the other hand, many studies have pointed out the role of oxidants as triggers of apoptosis in various chemotherapeutic agent-treated cancer cells [30–32]. The TMZ/VPA combination in the literature supports a link between apoptosis and GSH depletion that may be through γ-GCS inhibition (Figs. 3 and 4).

In conclusion, our study demonstrates that the use of a combination of clinical concentrations of TMZ and VPA synergistically sensitized glioma cells to induce apoptosis. Our results indicate that HO-1 and γ-GCS expression may result in drug resistance to TMZ-induced apoptosis. Overexpression of Bax, depletion of GSH, and inhibition of HO-1 and γ-GCS played important roles in the observed synergism-mediated cell death. Figure 7 shows our proposed model of how the TMZ/VPA combination treatment enhanced apoptosis in U87MG cells.

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