



## Temozolomide and tranilast synergistic antiproliferative effect on human glioblastoma multiforme cell line (U87MG)

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### Abstract

**Background:** Glioblastoma multiforme (GBM) is the most malignant primary brain tumor. Temozolomide (TMZ) is a chemotherapeutic agent that has been used in GBM treatment. Resistance to TMZ is a major obstacle to successful GBM treatment. The aim of the present study was to investigate the effect of TMZ and tranilast on human GBM cell line (U87MG).

**Methods:** In this in vitro experimental study, the effect of TMZ and tranilast on cell proliferation was measured using the MTT assay. Median effect analysis was performed to determine the TMZ and tranilast interaction. Lactate dehydrogenase assay was used to determine TMZ and tranilast cytotoxicity. Cell fluorescent staining and real-time PCR were used for apoptosis evaluation. The effect of TMZ and tranilast on U87MG nitric oxide (NO) production was evaluated by Griess assay.

**Results:** TMZ and tranilast had a significant dose- and time-dependent inhibitory effect on cell proliferation. The mean combination index values represented a synergistic effect, and dose reduction index values suggested the advantages of reducing the toxicity, adverse effects, and drug resistance in combination of TMZ and tranilast. Apoptosis cell death was induced by TMZ and/or tranilast in cells. TMZ and tranilast reduced NO production in cells.

**Conclusion:** TMZ and tranilast combination inhibited the GBM cells growth effectively.

**Keywords:** Drug combination, Drug resistance, Glioblastoma multiforme, Temozolomide, Tranilast

**Conflicts of Interest:** None declared

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### Introduction

Glioblastoma multiforme (GBM, classified as grade IV gliomas) is the most common malignant form of brain tumor in adults, accounting for 16% of all diagnosed primary tumors (1). Current standard treatment options for GBM include maximal surgical resection followed by radiotherapy and systemic chemotherapy (2). In spite of the recent advances in cancer treatment, the prognosis of GBM still remains very poor with median survival of about one year after start of treatment (3).

Alkylating agents were the first class of compounds identified for cancer treatment (4). Temozolomide (TMZ) is an orally administered alkylating agent used for GBM treatment. It readily passes the blood-brain barrier because of its small size and lipophilic properties, then,

methyates DNA at the specific sites on guanine and adenine bases, and produces the DNA adducts O6 methyl-guanine, which activates the mismatch repair system, causing double strand breaks in the DNA, disrupting the subsequent DNA replication, arresting cell cycle in G<sub>2</sub>/M phase, and ultimately activating apoptosis pathway (5). However, intrinsic and acquired resistance to TMZ is a major obstacle to successful treatment of GBM (6).

Tranilast is an antiallergic drug that was approved for use in the treatment of allergic disorders such as asthma, allergic rhinitis, and atopic dermatitis. Previous studies have shown the antiproliferation ability of tranilast in various tumor cell lines, including glioma, uterine leiomyoma, oral squamous cell carcinoma, and pancreatic carci-

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#### ↑What is “already known” in this topic:

Tranilast exerted an inhibitory effect on glioma tumor cells proliferation.

#### →What this article adds:

Combination of temozolomide and tranilast had a synergistic inhibitory effect on glioblastoma multiforme cells proliferation.

noma cells (7). Tranilast inhibits glioma cell growth through alteration of transforming growth factor-beta releases and reduces migration and invasion (8).

There has been a growing interest for combination therapy using multiple anticancer agents as a suitable solution to overcome drug resistance. Different anticancer drugs affect different targets and cell subpopulations, and therefore can enhance the therapeutic effects, reduce dose and side effects, and prevent or delay the induction of drug resistance (9).

Our previous studies indicated that combination of TMZ with thymoquinone (a natural compound) enhanced the therapeutic effect of TMZ (10-12). The present study was conducted to evaluate the effect of tranilast on the TMZ cytotoxicity on human glioblastoma cell line.

## Methods

### Cell line and reagents

For this in vitro experimental study, human GBM cell line (U87MG) was obtained from the National Cell Bank of Iran (NCBI). TMZ, tranilast, trypsin, paraformaldehyde, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Triton X-100, propidium iodide (PI), zinc sulfate, vanadium chloride (III), sulfanilamide, Acridine orange (AO), Ethidium bromide (EB), and N-(1-naphthyl) ethylenediamine dihydrochloride were purchased from Sigma-Aldrich Chemical Co. (Germany). Dulbecco's modified eagle medium/ham's F12 nutrient mixture (DMEM/F12) and fetal bovine serum (FBS) were purchased from Gibco (Gibco Company, Germany). All experiments were done in triplicate and repeated independently at least 3 times.

### Cell culture

U87MG cell line was cultured in T75 flasks containing DMEM/F12 supplemented with 10% FBS without antibiotics. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. TMZ and tranilast were dissolved in sterile DMSO at a stock concentration of 100 mM and stored at -20 °C until use. The final concentration of DMSO did not exceed 0.2% (v/v) in the culture medium.

### Proliferation assay

MTT is an in vitro colorimetric assay used for measuring cell proliferation. This technique is based on the potential of mitochondrial dehydrogenase enzymes in living cells for conversion of tetrazolium dye to a purple color product. U87MG cells were seeded at a density of  $15 \times 10^3$  cells/well in 96-well plate and incubated overnight. Then, the medium was replaced with a serum-free medium containing TMZ, tranilast. The concentrations used for treatment were as follow: TMZ: 12.5, 25, 50, 100, and 200 µM; tranilast: 12.5, 25, 50, 100, and 200 µM. Control cells were treated with medium containing an equivalent amount of DMSO. Cells were incubated for 24, 48, 72, and 96 hrs. at 37 °C. Then, the medium was removed, well washed with PBS, and 20 µL MTT solution (5 mg/mL in PBS) was added to each well. After a 4-hour incubation at 37°C, to dissolve the formed purple forma-

zan crystals, 100 µL dimethyl sulfoxide (DMSO) was added to each well. The absorbance at 570 nm was recorded using a plate reader spectrophotometer. MTT assay was performed in the dark because the MTT reagent is sensitive to light. The percentage of proliferation was calculated as follows (13):

Cell proliferation (%) = (absorbance of treated cells / absorbance of control cells) × 100.

The half maximal inhibitory concentration (IC<sub>50</sub>) values of TMZ and tranilast against U87MG cells were calculated using GraphPad Prism 5 (GraphPad Software Inc, San Diego, USA).

### Median effect analysis

The method proposed by Chou and Talalay was used to evaluate the nature of TMZ and tranilast interaction (synergistic, additive, or antagonistic). The combination of TMZ and tranilast in constant concentration ratio (1:1.4), based on their corresponding IC<sub>50</sub>s (104.46 and 146.09 µM for TMZ and tranilast, respectively) in 2-fold serial dilutions above and below the IC<sub>50</sub> values, was done; then, the MTT assay was performed again. The combination index (CI) and dose reduction index (DRI) values were calculated using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). The CI values were interpreted as additive (CI = 1), synergistic (CI <1), and antagonistic (CI >1). DRI value represents the degree to which the concentration of a compound can be reduced when used in combination with another compound to maintain an equivalent effect, and Fa is the fraction of cell death ranging from 0 (no cell killing) to 1 (100% of cell killing). Classical isobolograms were also obtained by plotting drugs concentrations (alone and in combination) that inhibit 50%, 75% and 90% viability. In this plot, if the combination data points fall on the hypotenuse, lower left or upper right, an additive, synergism, or antagonism effect is suggested, respectively (14).

### Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme that is released into the extracellular space when the plasma membrane is damaged. To quantify the TMZ and tranilast cytotoxicity on U87MG cell line, LDH activity in culture medium was measured using colorimetric LDH assay kit (Abcam, Cambridge, MA, USA). Briefly, GBM cells ( $7 \times 10^4$ ) were seeded in 24 well plates and after adhesion they were treated with 0.5 mL medium containing 20 µM TMZ, the maximum concentration of TMZ in the brains of patients with glioma (10), and/or 100 µM tranilast, a concentration below tranilast IC<sub>50</sub>. After a 72-hour incubation period, the supernatant was collected and centrifuged at 250 ×g for 10 minutes and LDH activity was measured in accordance with manufacturer's instructions.

### Acridine orange / Ethidium bromide double staining

AO/EB cell staining is a reliable method for evaluating the nuclear morphology, DNA fragmentation, and chromatin condensation. AO is able to pass cell membrane, intercalate to DNA bases, and emit green fluorescence,

whereas EB is only taken up by cells whose cytoplasmic membranes are damaged and emits red fluorescence by intercalation into DNA. The fluorescence emission of EB is dominated over AO, so after staining, live cells show normal green nucleus, early apoptotic cells display green nucleus with condensed or fragmented chromatin, late apoptotic cells have red nucleus with condensed or fragmented chromatin and necrotic cells show normal red nucleus (15). U87MG cells were seeded at a density of  $7 \times 10^4$  in 24 well plates and allowed to adhere overnight, and then treated with TMZ (20  $\mu$ M) and/or trinitilast (100  $\mu$ M) for 72 hours. Then, medium containing drugs was removed, each well was washed with PBS and stained by adding 100  $\mu$ L AO/EB dye mix (1 part of 100  $\mu$ g/mL of AO and 1 part of 100  $\mu$ g/mL of EB in PBS). After 15 minutes, cells were washed 3 times with PBS and observed under the fluorescent microscope (16).

#### Terminal transferase dUTP nick end labeling assay

The effect of TMZ and trinitilast on the U87MG cells apoptosis was investigated by terminal transferase dUTP nick end labeling (TUNEL) assay using an in-situ cell death detection kit, (Roche Diagnostics; Germany), according to the manufacturer's instructions. Briefly, after a 72-hour incubation with TMZ (20  $\mu$ M) and/or trinitilast (100  $\mu$ M) in 96 well plate, the cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes on ice, and incubated with 50  $\mu$ L of TUNEL mixture solution (label solution and enzyme solution) for 1 hour. The PI staining was used for differential staining of the cells and was incubated for 5 minutes at room temperature. Finally, the cells were observed under a fluorescence microscope. All the described stages were performed in dark condition. The apoptotic index of the cells was calculated as follows (17):

Apoptotic index (%) = (number of apoptotic cells/total number of cells)  $\times$  100.

#### Nitric oxide measurement

Griess assay was used to evaluate the TMZ and/or trinitilast effect on NO production levels in U87MG cells. After treatment with TMZ (20  $\mu$ M) and/or trinitilast (100  $\mu$ M) for 72 hours 400  $\mu$ L of the culture media were collected and deproteinized by adding 6 mg of zinc sulfate and centrifuging at 10 000 g for 10 minutes at 4°C. Then, 100  $\mu$ L of each deproteinized sample was transferred to microplate wells, 100  $\mu$ L vanadium (III) chloride (8 mg/mL in HCl 1 M), 50  $\mu$ L 2% sulfanilamide (in 5% HCl), and 50  $\mu$ L of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (in deionized water) were added, respectively; then, samples were incubated for 30 minutes at 37°C. The optical density of each well was measured at 540 nm with reference reading at 630 nm using a microplate reader. Sodium nitrite standard curve was used to determine the NO concentrations (18).

#### Real-Time PCR

The effect of TMZ and/or trinitilast on the expression level of 4 apoptosis related genes (BAX, BCL-2, TP53 and CASPASE 3) was analyzed by real-time PCR. RNA

from GBM cells, treated with TMZ and/or trinitilast for 72 hours, was extracted using total RNA isolation kit (DENAZist, Tehran, Iran). Complementary DNA (cDNA) synthesis was done using cDNA synthesis kit (Vivantis Technologies, Selangor DE, Malaysia). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as an internal control and the fold change in relative expression of each target mRNA was calculated based on comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method. Thermal cycler conditions were as follow: 15 minutes at 50°C for cDNA synthesis, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C to denature the DNA, and 45 seconds at 60°C to anneal and extend the template. Real-time PCR was performed using SYBR Premix Ex Taq Technology (Takara Bio Inc., Shiga, Japan) on the Applied Biosystems StepOne Real-Time PCR System. The primer sequences were as follow: for BAX

Forward 5'-CCTGTGCACCAAGGTGC CGGAACT-3', reverse 5'-CCACCCTGGTCTTGGATCC AGCCC-3', for BCL-2

forward 5'-TTGTGGCCTTCTT TGAGTTCGGTG-3', reverse 5'-GGTGCCGGTTCAG GTACTCAGTCA-3', for TP53

Forward 5'-TAACAG TTCCTGCATGGGCGGC-3', Reverse: 5'-AGGACAGG CACAAACACGCACC-3', for CASPASE

Forward: 5'-CAA ACTTTTTCAGAGGGGATCG-3', Reverse: 5'-GCAT ACTGTTTCAGCATGGCAC-3' 3 and for GAPDH

forward CAATGACCCCTTCATTGACC, and reverse TTCACACCCATGACGAACAT.

#### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD). Statistical evaluation was performed using one-way analysis of variance, followed by Tukey's post hoc test with SPSS16.0 (SPSS Inc., Chicago, IL, USA), and differences were considered to be statistically significant at  $p < 0.05$ . Dose effect curve parameters, CI plot, DRI plot, and classical isobologram were obtained using CompuSyn program (CompuSyn Inc, Paramus, NJ, USA).

#### Results

##### Proliferation assay

The results of viability assays indicated that both drugs were moderate inhibitors of U87MG cell growth (Fig. 1 a and b). The IC<sub>50</sub> values for TMZ and trinitilast were calculated and presented in Table 1.

Combination of TMZ and trinitilast reduced the U87MG cell proliferation, which was greater than each agent alone (Fig. 1 c). CI and DRI values were calculated by CompuSyn program at IC<sub>50</sub>, IC<sub>75</sub> and IC<sub>90</sub>; also, CI plot (Fig. 1 d), DRI plot (Fig. 1 e), and the isobologram (Fig. 1 f) were constructed. The results showed that the CI values were 0.42, 0.38, and 0.34 at IC<sub>50</sub>, IC<sub>75</sub>, and IC<sub>90</sub>, indicating a synergistic effect. The DRI values were 4.71 for TMZ and 4.72 for trinitilast at IC<sub>50</sub>, 5.76 for TMZ, 4.83 for trinitilast at IC<sub>75</sub>, 7.05 for TMZ, and 4.95 for trinitilast at IC<sub>90</sub>.

Table 1. IC<sub>50</sub> values for temozolomide and tranilast in the glioblastoma multiforme U87MG cell line

| Drug           | 24 hours | 48 hours | 72 hours | 96 hours |
|----------------|----------|----------|----------|----------|
| TMZ (uM)       | 1631.00  | 495.124  | 104.46   | 57.48    |
| Tranilast (uM) | 4603.59  | 592.36   | 146.09   | 94.86    |

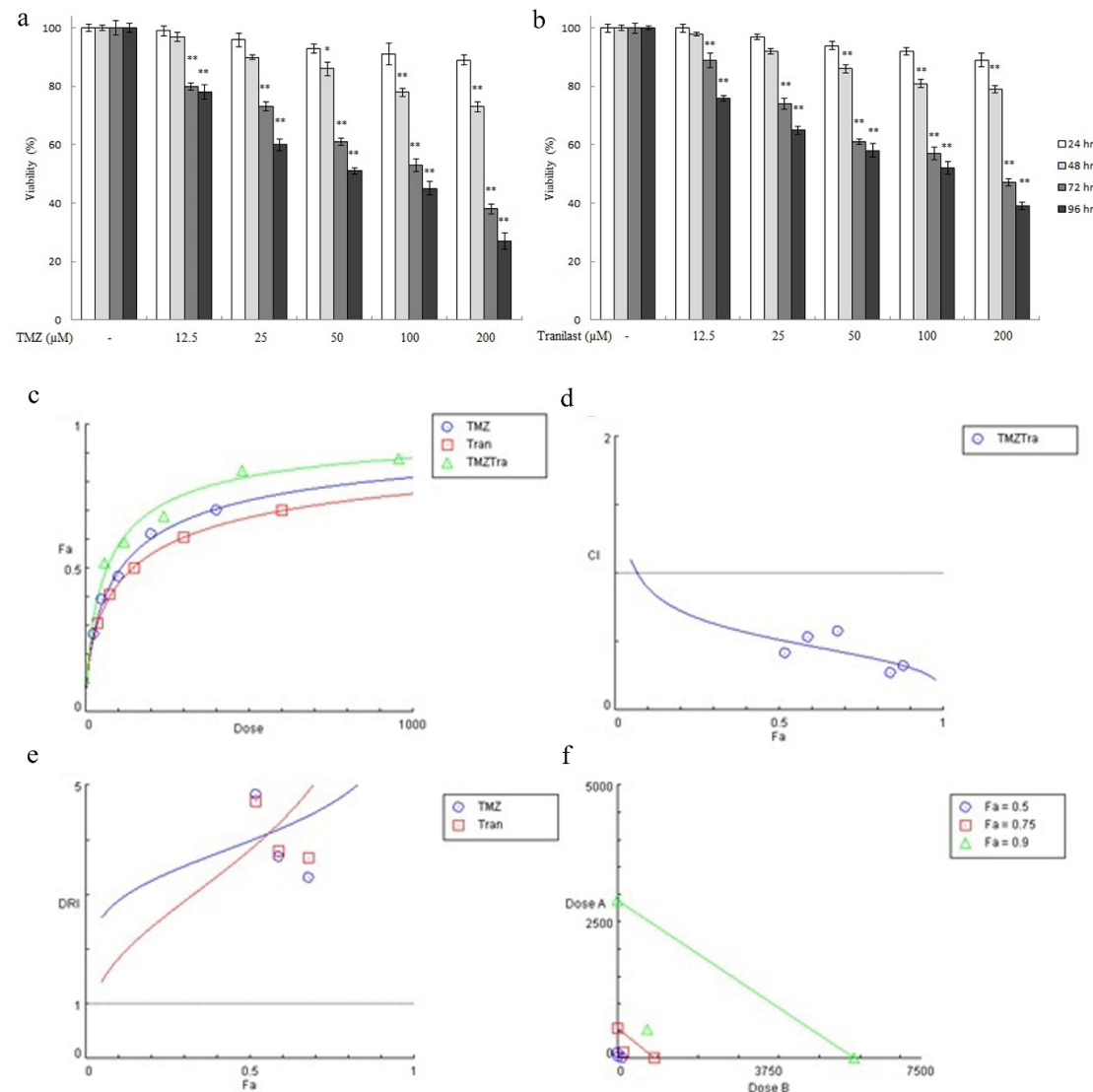


Fig. 1. The effect of temozolomide and/or tranilast on proliferation of U87MG cells. Cells were treated with TMZ (a) and tranilast (b) for 24, 48, 72, and 96 hours, and proliferation was measured by MTT assay. Control wells were treated with equivalent amount of medium alone. The results showed the mean±SD from triplicated experiments (\*P < 0.05; \*\* P < 0.01 compared to controls). (c) Dose-effect curves for temozolomide, tranilast, and their combination after a 72-hour treatment. (d) Combination index plot: The combination index is plotted as a function of Fa. (e) Dose reduction index plot for combination: Dose reduction index values at different Fa values for each drug in the combination. (f) Isobologram for combination: Classic isobologram at IC<sub>50</sub>, IC<sub>75</sub>, and IC<sub>90</sub>.

### Cytotoxicity assay

Measurement of LDH activity in the cell culture medium revealed that TMZ and/or tranilast significantly increased LDH release after a 72-hour incubation. Combination of TMZ and tranilast increased medium LDH activity (~69%) greater than either TMZ (~29%) or tranilast (~43%) alone (Fig. 2 a). Therefore, cell death, accompanied by plasma membrane damage, was greater in combination treatment.

### NO measurement

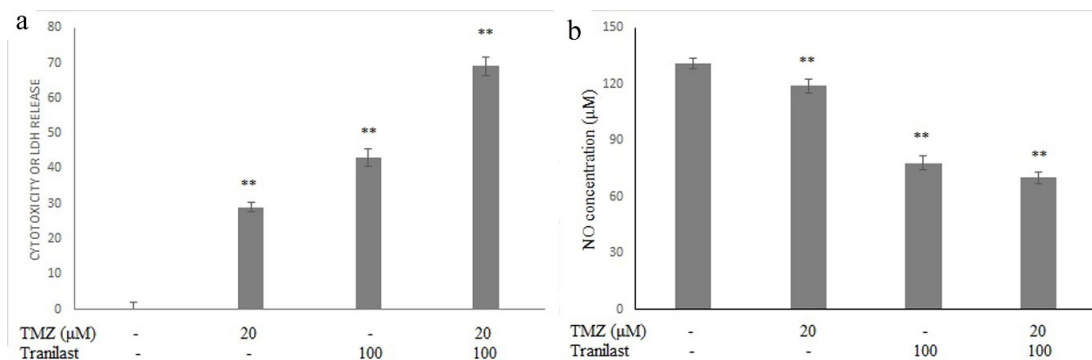
The results of Griess assay showed that treating U87MG cells with TMZ and tranilast as a single treatment for 72 hours significantly decreased NO concentration in cell

culture medium was reduced 10% and 40% after treatment with TMZ and tranilast, respectively. The minimum NO level was observed as an effect of combination treatment (Fig. 2 b).

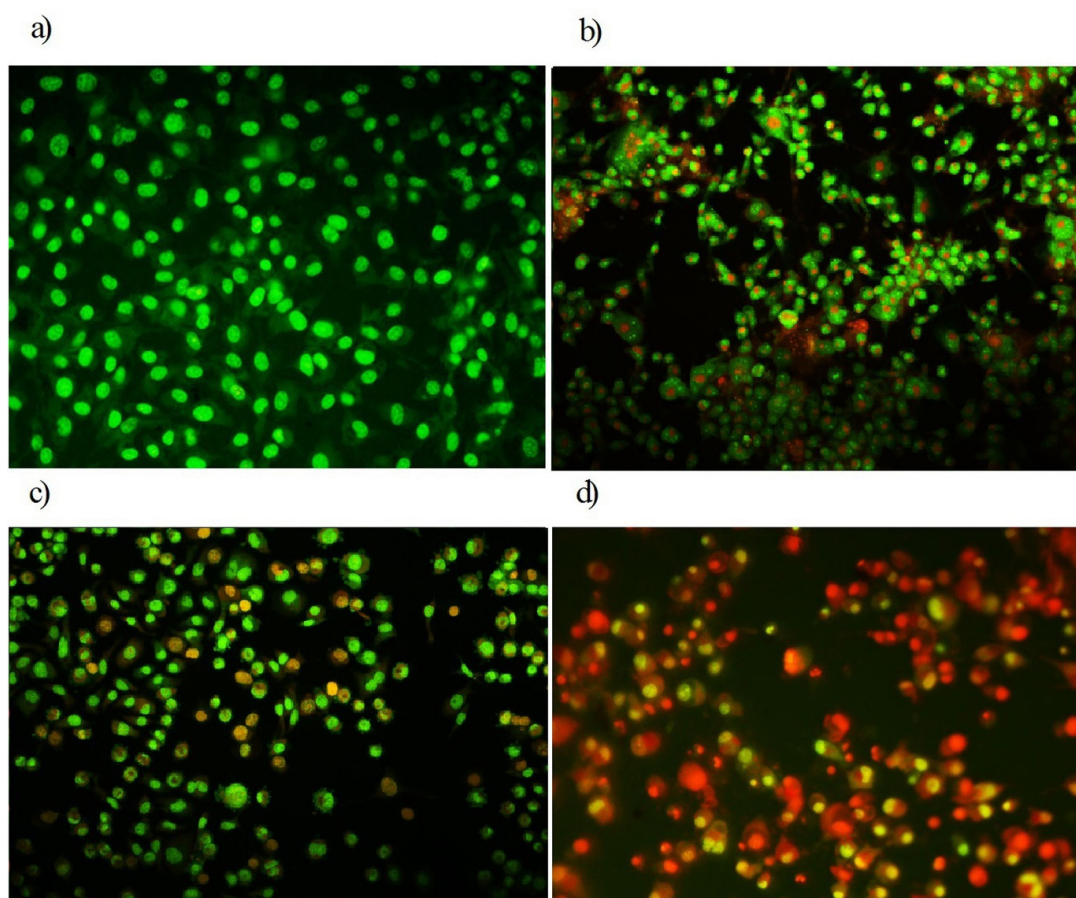
### AO/EB staining

The results from AO/EB fluorescence staining are shown in Figure 3. Live cells with normal morphology were abundant in the control group. Early apoptosis occurred in the group treated with TMZ, both early and late apoptosis occurred in the group treated with tranilast, and nearly all the cells were in late stage of apoptosis in combination treatment group and few cells were present in





**Fig. 2.** (a) Temozolomide and tranilast cytotoxicity for U87MG cells. Cells were treated with temozolomide and tranilast for 72 hours, and activity of lactate dehydrogenase was measured. Control wells were treated with equivalent amount of medium alone. (b) Effect of TMZ and/or tranilast on NO production in U87MG cells was measured by Griess reaction. P-values were determined using one-way ANOVA (\* $p < 0.05$  compared to controls; \*\* $p < 0.01$  compared to controls).



**Fig. 3.** U87MG cells were stained by AO/EB and observed under fluorescence microscope: (a) control group; (b) in the presence of 20 μM TMZ; (c) in the presence of 100 μM tranilast; and (d) in the presence of in combination both. Green live cells show normal morphology; green early apoptotic cells show nuclear margination and chromatin condensation. Late orange apoptotic cells show fragmented chromatin and apoptotic bodies.

early stage. Necrosis cell death was negligible in all groups.

#### TUNEL assay

The results of TUNEL assay showed the presence of apoptotic cell in U87MG cell treated with TMZ and tranilast alone and in combination (Fig. 4 a-f). The number of apoptotic cells were calculated and presented as percentage (Fig. 4 e). After a 72-hour treatment with TMZ and tranilast, as much as 16% and 39% of cells were apoptotic, respectively, whereas 51% of cells were apop-

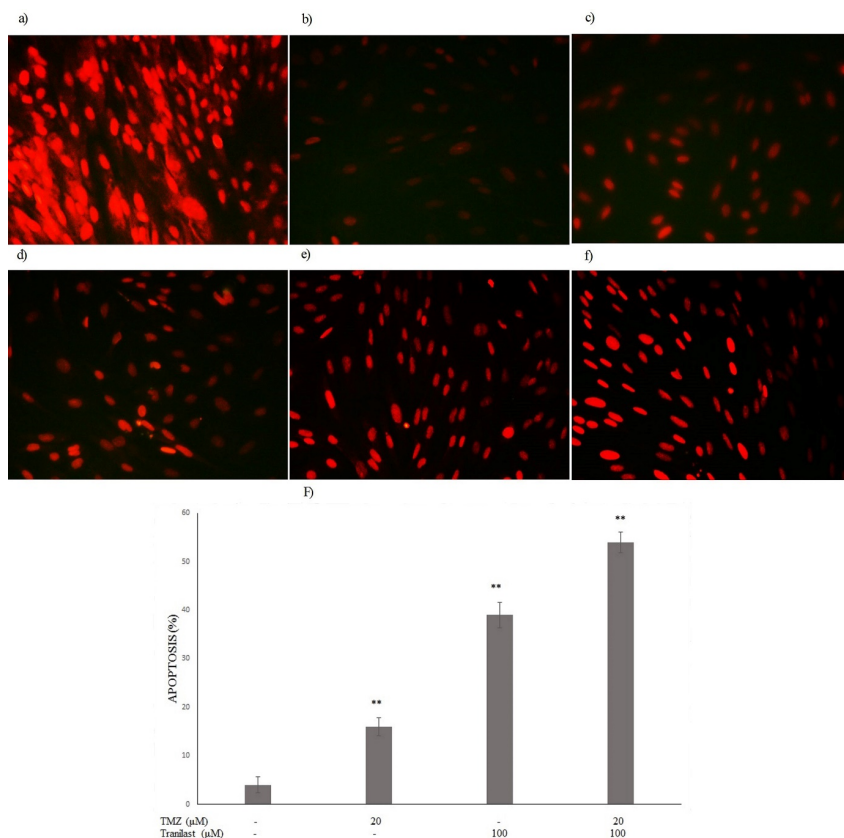
totic after combination treatment.

*TP53*, *BAX*, *Bcl-2*, and *CASPASE 3* mRNA expression

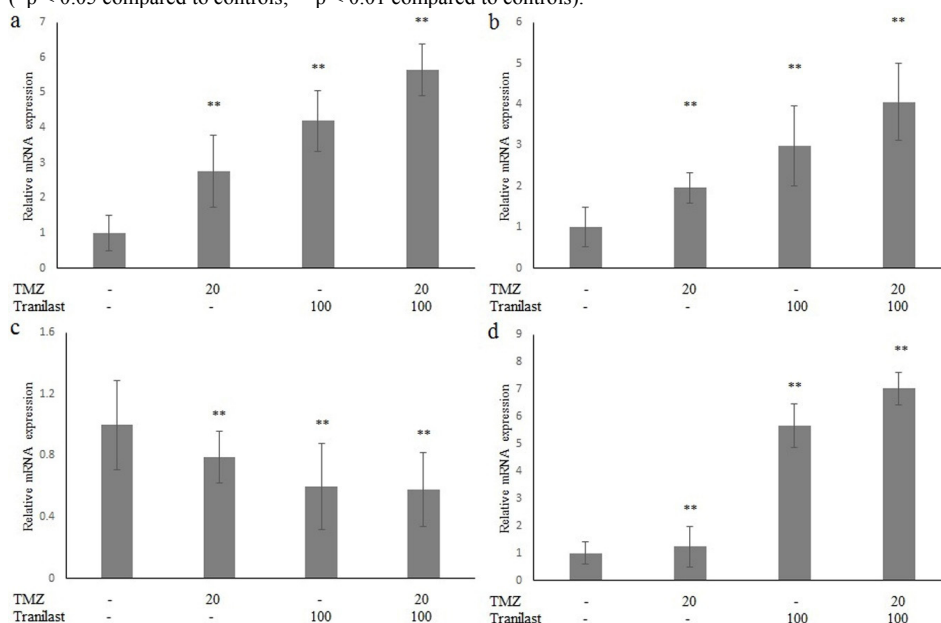
Expression of 4 apoptosis related genes (*TP53*, *BAX*, *BCL-2*, and *CASPASE 3*) were evaluated using real-time PCR. As shown in Figure 5 a, *TP53* was upregulated by 2.76-, 4.19-, and 5.65-fold by TMZ, tranilast, and combination of both, respectively, as compared to levels in control cells. Results of real-time PCR also showed the down-regulation of *BCL-2* and upregulation of *BAX* expression at 72-hour treatment (Fig. 5 b and c). *BAX* to *BCL-2* ratio was increased by 2.48, 4.98, and 6.98 in U87MG cells

treated with TMZ, tranilast and combination of both, respectively. Expression level of *CASPASE 3* was evaluated by 1.23-, 5.66- and 7.03-fold by TMZ, tranilast, and com-

bination treatment, respectively (Fig. 5 d).



**Fig. 4.** Apoptotic potential of TMZ and/or tranilast detected by TUNEL staining in U87MG cells. (a) positive control group; (b) negative control group; (c) control group; (d) 20  $\mu$ M TMZ; (e) 100  $\mu$ M tranilast; (f) combination of 20  $\mu$ M TMZ and 100  $\mu$ M tranilast. (g) The graph of the mean percentage of apoptotic cells from 3 independent experiments performed in triplicate. The positive control was incubated with label solution without enzyme solution. P-values were determined using one-way ANOVA (\* $p < 0.05$  compared to controls; \*\* $p < 0.01$  compared to controls).



**Fig. 5.** Expression levels of apoptotic factors in U87MG cells after TMZ and/or tranilast after a 72-hour treatment was measured by real-time PCR. (a) TP53 (tumor suppressor); (b) BAX (pro-apoptotic); (c) BCL-2 (anti-apoptotic); and (d) CASPASE 3 (required enzyme for execution of apoptosis). BAX/BCL-2 values  $> 1$  means that the cell line enters apoptosis. P-values were determined using one-way ANOVA (\*\* $p < 0.01$  compared to controls).

## Discussion

In the present study, for the first time, the synergistic inhibition of U87MG cell proliferation by the combination of TMZ and tranilast was reported. The results showed that TMZ and tranilast significantly decreased cell proliferation in a time- and dose-dependent manner. Combination treatment showed a significant growth inhibition effect with a combination index values, indicating synergistic effect at  $IC_{50}$ ,  $IC_{75}$ , and  $IC_{90}$ . This combination also resulted in a greater 1 DRI for both TMZ and tranilast. When DRI is more than 1, it allows a dose reduction that leads to toxicity reduction in the therapeutic applications.

This combination reduced  $IC_{50}$ s, 2.04 folds for TMZ and 2.86 folds for tranilast. A reduction in the dose of temozolomide for a particular effect is clinically important because this reduction is associated with a decrease in the general chemotherapy side effects.

TMZ is an approved chemotherapeutic drug by the Food and Drug Administration and is used as a first-line treatment for GBM. TMZ resistance is a major issue in the management of glioblastoma and limits the median survival of the patient to about 1 year (19). Currently combination therapy with multiple drugs has become the primary strategy to treat drug-resistant cancers. Previous in vitro studies indicated that therapeutic activity of TMZ was enhanced when combined with other anticancer agents (20-24). Moreover, in clinical trials, the combination of carmustine and TMZ as a neoadjuvant therapy exhibited promising activity with a good safety profile in GBM (25), so drug combination therapy can be a beneficial approach for GBM treatment.

Previous studies showed that tranilast caused concentration-dependent inhibition of proliferation, migration, and invasiveness of 2 human malignant glioma cell lines (LN-18 and T98G) (8).

The present study showed that tranilast enhanced the cytotoxicity of TMZ by increment of apoptosis cell death. TP53 is a transcription factor in nucleus that acts as a regulator of the expression level of genes involved in cell cycle and apoptosis. Expression of *BAX* relative to *BCL-2* was elevated by increased expression of TP53 (26). Alteration in the levels of pro- and antiapoptotic proteins is known to regulate apoptotic pathway. TMZ induced apoptosis in U87MG cells by increasing the level of BAX (proapoptotic) to *BCL-2* (antiapoptotic) proteins ratio (27).

Results of this study showed that both TMZ and tranilast increased the TP53 expression levels and *BAX/BCL-2* ratio; therefore, treatment of U87MG cells with tranilast augmented TMZ induced apoptosis, indicating that tranilast worked as synergistic agent in TMZ-induced apoptosis in human glioblastoma cells.

Apoptosis may occur via extrinsic (mitochondrial) or intrinsic (death receptor) pathways. The intrinsic pathway is mediated by *BCL-2* family proteins, which regulate the mitochondrial membrane disruption, cytochrome c release to the cytosol and *CASPASE 9* activation. The extrinsic pathway of apoptosis is triggered through death receptors activation and the subsequent *CASPASE 8* activation.

Eventually both intrinsic and extrinsic pathway converge will form on the same terminal or execution path-

way.

*CASPASE 3* is a convergence point for 2 apoptosis pathways. It catalyzes the specific cleavage of many key cellular proteins (28). The data in this study showed that TMZ and tranilast increased the expression level of *CASPASE 3* in U87MG cells, suggesting involvement of the *CASPASE*-dependent apoptotic pathway in the anti-proliferative effect.

Several studies have been done on the combined treatments of TMZ and different apoptotic agents. A study reported the synergy of lidamycin (a member of the enediyne antitumor antibiotic family) and TMZ in cytotoxicity and apoptosis. Similar to the present study, the expression levels of *TP53* and *BAX* were significantly upregulated, whereas the expressions of *CASPASE 3* and *BCL-2* were dramatically downregulated by the combination, compared to a single drug (29). Also, the synergistic cytotoxic and apoptotic effect of combination of temozolomide and chloroquine treatment with increased sub-G1 hypodiploid cells and *CASPASE* activation was reported (30). Results of another study showed that the combined administration of N-(2-hydroxyphenyl) acetamide and temozolomide significantly enhanced the cell growth inhibition and apoptosis compared to each agent alone. Furthermore, the data revealed that this cooperative apoptosis induction was associated with increased ratio of *BAX* to *BCL-2* and active *CASPASE 3* expression (31).

Nitric oxide (NO) exhibits ambiguous roles in cancer cells. Studies indicated a tumor-promoting role for NO. Some effects of NO in cancer include chemotherapeutic resistance, inhibition of apoptosis, and enhanced cell proliferation. NO has an important role in glioma pathophysiology. A protumorigenic role has been suggested for NO in gliomas. Also, NO has a role in facilitating glioma cell growth. One study indicated that NO, in the endothelium or in glioma cells, may represent mechanisms by which the vasculature and neoplastic cells interact with each other to affect glioma growth and response to therapy (32). In the present study, it was found that TMZ and tranilast significantly reduced NO production and combination of both had a stronger effect on the reduction of NO level than each agent alone.

Despite recent improvements in cancer treatment, GBM is still associated with a poor prognosis and short-term survival of the patients mainly because of drug resistance. Hence, development of new strategies for overcoming this problem is necessary. Combination therapies are the most effective of the current strategies against cancer, as multiple drugs affect multiple targets and cell subpopulations, and therefore can enhance the therapeutic effects, reduce dose and side effects, and prevent or delay the induction of drug resistance.

## Conclusion

The studies on drug combination indicated that other anticancer agents can improve TMZ-mediated cytotoxicity. Data of this study showed that combination treatment with TMZ and tranilast resulted in synergistic antitumor effect in GBM cells. Also, it was found that the mechanism of reducing cell resistance to TMZ by tranilast might be at-



tributed to apoptosis induction. Thus, the combination of TMZ and tranilast for the treatment of glioblastoma patients may result in a desirable clinical outcome. Therefore, more in vivo studies should be conducted on TMZ/tranilast synergy and future clinical trials should evaluate the efficacy of TMZ and tranilast combination therapy among GBM patients.

### Conflict of Interests

The authors declare that they have no competing interests.

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