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Original article

Investigation of the anticancer effects of tschimgin alone or in combination with temozolomide against glioblastoma multiforme: an *in vitro* study

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Abstract

Objective: Glioblastoma (GB) poses a significant clinical challenge due to its aggressive nature and resistance to temozolomide (TMZ). This study investigated the natural compound tschimgin, alone and in combination with TMZ, as a potential therapeutic strategy against TMZ-resistant GB cells.

Materials and methods: The study assessed the effects of tschimgin and TMZ, alone and combined, on TMZ-resistant glioblastoma cells (T98G) and normal fibroblasts (HFF) using MTT assays, combination index analysis, cell cycle analysis, reactive oxygen species (ROS) measurements, caspase activity tests, and qRT-PCR for *Bax/Bcl-2* expression.

Results: Tschimgin significantly enhanced TMZ-induced cytotoxicity in T98G cells, with minimal impact on HFF cells. The combined treatment resulted in a substantial reduction in cell survival compared to either treatment alone. Moreover, findings revealed that both compounds, particularly in combination, induced cell cycle arrest at the sub-G1 and G2/M phases, increased ROS production, increased caspase 3/7 activity, and upregulated the *Bax/Bcl2* ratio. The increase in the *Bax/Bcl-2* ratio was driven primarily by significant upregulation of Bax, while *Bcl-2* levels remained largely unchanged.

Conclusion: The combination of tschimgin and TMZ exhibits a synergistic effect on TMZ-resistant GB cells, suggesting it as a potential therapeutic strategy for patients with resistant tumors. These findings suggest that tschimgin may serve as an adjunct therapeutic agent to enhance the efficacy of TMZ in resistant GB. However, further mechanistic, *in vivo*, and clinical studies are warranted to evaluate the effectiveness and safety of this compound.

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Introduction

Glioblastoma (GB) represents a particularly devastating form of brain cancer, distinguished by its aggressive behavior and inherent resistance to many standard treatments. This malignancy is marked by rapid cell division and a tendency to infiltrate surrounding brain tissue, making complete surgical removal extremely difficult (Hanif et al. 2017; Pouyan et al. 2025). Although medical science has made progress in surgical methods, radiation therapy, and chemotherapeutic agents, including the widely used temozolomide (TMZ), the prognosis for patients diagnosed with GB continues to be poor. The median survival time for patients with this condition typically does not extend beyond 15 months, underscoring the urgent need for more effective therapeutic interventions (Angom et al. 2023; Jezierzanski et al. 2024). A major obstacle in the successful management of GB is the frequent emergence of resistance to conventional treatment approaches. This resistance significantly limits the effectiveness of existing therapies, leading to disease progression and poor patient outcomes (Yalamarty et al. 2023). The challenge of treatment resistance has spurred an intense search for innovative therapeutic strategies that can circumvent these mechanisms and improve patient survival (Garg et al. 2024).

In response to the limitations of current therapies, increasing research has been initiated to investigate the therapeutic potential of various compounds, with a particular focus on agents capable of overcoming drug resistance in GB cells. Meanwhile, compounds belonging to the alkaloid family have shown promising anticancer properties in preclinical studies. Notably, alkaloid compounds have shown the potential to counteract drug resistance mechanisms, suggesting that they could enhance the efficacy of existing treatments when used in combination (Habli et al. 2017). Plant-derived alkaloids have demonstrated anticancer activity in both *in vitro* and *in vivo* studies. Despite their limited solubility, these compounds hold promise as potential antitumor agents (Habli et al. 2017; Laines-Hidalgo et al. 2022). Benzophenanthridine alkaloids, a subclass of the benzylisoquinoline alkaloid family exemplified by sanguinarine, exhibit a broad spectrum of pharmacological activities and are consequently under investigation for their therapeutic potential in the management of various human diseases, including cancer (Laines-Hidalgo et al. 2022).

Tschimgine is a naturally occurring compound belonging to the alkaloid family and structurally classified as an ester-type monoterpene derivative. It has the molecular formula $C_{17}H_{22}O_3$ and a molecular weight of 274.35 g/mol. Its chemical structure consists of a monoterpenoid core linked to an ester functional group, a configuration that contributes to its diverse biological activities. Pharmacologically, tschimgine is categorized among monoterpene derivatives and exhibits several notable effects, including cytotoxic activity against the melanoma SK-MEL-28 cell line, potent inhibition of acetylcholinesterase, a key therapeutic target in Alzheimer's disease, and modulation of estrogen receptor activity (Karimi et al. 2010; Kojo et al. 2018; Valiahdi et al. 2013). In addition, structurally related compounds such as stylosin demonstrate comparable cytotoxic and pro-apoptotic properties, underscoring the therapeutic potential of this class of monoterpene-based alkaloids (Karimi et al. 2010; Tazik et al. 2020).

In light of the above, this study was designed for the first time to specifically investigate the combined effects of tschimgin, an alkaloid, and TMZ on the T98G GB cell line, which demonstrates TMZ resistance (Lee 2016). The main objective is to evaluate the synergistic potential of these two agents.

Materials and Methods

Cell line and reagents

T98G cells, resistant to TMZ, and HFF cells, a normal fibroblast cell line, were obtained from the National Cell Bank of Iran (Pasteur Institute). Culture reagents, including fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic Acid (EDTA), penicillin-streptomycin, and high-glucose Dulbecco's Modified Eagle Medium (DMEM), were procured from Gibco. Reactive oxygen species (ROS) detection kits (DCFDA/H2DCFDA) were supplied by Abcam. Propidium iodide (PI), resazurin, tschimgin, and TMZ were obtained from Sigma-Aldrich, Golexir Iran Company, and Cayman Chemical, respectively. Tschimgin was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 50 mM, with a final DMSO concentration of 0.1% (v/v) in all treatment conditions. Vehicle controls containing 0.1% DMSO were included in all experiments. All cell lines were routinely tested for *Mycoplasma* contamination, and only *Mycoplasma*-free cultures were used.

Cell culture and cell viability assay

T98G and HFF cells were cultured in high-glucose DMEM supplemented with 10% of FBS and 1% of penicillin-streptomycin (100X). Cell viability was evaluated using the resazurin assay. A total of 12,000 cells per well were seeded into 96-well plates and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. Cells were then treated with varying concentrations of TMZ (62.5–20,000 µM) and tschimgin (7–500 µM) for 24, 48, and 72 hr. After treatment, 20 µl of resazurin solution was added, and the mixture was incubated for an additional 2-3 hr. Fluorescence intensity was recorded at 550 nm excitation and 590 nm emission using a Victor X5 microplate reader. Half-maximal inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism software. All experiments were conducted in triplicate to ensure reproducibility.

Combination index calculation

T98G cells (12,000 per well) were exposed to tschimgin and temozolomide (TMZ) in 96-well culture plates, either individually or in combination, for 72 hr. Following treatment, cell viability was evaluated, and drug interaction effects were analyzed using CalcuSyn software to compute combination index (CI) values; CI < 1 indicates synergism, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism.

Cell cycle analysis

T98G cells (30×10^4) were seeded in 12-well plates and treated for 72 hr with tschimgin (30 µM), TMZ (250 µM), or a combination of both. Following treatment, cells were fixed in 70% ethanol, incubated with RNase and PI solution (50 µg/ml PI, 1 mg/ml sodium citrate, and 0.1% Triton X-100) Cell cycle analysis was performed using a BD FACSCalibur™ flow cytometer (Becton Dickinson, USA), and data were processed and analyzed with FlowJo software version 10 (FlowJo LLC, OH, USA) to determine cell cycle phase distribution.

Reactive oxygen species (ROS) assay

T98G cells were seeded at a density of 2.5×10^4 cells per well in 96-well plates and allowed to adhere overnight. Following a washing step, cells were incubated with 25 µM H2DCFDA for 45 min, then washed three times before treatment with 30 µM of tschimgin, 250 µM of TMZ, a combination of 30 µM of tschimgin and 250 µM of TMZ, N-acetylcysteine (NAC) (5 mM) alone, or in combination with 30 µM of tschimgin, 250 µM of TMZ, and a combination of 30 µM of tschimgin and 250 µM of TMZ for 4 hr. Then, 100 µM Tert-butyl hydroperoxide (TBHP) was used as a positive control. Finally, ROS production was quantified by measuring fluorescence intensity (excitation/emission: 485/535 nm) using a Victor X5 Multiplate Reader. All treatments were performed in triplicate. In

addition, cell viability in all treatment groups was evaluated using a Resazurin assay kit. All treatments were performed in triplicate.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from T98G cells treated for 72 hr with tschimgin (30 µM), TMZ (250 µM), or their combination, using the Favorgen RNA Extraction Kit. RNA concentration and purity were assessed by agarose gel electrophoresis and UV spectroscopy. Complementary DNA (cDNA) was synthesized using the Yekta Tajhiz cDNA Synthesis Kit. The qRT-PCR was carried out using RealQ Plus 2X MasterMix Green (without Rox™), gene-specific primers for *Bax*, *Bcl-2*, and *B2M*, and a LightCycler® 96 system. NCBI Primer-BLAST confirmed primer specificity against the human RefSeq mRNA database. Both forward and reverse primers aligned specifically to the *Bax, Bcl-2, and B2M* reference sequence with full-length identity and no significant off-target matches. All experiments were performed in triplicate. The primer sequences (Metabion, Germany) are provided in Table 1. Relative mRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ (Livak) method.

Caspase-3/7 activity evaluation

Caspase-3/7 activity was measured to evaluate apoptosis in T98G cells following treatment. After a 72-hr incubation with tschimgin (30 µM), TMZ (250 µM), or their combination, the cells were processed according to the manufacturer's protocol for a colorimetric Caspase-3/7 assay kit (Kiazist Life Sciences, Iran). Briefly, the cells were rinsed with cold phosphate-buffered saline (PBS) and lysed using the provided lysis buffer. The lysates were then centrifuged at 10,000 × g for 10 min at 4°C to collect the supernatant. The protein concentration of each sample was determined using a BCA assay to normalize the results. For the enzymatic reaction, cell lysate was combined with reaction buffer containing dithiothreitol (DTT) and the colorimetric caspase-3/7 substrate (DEVD-pNA). The mixture was incubated at 37°C for 1.5 hr in a 96-well plate. During this incubation, active caspase-3/7 cleaves the p-nitroaniline (pNA) chromophore from the DEVD substrate, producing a yellow color. The absorbance of the released pNA was measured at 405 nm using a BioTek microplate reader (USA). A standard curve prepared with known concentrations of pNA was used to quantify the caspase-3/7 activity. The final results are expressed as mU/µg protein. All measurements were performed in triplicate.

Statistical analysis

Data from three independent experiments were analyzed using GraphPad Prism® version 8. First, data were checked for normality using the Shapiro–Wilk test. For data that followed a normal distribution, a parametric one-way ANOVA was performed, followed by Tukey's post-hoc test for multiple comparisons. For data that did not follow a normal distribution, a nonparametric Kruskal-Wallis test was used, followed by Dunn's post hoc test. Results are presented as mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

Table 1. The sequence of primers

Gene symbol	Gene name	Primers (5′ → 3′)
Bax	Bcl-2-associated X protein	Forward: AAACTGGTGCTCAAGGCCC
		Reverse: GTCCAATGTCCAGCCCATGA
Bcl-2	B-cell lymphoma 2	Forward: GTCATGTGTGTGGAGAGCGTC
		Reverse: CCGTACAGTTCCACAAAGGCATC
B2M	Beta-2-Microglobulin	Forward: AGGCTATCCAGCGTACTCCA
		Reverse: TGTCGGATGGATGAAACCCA

Results

Tschimgin and TMZ reduced the viability of T98G cells in a dose- and time-dependent manner

Tschimgin and TMZ inhibited T98G cell viability in a dose- and time-dependent manner, as determined by a resazurin assay. After 24, 48, and 72 hr, the IC_{50} values for tschimgin were 98.53, 78.61, and 62.89 µM, respectively (Figure 1A). After 24, 48, and 72 hr, the corresponding IC_{50} values for TMZ were >2000, ~1000, and 494.2 µM, respectively (Figure 1B). These results indicate that both compounds decrease T98G cell viability in a dose- and time-dependent fashion.

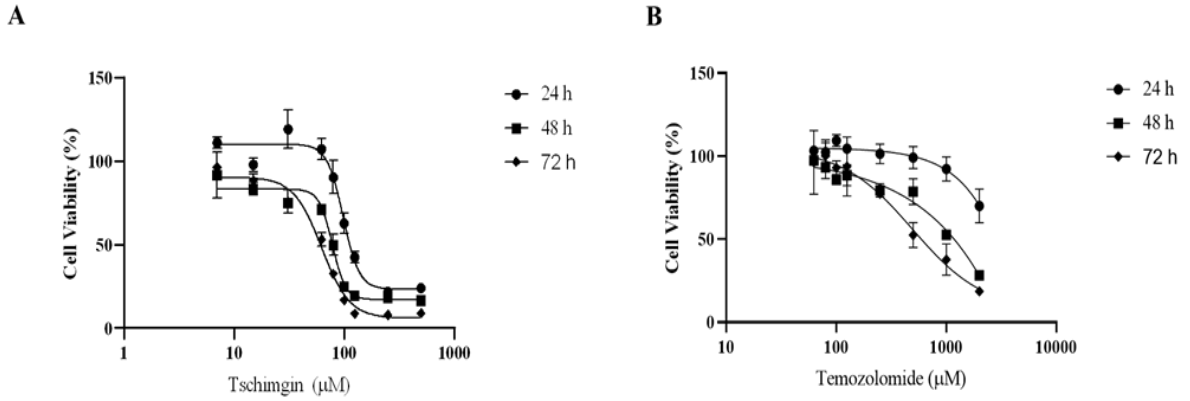


Figure 1. The effects of different doses of tschimgin (A) and TMZ (B) on T98G cell viability were assessed using the resazurin assay kit at 24, 48, and 72 hr after treatment. The results are presented as mean ± SD. (n=3)

Tschimgin exhibits less toxicity than normal HFF cells

Tschimgin toxicity to normal cells was evaluated by treating HFF fibroblast cells for 24, 48, and 72 hr. Microscopic analysis (Figure 2A) revealed minimal HFF cell shrinkage and detachment at the tschimgin IC50 (62.5 µM) after 72 hr, suggesting lower toxicity to HFF cells than to T98G cancer cells. Data in Figure 2B also show that the IC50 values for HFF cells were 108.8, 95.5, and 87.52 µM at 24, 48, and 72 hr, respectively, further confirming tschimgin's minimal cytotoxicity against fibroblasts compared to T98G cells (Table 2).

Table 2. IC50 values for T98G and HFF cells after treatment with TMZ and tschimgin for 24, 48, and 72 hr.

Drug	Cell	TIME (hr)	IC50 (µM)
TMZ	T98G	24	>2000
		48	~1000
		72	494.2
Tschimgin	T98G	24	98.53
		48	78.61
		72	62.89
	HFF	24	108.8
		48	95.5
		72	87.52

Tschimgin sensitizes T98G cells to TMZ

This study investigated the synergistic potential of combining tschimgin with TMZ, a standard treatment for GB, at the specific concentration presented in Figure 3C. T98G cells treated with tschimgin, TMZ, or both were assessed for viability using a resazurin assay. Resazurin assay results demonstrated that the combination of tschimgin and TMZ was more toxic to T98G cells across all concentrations than either treatment alone (Figure 3A). Moreover, CI analysis indicated a synergistic interaction (CI < 1) at all concentrations tested (Figure 3B and 3D).

The combination of tschimgin with TMZ induced significant sub-G1 and G2/M cell cycle arrest

Cell cycle analysis demonstrated that tschimgin and TMZ each induced G2/M arrest in T98G cells, with tschimgin exerting a greater effect (G2/M: 31.9%) than TMZ (22.2%) (Figure 4A). Tschimgin also increased the Sub-G1 population to 11.8%, whereas TMZ alone increased it by 10.1%. Notably, the combination of tschimgin and TMZ at their lower IC50

concentrations produced a marked enhancement of both Sub-G1 and G2/M arrest, elevating these phases to 14.1% and 39.6%, respectively (Figure 4B). These quantitative data further confirm that the combined treatment induces a stronger cell-cycle blockade than either compound alone.

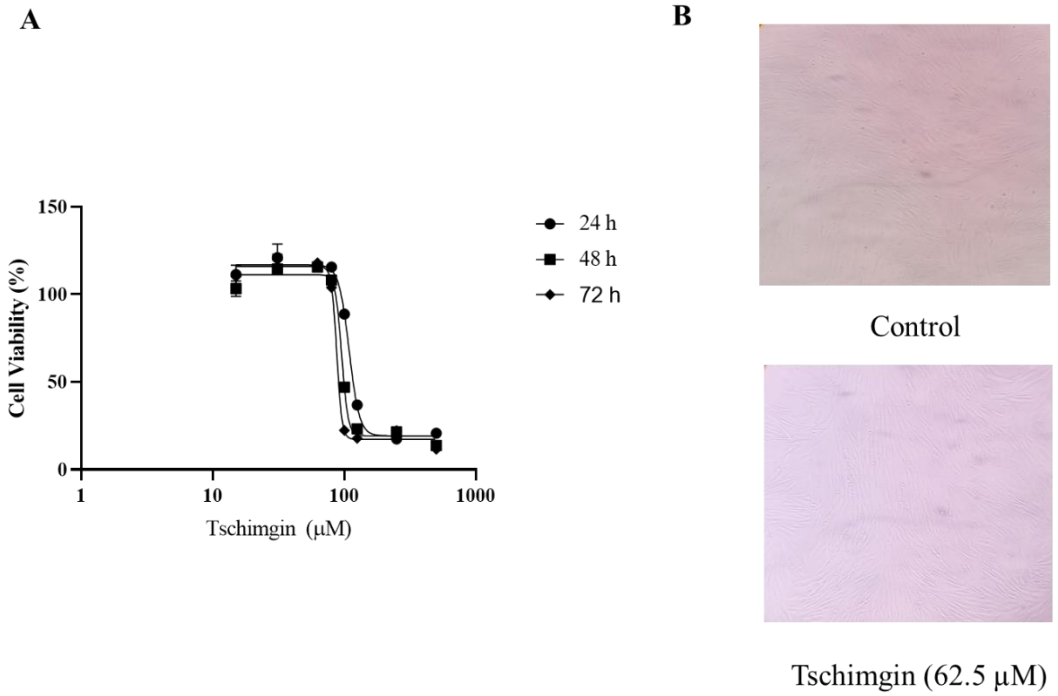
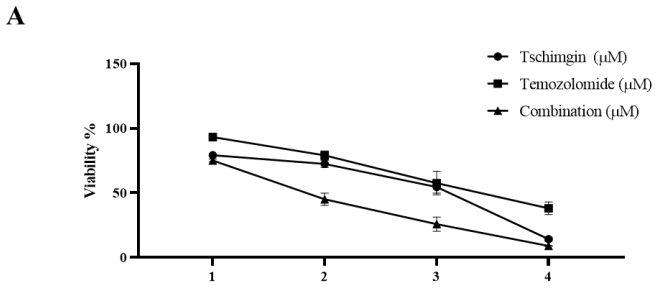
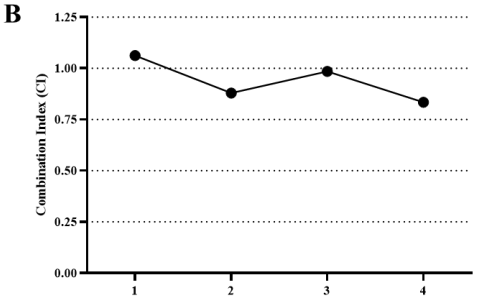


Figure 2. The effect of tschimgin on the viability of normal HFF fibroblasts was evaluated. (A) Cell viability was assessed after 24 hr of treatment using the resazurin assay (mean ± standard deviation, n=3). (B) Microscopic images show HFF cells after 72 hr of treatment with 62.5 µM tschimgin.





C

	Temazolomide (µM)	Tschimgin (µM)	Fa	CI
1	125	15.6	0.751	1.062
2	250	31.25	0.450	0.879
3	500	62.5	0.258	0.985
4	1000	125	0.089	0.834

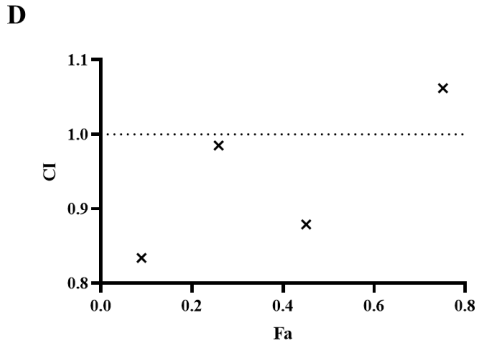


Figure 3. (A) Resazurin assay results demonstrating that the combination treatment with tschimgin and TMZ at (B) specific doses after 72 hr was significantly more toxic to T98G cells across all concentrations compared to either agent alone. (C) Combination index (CI) analysis further confirmed a synergistic interaction (CI<1) at all concentrations tested. (D) The Combination index (CI) - Fraction affected (Fa) plot (n=3).

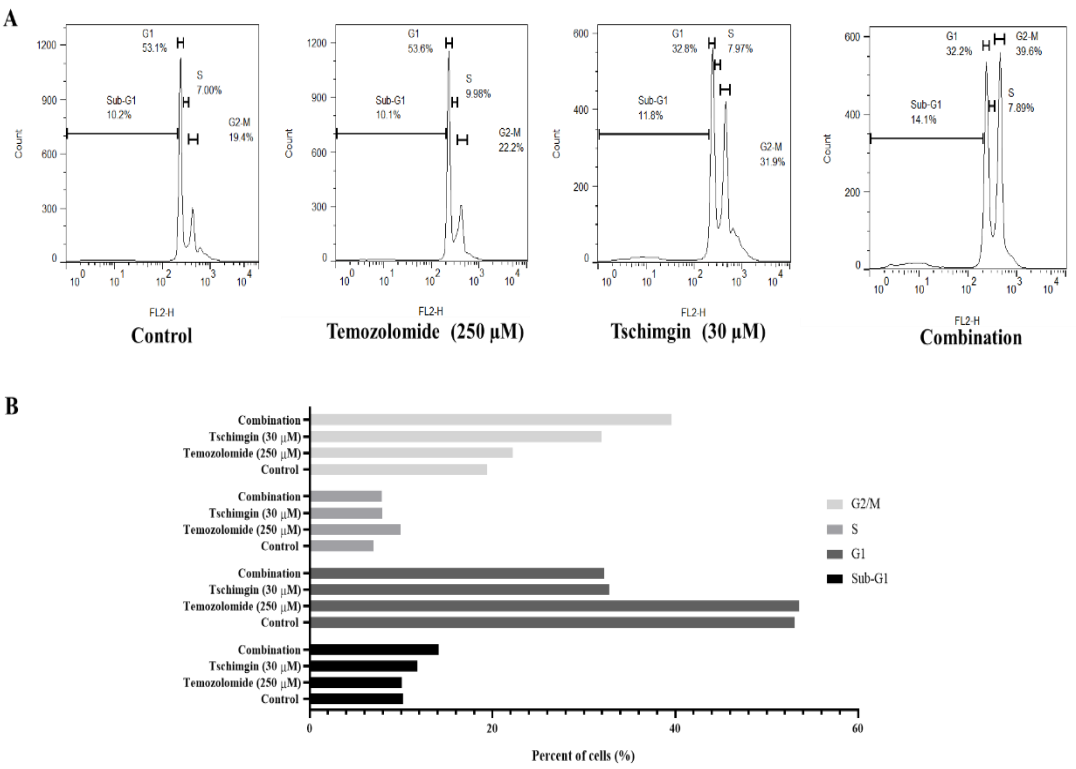


Figure 4. (A) Tschimgin (30 µM), TMZ (250 µM), and their combination were applied to T98G cells for 72 hr, and cell cycle analysis was performed. (B) The combined treatment induced notable sub-G1 (14.1 %) and G2/M (39.6 %) arrest.

The combination of tschimgin with TMZ resulted in significant and high ROS production

ROS levels were measured after four hours using a fluorometer to assess the effects of tschimgin, TMZ, and their combination. As shown in Figure 5A, both tschimgin and TMZ significantly increased ROS production. Combining tschimgin and TMZ significantly increased ROS levels in T98G cells compared with controls. As expected, the positive control TBHP also significantly increased ROS production. Co-treatment with NAC, tschimgin, and TMZ, and the combination group resulted in significantly lower ROS generation compared to each compound individually (Figure 5A). Interestingly, our data show that NAC reversed the reduction in cell viability at 72 hr in all tschimgin-, TMZ-, and combination-treated cells (Figure 5B). Hence, it is hypothesized that ROS is a major mechanism underlying tschimgin- and TMZ-induced cytotoxicity in GB cells.

The combination of tschimgin with TMZ increases the expression of apoptosis-inducing mRNAs in T98G cells

Tschimgin and TMZ alone or in combination significantly increased the expression of *Bax* mRNA in T98G cells, a gene known to induce apoptosis ($p<0.05$) (Figure 6A). While tschimgin, TMZ, and their combination decreased *Bcl-2* expression, these changes were not statistically significant (Figure 6B). In total, the combination of tschimgin and TMZ further elevated the *Bax/Bcl-2* expression ratio ($p<0.05$) (Figure 6C), with fold changes of 2.0, 1.1, and 2.3, respectively.

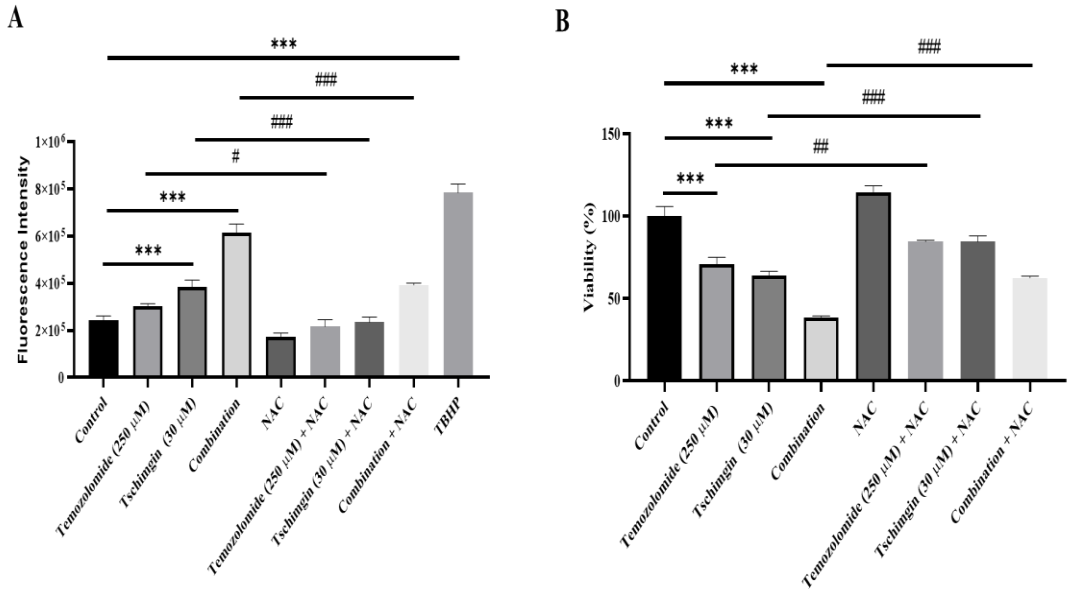


Figure 5. (A) ROS production in T98G cells was assessed after 4 hr of treatment with 30 μM tschimgin, 250 μM TMZ, or a combination of both, using DCFDA. TBHP was used as a positive control. A significant increase in ROS production compared to the control is shown in all treated groups (***p<0.001). The combination treatment with NAC also significantly decreased ROS production compared to non-NAC-treated groups (#p<0.05, ###p<0.001). (B) N-acetyl cysteine (NAC, 5 mM) combined with 30 μM tschimgin, 250 μM TMZ, or a combination of both increased the viability of the T98G cells 72 hr after treatment compared with each group in the same concentrations. Each column represents the mean ± standard error of the mean in the samples. (***p<0.001 as compared to control and ##p<0.01 and ###p<0.001 compared to each group in the same concentration (n = 3).

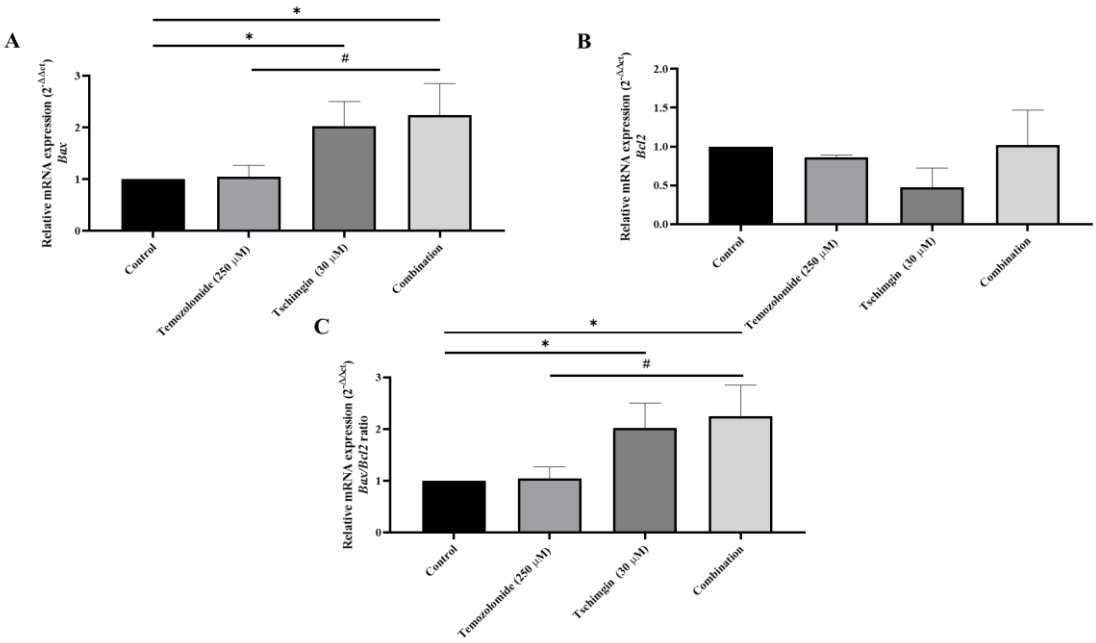


Figure 6. Quantitative RT-PCR of T98G cells was assessed to investigate (A) *Bax*, (B) *Bcl-2*, and (C) *Bax/Bcl-2* ratio after 72 hr of treatment with 30 μM tschimgin, 250 μM TMZ, or a combination of both compared to the control (*p<0.05). The combination treatment also significantly affected gene expression (#p<0.05).

The combination of tschimgin with TMZ resulted in significant high caspase 3/7 activity

Caspase-3/7 activation, a key biomarker of apoptosis induction, was measured. The data in Figure 7 indicates a significant increase in caspase 3/7 activity after treatment with TMZ, tschimgin, or the combination of tschimgin and TMZ at 72 hr compared to the control; however, the difference was statistically significant only for tschimgin and the combination therapy. Moreover, Combination therapy resulted in a significantly higher level of Caspase-3/7 activation compared to either tschimgin or TMZ alone.

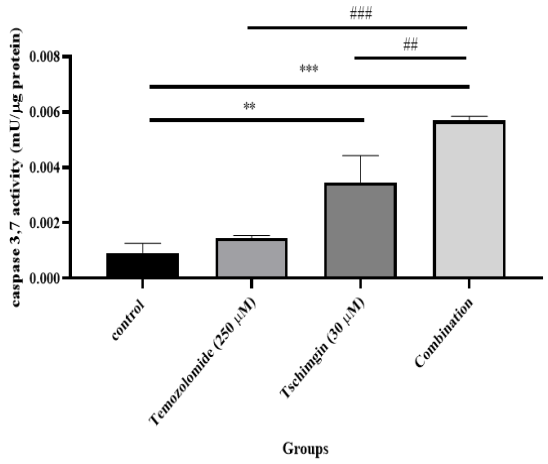


Figure 7. The effects of tschimgin (30 µM), TMZ (250 µM), or their combination on caspase 3/7 activity. Results are reported as mean±SD. **p<0.01 and ***p<0.001 compared to the control. ##p<0.01, ###p<0.001 compared to combination therapy (n=3).

Discussion

The present study demonstrated that tschimgin and TMZ inhibited T98G cell viability in a dose- and time-dependent manner, with tschimgin displaying greater potency than TMZ. Tschimgin exerted minimal cytotoxicity on normal HFF fibroblasts at concentrations effective against T98G cells, indicating selective toxicity. Combination treatment with tschimgin and TMZ exhibited synergistic effects, significantly reducing cell viability and enhancing morphological signs of cytotoxicity. Cell cycle analysis revealed that the combination induced substantial sub-G1 and G2/M phase arrest, implicating both apoptotic and mitotic disruption mechanisms. Furthermore, the combinatorial regimen markedly elevated intracellular ROS levels and increased *Bax* expression, while downregulating *Bcl-2*, thereby enhancing the pro-apoptotic *Bax/Bcl-2* ratio. These findings suggest that tschimgin not only possesses anticancer activity but also sensitizes GB cells to TMZ by inducing oxidative stress and activating the apoptotic pathway.

Compared to other well-characterized benzophenanthridine alkaloids, tschimgin exhibits distinctive quantitative and mechanistic features. For example, sanguinarine strongly inhibits proliferation in various cancer cell lines, showing potent cytotoxicity with IC_{50} values in the micromolar range (approximately 0.2 µM). It induces apoptosis by promoting excessive ROS generation, disrupting mitochondrial membrane potential, releasing cytochrome c, and activating caspase-9 and caspase-3 (Choi et al. 2008; Vavreckova et al. 1996). Chelerythrine shows cytotoxicity at low micromolar concentrations (≈5–20 µM), elevates intracellular ROS, and in some contexts induces necroptosis rather than classical apoptosis (Liu et al. 2023). By contrast, tschimgin effectively reduced the viability of T98G glioblastoma cells at micromolar concentrations while preserving normal fibroblasts, indicating superior selectivity. Moreover, in combination with TMZ, tschimgin synergistically suppressed cell viability, significantly increased ROS levels, upregulated *Bax*, and downregulated *Bcl-2*, thereby shifting the *Bax/Bcl-2* ratio toward apoptosis. In contrast to chelerythrine, which can induce necroptotic cell death depending on the cell type (Matkar, Wrischnik, and Hellmann-Blumberg 2008; Chen et al. 2022), tschimgin + TMZ consistently activated classical apoptotic signaling, as evidenced by sub-G1 accumulation and modulation of

Bax/Bcl-2. These features position tschimgin collectively as a multi-targeted agent with both selective cytotoxicity and chemosensitizing potential, quantitatively and mechanistically distinct from other benzophenanthridines.

In a study related to our investigation but lacking potentiation, novel phenanthrolindolizidine alkaloid (PA) derivatives, YS306 and YS206, were synthesized and evaluated for antitumor activity *in vitro*. These compounds effectively suppressed the proliferation of HepG2 liver cancer cells and HCT116 and HT29 colon cancer cells at micromolar concentrations. They induced G2/M cell cycle arrest and inhibited cellular migration, without inducing apoptosis. These findings suggest that structural modification of natural PAs may yield promising chemotherapeutic agents (Liu et al. 2017). Another investigation assessed the cytotoxic and anticancer properties of 13 Papaver alkaloids against HeLa cervical cancer cells and normal Vero cells using the MTT assay. Cells were treated with alkaloids at concentrations ranging from 1–300 µg/ml for 48 hr, and IC50 values were calculated. Berberine and macranthine demonstrated the highest anticancer efficacy, with IC50 values of 12.08 µg/mL and 24.16 µg/mL, respectively, against HeLa cells. Notably, macrantin exhibited a selectivity index (SI) of 12.42, and berberine an SI of 5.89, indicating selective toxicity toward cancer cells. In contrast, salutaridine showed no cytotoxicity (Demirgan et al. 2016).

Consistent with our findings, Beljanski and Beljanski examined the synergistic effects of alkaloid compounds combined with chemotherapeutic agents. It was demonstrated that hemanalstonine, serpentine, and sempervirine, at defined doses, significantly suppressed tumor progression in BALB/C mice bearing YC8 lymphoma and Swiss mice with Ehrlich carcinoma. These alkaloids, when combined with chemotherapeutics such as 5-fluorouracil (5-FU), daunorubicin, lomustine (CCNU), or cyclophosphamide, inhibited the growth of ascites and solid tumors without exacerbating toxicity (Beljanski and Beljanski 1986). Also, a recent study has further highlighted the therapeutic relevance of plant-derived alkaloids by investigating the antiangiogenic potential of berbamine in GB models. Berbamine effectively inhibited vascular endothelial growth factor (VEGF)- and brain-derived neurotrophic factor **(**BDNF)-induced angiogenic processes—including proliferation, adhesion, invasion, tube formation, and ROS production—in HUVECs, without affecting cell viability. These effects were associated with suppression of VEGF/VEGFR2/CaMKIIγ and BDNF/TrkB/CaMKIIγ signaling pathways. Berbamine also reduced hypoxia-inducible factor 1-alpha (HIF-1α) and VEGF expression in U87MG cells and inhibited neovascularization and tumor growth in a chick embryo CAM model. These results support the role of berbamine as a novel antiangiogenic agent for GB therapy. While that study emphasizes inhibition of angiogenesis, our findings focus on enhancing chemosensitivity and promoting apoptosis, thereby illustrating the multi-targeted potential of natural compounds in GB treatment (Kim et al. 2021).

Despite the encouraging outcomes, several limitations must be addressed before clinical application. Comprehensive pharmacokinetic and toxicological analyses of the tschimgin/TMZ combination are essential. These include characterizing absorption, distribution, metabolism, excretion, and evaluating systemic toxicity (Ortiz et al. 2021). Given the intrinsic heterogeneity of GB, it is also imperative to identify predictive biomarkers that enable personalized therapeutic strategies. Such biomarkers would facilitate patient stratification and optimize treatment selection based on tumor-specific molecular profiles (Rončević et al. 2025).

Future investigations should prioritize extensive *in vivo* studies to corroborate the

in vitro findings. Evaluating the tschimgin/TMZ combination in animal models that recapitulate the GB microenvironment will yield more clinically relevant data regarding efficacy and safety. These studies should assess tumor progression, survival outcomes, and off-target toxicities. Ultimately, rigorously designed clinical trials are warranted to determine the therapeutic potential and safety profile of this combination in treatment-resistant GB. Trials should focus on tumor response, overall survival, and adverse event monitoring.

Future studies should also incorporate mechanistic analyses to elucidate the molecular pathways underlying the tschimgine/TMZ synergism. In particular, examining the involvement of key regulatory nodes such as p53-mediated apoptosis, NF-κB–driven inflammatory and survival signaling, and MAPK pathway modulation could clarify how this combination influences GB cell fate (Sahebi et al. 2022). Such mechanistic insight would not only validate observed therapeutic effects but also help identify predictive biomarkers and potential targets for further therapeutic optimization.

In conclusion, the present study demonstrated that tschimgin effectively inhibits the viability of T98G glioblastoma cells in a dose- and time-dependent manner, with lower cytotoxicity toward normal HFF fibroblasts. When combined with temozolomide, tschimgin exhibited synergistic anticancer effects, significantly enhancing cytotoxicity and reducing cell viability. This combination induced marked sub-G1 and G2/M cell cycle arrest, elevated intracellular ROS production, and increased pro-apoptotic *Bax* mRNA expression while reducing *Bcl-2* levels. Collectively, these findings suggest that tschimgin may potentiate the therapeutic efficacy of TMZ in resistant GB cells and support its further evaluation as part of a combinatorial treatment strategy. Nonetheless, further *in vivo* and clinical investigations are essential to assess the therapeutic efficacy and safety profile of this combination. In addition, while our cytotoxicity screening in HFF fibroblasts provides a foundational understanding of the test compounds' general cellular toxicity, using a standard model referenced in the field, a recognized limitation is that fibroblasts are not representative of neural tissue. To directly assess neurotoxicity and improve translational relevance, future work should include evaluations in normal glial or astrocyte cell lines, thereby strengthening the translational relevance of our neurotoxicity findings. By thoroughly investigating the molecular mechanisms underlying the interactions between tschimgin and TMZ in resistant GB cells, this research aims to provide valuable insights into innovative targeted-therapy approaches for this challenging and aggressive malignancy. The ultimate goal is to identify strategies that can improve survival and quality of life for patients battling this devastating disease. Such studies are critical to advancing personalized treatment options and improving clinical outcomes for GB patients.

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Conflicts of interest

The authors declare no conflict of interest.

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Ethical Considerations

Evaluated by the Research Ethics Committees of School of Medicine, Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1403.361)

Code of Ethics

IR.MUMS.MEDICAL.REC.1403.361

Availability of data and materials

Data can be supplied upon reasonable request.

Authors' Contributions

FA-M, MMV, MGH: Conceptualization, Methodology, Formal analysis, MT: Methodology, PH, SGH, FD: Write Original Draft, Formal analysis, Validation. MJ-N: Conceptualization, Validation, Writing - Review & Editing, Supervision, Funding acquisition

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