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ORIGINAL ARTICLE

Overcoming Intrinsic and Acquired Temozolomide Resistance in Glioblastoma: Fisetin as a Potential Strategy to Enhance Sensitivity via ZEB1 Modulation

Sena FERAH[1], Mine ÇAMLIBEL[1], Melis ERÇELİK[1], Çağla TEKİN[1], Gülçin TEZCAN[2], Melisa GÜRBÜZ[1], Ahmet BEKAR[3], Hasan KOCAELİ[3], Berrin TUNCA[1]*

[1]Bursa Uludağ University Faculty of Medicine, Department of Medical Biology, Bursa, Türkiye
[2]Bursa Uludağ University Faculty of Dentistry, Department of Fundamental Sciences, Bursa, Türkiye
[3]Bursa Uludağ University Faculty of Medicine, Department of Neurosurgery, Bursa, Türkiye

ABSTRACT

Objectives: Glioblastoma (GB) is the most aggressive type of brain tumor in adults, and the chemical agent temozolomide (TMZ) is widely used for its treatment. However, TMZ resistance can lead to therapeutic failure. The aim of this study was to investigate the effect of the bioflavonoid fisetin on GB cell growth and on overcoming TMZ resistance in TMZ-sensitive, inherited-resistant, and acquired-resistant GB cells the effect of fisetin on TMZ efficacy evaluin primary GB cells.

Materials and Methods: GB cell lines (T98G; intrinsic TMZ-resistant, A172; TMZ-sensitive, A172-R; acquired TMZ-resistant) and primary GB cells derived from patient samples were treated with effective doses of TMZ (ranging from 900 to 1000 μM), fisetin (ranging from 13.78 to 16.40 μM), or a combination of both. TMZ resistance was acquired in A172 cells through stepwise increases in TMZ concentration. Real-time cell proliferation was measured using the xCELLigence system. The migratory capacity of the cells was evaluated using a wound-healing assay. The RNA expression of the epithelial-to-mesenchymal transition (EMT)-inducing transcription factor E-box-binding homeobox 1 (ZEB1) was assessed by quantitative polymerase chain reaction. Cell assays were analyzed by analysis of variance, and ZEB1 expression was analyzed by t-test.

Results: Fisetin substantially enhanced the effect of TMZ in all the cell lines included in the present study, as evidenced by significant decreases in cell proliferation and wound-healing, and in ZEB1 expression ($p<0.0001$). In addition, TMZ+fisetin reduced ZEB1 expression in primary GB tumors but not in butterfly GB cells.

Conclusion: Fisetin alone was effective against GB; importantly, the TMZ+fisetin combination demonstrated greater efficacy than TMZ alone by enhancing sensitivity to TMZ through downregulation of ZEB1 in various resistant models, including patient-derived samples. Since ZEB1 is associated with EMT and drug resistance, fisetin may be a promising anticancer candidate to improve chemotherapeutic efficacy in resistant GB and to shed light on personalized treatments, pending further preclinical research.

Keywords: Glioblastoma, temozolomide resistance, fisetin, epithelial-to-mesenchymal transition, ZEB1

***Correspondence:** btunca@uludag.edu.tr, **ORCID-ID:** orcid.org/0000-0002-1619-6680
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INTRODUCTION

Glioblastoma (GB) is the most prevalent and aggressive brain tumor,[1] and it exhibits an alarmingly high 5-year mortality rate (up to 95%).[2] The World Health Organization statistics from the year 2020 indicate a significant burden of brain tumors, accounting for approximately 1.6% of all cancer cases and 2.5% of cancer-related deaths; incidence rates are rising, reaching up to 10 per 100,000 population.[2,3] Standard treatment for GB consists of surgical resection, radiotherapy, and chemotherapy. Despite this treatment protocol, patients have a median survival of only 12.6 months.[4]

Maximum surgical resection is an effective treatment method that prolongs the survival of patients with this disease. However, due to the invasive nature of GB, maximum resection cannot be performed in many cases, which increases the risk of recurrence.[5] Temozolomide (TMZ), an alkylating agent, is commonly used to treat GB.[6] TMZ is indispensable in the treatment of GB because it effectively crosses the blood-brain barrier (BBB), the main obstacle to GB therapy.[6] However, because these tumors are highly heterogeneous, resistance to TMZ therapy is common among GB patients [7] TMZ resistance can be mainly due to O6-methylguanine-DNA methyltransferase (MGMT) methylation[8] and alterations in other signaling pathways involved in DNA repair systems[9] and stem-like cell growth.[10] TMZ resistance can be inherited or acquired after a period of treatment.[11] Although the intrinsic factors and mechanisms affected during tumor acquisition differ, tumor aggressiveness, recurrence, and metastasis, which can result from enhanced epithelial-to-mesenchymal transition (EMT), are observed in both cases.[11] Zinc finger E-box-binding homeobox 1 (ZEB1) is a transcription factor that plays a role in the induction of EMT.[12] In addition, ZEB1 has been shown to contribute to chemotherapy resistance by promoting the acquisition of cancer stem cell properties.[13] Therefore, targeting ZEB1 may be an effective strategy to interrupt EMT and maintain stem-like cells.

To date, promising findings have been obtained from including natural compounds in research on new therapies for many cancer types, considering their antioxidant and cytotoxic effects.[14] In addition, the combined use of some natural compounds with certain chemotherapeutic agents has been shown to increase the effectiveness of these agents and reduce their side effects.[15,16] Fisetin, a flavonoid found mainly in strawberries, apples, onions, wine, and tea, is also commercially available and exhibits various bioactivities, including antioxidant, anti-inflammatory, and anticancer effects.[17] Studies have shown that fisetin can modulate key pathways implicated in GB and in drug resistance, including MSH2, ZEB1, P53, PI3K, and Bax/Bcl2, across different cancers.[18,19] Particularly, fisetin has shown inhibitory effects on ZEB1 and ZEB1-mediated invasion in melanoma, thereby enhancing sorafenib efficacy by modulating EMT.[20] Although fisetin has been investigated in other cancer models, its effects on GB and TMZ remain underexplored. While previous studies have reported the senolytic and pro-apoptotic actions of fisetin[21] and its influence on GB senescence in GB cell lines,[22] its specific role in modulating cellular aggressiveness and resistance, especially when combined with TMZ, remains unelucidated. In this study, we aimed to determine the effect of fisetin on GB cancer cell aggressiveness, TMZ response, and ZEB1 expression *in vitro*. To address varying TMZ sensitivities observed in GB, we included intrinsically TMZ-resistant T98G and TMZ-sensitive A172 cells in this study. Notably, this study is the first to investigate the effects of fisetin on cell lines that acquired resistance following long-term TMZ exposure. This study aims to investigate the therapeutic potential of fisetin in overcoming existing and acquired TMZ resistance in GB. Specifically, we evaluated the effects of fisetin, both alone and in combination with TMZ, on cell proliferation, migratory capacity, and the expression of the EMT-related transcription factor ZEB1 across various models, including TMZ-sensitive, intrinsic-resistant, and acquired-resistant GB cell lines, as well as patient-derived primary tumor samples with different pathological features. We hypothesize that fisetin enhances TMZ efficacy by modulating the EMT process, thereby providing a potential strategy to circumvent drug resistance in GB treatment.

MATERIALS AND METHODS

The experimental procedure was established to evaluate the effects of TMZ and fisetin treatments on GB cells with varied resistance profiles. TMZ-sensitive (A172) and TMZ-resistant (T98G) GB cell lines, a resistant subline (A172-R) generated by exposing A172 cells to increasing doses of TMZ for 18 months, a primary tumor culture obtained from tumor tissues removed from three GB patients at surgery, and a healthy fibroblast cell line (L929) were used to represent GB cells with varying resistance properties. Treatment of cells with various concentrations of fisetin and TMZ, assessment of cell viability using a real-time cell analyzer [xCELLigence real-time cell analyzer (RTCA)], evaluation of cell migration by a wound-healing assay, quantification of ZEB1 gene expression by real-time-quantitative polymerase chain reaction (qPCR), and statistical comparison of treatment-group differences using analysis of variance (ANOVA) and t-test were performed in this study.

Reagents

TMZ (C6H6N6O2) was obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA; Cat. no.: T2577). Fisetin (3,3′,4′,7-tetrahydroxyflavone) (≥96% purity) was obtained from Tokyo Chemical Industry Co. (TCI) (Cat no.: T0121; Tokyo, Japan). All reagents were dissolved in dimethyl sulfoxide (Sigma-Aldrich, MO, USA) and stored at -20 °C until use.

Cell lines and primary GB cells

The fibroblast-like GB cell lines, TMZ-resistant T98G[23] and TMZ-sensitive A172[24], were obtained from Dr. Tuğba Bağcı Önder, Koç University, İstanbul, Türkiye, and the healthy fibroblast line L929[25] was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). In addition, primary tumors from three GB patients who underwent surgery as part of their therapy were included in the study. Tumor samples

were collected during surgical procedures at the Neurosurgery Department of Bursa Uludağ University and were characterized by a pathologist. The collection of tumor samples was approved by the Uludağ University Faculty of Medicine Clinical Research Ethics Committee (approval number: 2023-3/43, dated: 14.02.2023). Written informed consent was obtained from all patients prior to their inclusion in the study. GB patients were included based on the following criteria: primary high-grade brain tumors (GB) that were surgically resected, non-metastatic, and treatment-naive (no prior chemotherapy/radiotherapy). Of all patients, those who did not meet the inclusion criteria were excluded. The primary tumor samples were cultured *in vitro* as described previously.[26] All cell lines and primary GB cells were maintained in Dulbecco's modified Eagle's medium-F12 (HyClone, UT, USA) supplemented with 10% fetal bovine serum (Gibco, MA, USA), 1% antibiotic/antimycotic solution (Capricorn, Ebsdorfergrund, Germany), 1 mM sodium pyruvate (Gibco, MA, USA), and 2 mM L-glutamine (Gibco, MA, USA) in a 5% CO_2 humidified incubator at 37 °C. All samples were stored in the cryotube at -152 °C.

Cell viability

An xCELLigence- RTCA (ACEA Biosciences, San Diego, CA, USA) was used to visualize cell proliferation. Briefly, 15×10^3 cells were seeded into an E-plate 16. After determining the log phase of the growth curve, TMZ-resistant T98G cells, TMZ-sensitive A172 cells, and noncancerous L929 fibroblasts were treated with seven concentrations of fisetin (5–200 µM) and a range of TMZ concentrations for 24–72 hours. The time-dependent half-maximal inhibitory concentration (IC_{50}) was considered the effective dose for both fisetin and TMZ. The experiments were repeated three times for each dose.

TMZ resistance acquisition

TMZ-sensitive A172 cells were exposed to seven increasing cycles of TMZ (100, 200, 350, 450, 550, 750, and 900 µmol/L) every 2-3 weeks for 18 months using a combination of methods previously described by Lee et al.[27] and St-Coeur et al.[28] After treatment with TMZ at the same concentration, the surviving cells were allowed to proliferate and were re-treated. The same cycle was repeated for 2-3 weeks, followed by a resting period in fresh medium without TMZ treatment. After the recovered cells reached 70% confluency, they were treated with increasing concentrations of TMZ. After treatment with increasing doses of TMZ for 18 months, the resulting cells were named A172-R. Cells that did not reach 70% confluence after 4 weeks were excluded from the experiment. The acquired TMZ insensitivity was assessed by measuring cell viability and wound-healing rate.

Migration assay

Cells were grown in a 6-well culture plate until they reached 70% confluency. A 10 µL pipette tip was used to vertically scratch the monolayers at three different locations, and the debris was removed. The wounded monolayers were treated with the IC_{50} concentrations of fisetin, TMZ, or TMZ+fisetin. The rate of gap closure along scratches reflected the migratory ability of the cells. The wound gap was visualized under an inverted microscope at 0, 6, 12, 18, and 24 hours and measured using NIH ImageJ software v1.52a (National Institutes of Health, Bethesda, MD, USA). Each experiment was performed with three technical replicates.

Reverse transcription PCR analysis

RNA extraction was performed following the protocol of the Zymo RNA Isolation Kit (Zymo Research; Irvine, CA, USA). The concentration and purity of the RNA samples were determined from the A260/A280 ultraviolet/visible absorbance ratio obtained with a Maestro Nano Micro-Volume spectrophotometer (Maestrogen Inc., Las Vegas, NV, USA). RNA samples with a ratio of ~1.8-2.0 were converted to complementary DNA (cDNA) using the ProtoScript® II First Strand cDNA Synthesis Kit (New England Bioscience; Ipswich, MA, USA). cDNA (200 ng) was used for real-time PCR. The expression of ZEB1 (F: AGTGTTACCAGGGAGGAGCAGTG, R: TTTCTTGCCCTTCCTTTCCTGTGTC; annealing: 55 °C)[29] was analyzed by qPCR using SYBR Green GoTaq® qPCR master mix (Madison, WI, USA). The RNA input was normalized to the housekeeping gene β-actin with primers (F: GACAGGATGCAGAAGGAGATTACT, R: TGATCCACATCTGCTGGAAGGT) and an annealing temperature of 60°C.[30] The threshold cycle (Ct) for RNA expression was determined using the StepOne real-time-qPCR System (Thermo Fisher, CA, USA). The $2-\Delta\Delta Ct$ method was used to calculate fold changes from Ct values.

Statistical analysis

In each sample, the untreated group served as the control. To evaluate the effects of fisetin and TMZ, each treatment group was analyzed in comparison to its respective control. The normality of the data was tested using the Shapiro-Wilk normality test in GraphPad Prism (v8.0). Since the data followed a normal distribution ($p>0.05$), they are presented as mean ± standard deviation (SD) and analyzed using parametric tests. One-way ANOVA and Tukey's test were used to analyze the effects of fisetin, TMZ, and TMZ+fisetin on cell viability. Two-way ANOVA was used to detect differences between sample groups in the wound healing analysis. An independent-samples t-test was used to assess differences in RNA expression levels. Statistical analyses were performed using SPSS version 20.0 (IBM SPSS Inc., Armonk, NY, USA), and the data were visualized using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). A *p*-value less than 0.05 was considered statistically significant at the 95% confidence level.

RESULTS

Fisetin decreased GB cell viability

The proliferation of T98G and A172 cells significantly decreased after incubation for more than 48 hours, regardless of fisetin treatment ($p<0.05$). This decline was attributed to the cells reaching maximum confluency within the E-plate wells. Therefore, data from incubation periods exceeding 48 hours were excluded from further analysis.

In T98G cells, 5 µM fisetin led to a 14.5±3.2% reduction after 24 hours and a 19.8±1.6% reduction after 48 hours. In contrast, treatment with 200 µM fisetin resulted in reductions of 75.3±0.6% and 79.1±2.2% in T98G cells after 24 and 48 hours, respectively (Figure 1a). The IC_{50} of fisetin for T98G cells was 16.40 µM at 24 hours and 12.26 µM at 48 hours ($p<0.001$) (Figure 1b).

In A172 cells, 5 µM fisetin reduced the proliferation rate by 26.3±1.0% and 23.6±1.9% at 24 and 48 hours, respectively (Figure 1c and d). A concentration of fisetin greater than 100 µM reversed the inhibitory effect on proliferation and reduced proliferative activity after 48 h. After 24 h of incubation with 75 µM fisetin, cell inhibition was 45.8±1.9%, whereas within 48 h it was 17.7±1.2%. In addition, 200 µM fisetin induced 40.0±5.0% cell inhibition at 24 hours, which decreased to 6.6±0.5% at 48 hours. The IC_{50} of fisetin was 13.78 µM at 24 hours and 10.34 µM at 48 hours in A172 cells ($p<0.001$) (Figure 1d). At concentrations between 50 µM and 75 µM—five times higher than the average IC_{50} observed in GB cell lines, fisetin did not reduce viability in the L929 fibroblast cell line and preserved 90.0±1.0% cell viability (Figure 1e and f). These findings demonstrated the safety of fisetin in noncancerous cells at the IC_{50} values determined for T98G and A172 cells. Therefore, T98G and A172 cells were treated with 16.40 µM and 13.78 µM fisetin, respectively, for 24 hours.

A172 cells gradually became resistant to the IC_{50} of TMZ

The IC_{50} of TMZ was determined to be 900 µM in A172 cells in our previous study.[25] Therefore, A172-R cells, resistant to 900 µM TMZ, exhibited morphological changes (Figure 2c) and were used in all analyses. To generate a 900 µM TMZ-resistant cell series, A172 cells were gradually exposed to increasing concentrations of TMZ (350, 450, 550, 750, and 900 µM). A decrease in TMZ sensitivity of A172-R cells (A172-350-R to A172-900-R) is shown in Figure 2a and 2b. Ultimately, 900 µM TMZ reduced the viability of the A172-900-R (A172-R) cells by 35%, whereas it reduced the parental A172 cells by 63% ($p<0.001$) (Figure 2a and b). Figure 2c shows the morphology of cells that became resistant to TMZ; these cells are characterized by increased aggressiveness and enhanced cell-cell interactions.

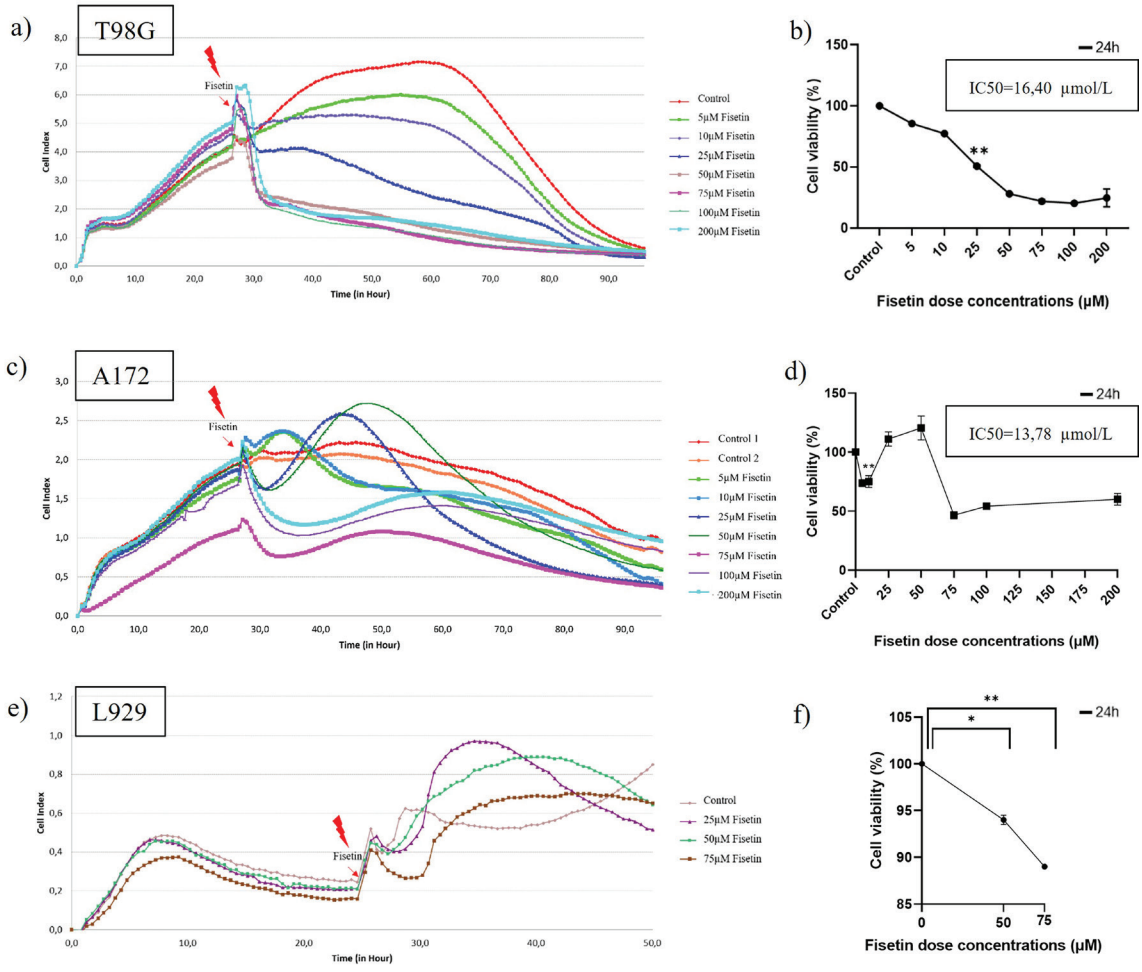

Figure 1. Effect of fisetin on GB cell proliferation. The effect of different doses of fisetin on real-time proliferation and its IC_{50} on cell viability in (a, b) T98G, (c, d) A172, and (e, f) L929 cells. *p* values were calculated using one-way ANOVA. The experiment was performed in three biological replicates. *$p<0.05$, **$p<0.0001$. Bars represent the mean ± standard deviation

ANOVA: Analysis of variance, IC: Inhibitory concentration

Fisetin additionally affects TMZ in GB cell lines

In our previous study, the IC_{50} values of TMZ were 1000 µM for T98G cells and 900 µM for A172 cells, respectively.[25] Accordingly, we treated T98G, A172, and A172-R cells with TMZ and with fisetin at their respective ICs_{50} to analyze the potential contribution of fisetin to TMZ therapy. Compared with TMZ alone, fisetin did not increase TMZ-mediated cell inhibition in T98G cells (Figure 3a and b). In contrast, combined treatment with TMZ + fisetin reinforced TMZ-mediated inhibition of A172 and A172-R cells (p<0.0001) (Figure 3c and f).

Fisetin cotreatment suppressed the migration ability of GB cell lines treated with TMZ via different resistance mechanisms.

The wound closure rate was 78.74% after 24 hours in untreated T98G cells. After TMZ treatment, the wound closure rate exceeded 72%. In addition, compared with no treatment, fisetin treatment decreased the wound area by up to 90.07%. In contrast, the inhibitory effect of TMZ + fisetin on migration was greater than that of TMZ alone (p<0.001) (Figure 4a).

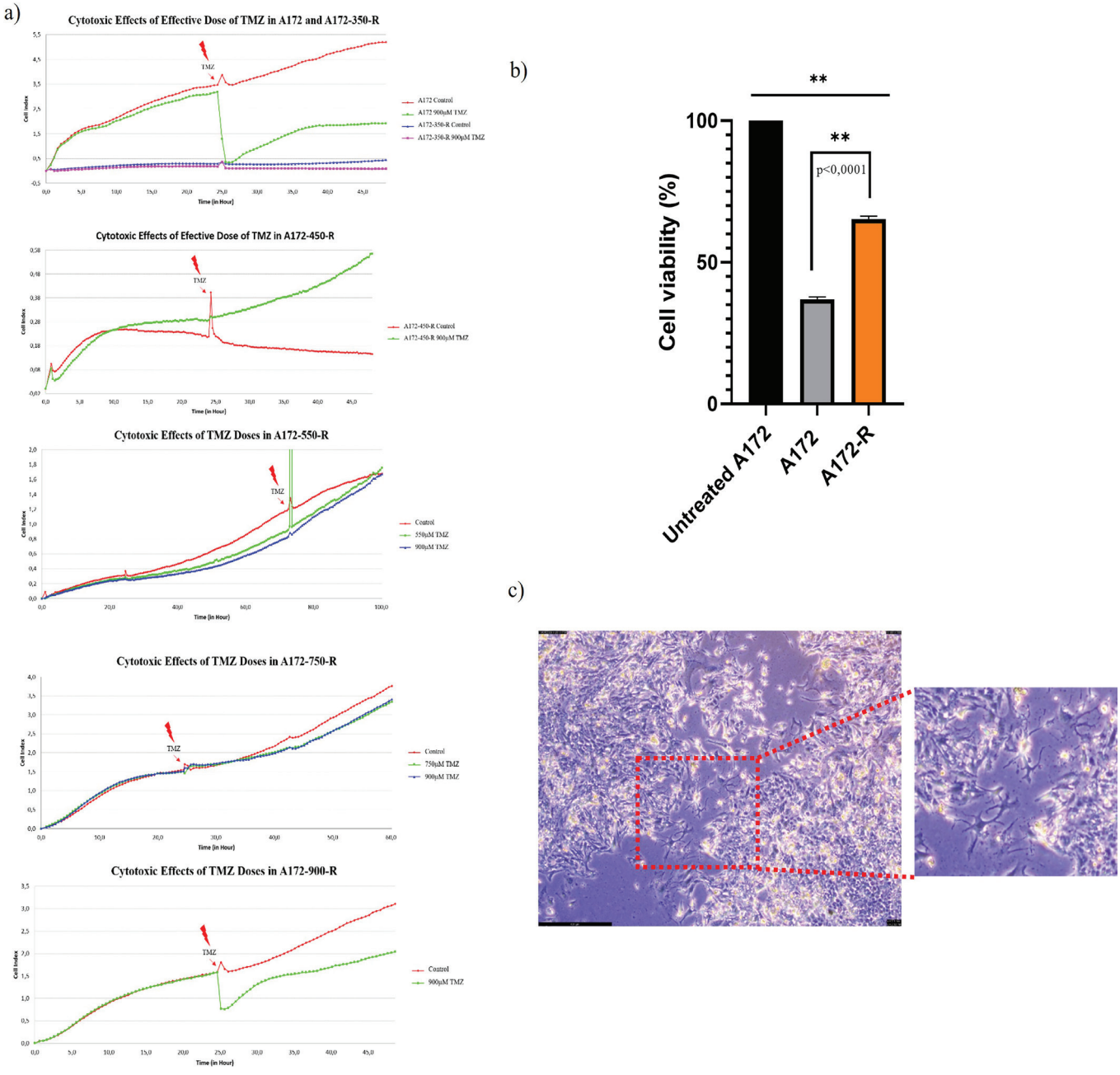

Figure 2. Effect of TMZ concentration on A172-R cells. (a) The representation of the gradually increasing TMZ resistance of A172-R cells during the acquisition of TMZ resistance. (b) The relative change in the viability of A172 and A172-R cells treated with 900 µmol/L TMZ. Each column represents the inhibitory effect of TMZ on cell viability compared with that of the corresponding untreated cell lines. Untreated A172 cells served as the negative control. (c) Microscopic representation of the morphology of acquired TMZ-resistant A172-R cells and cell-to-cell connections under a 4X objective (scale bar: 620 µm)

TMZ: Temozolomide

In untreated A172 cells, 73% of the wounded area was closed within 24 hours. Compared with no treatment, TMZ led to only a 32% decrease in wound-area recovery in A172 cells (p<0.001). Fisetin treatment of A172 cells resulted in a 27% recovery of the wound area (p<0.001). TMZ+fisetin treatment reduced wound recovery of A172 cells by 41.49% compared with cells treated with TMZ alone (p<0.001) (Figure 4b), indicating that fisetin enhances the efficacy of TMZ in A172 cells.

In untreated A172-R cells, wound-area closure was 69.49% after 24 h of treatment. After TMZ-only treatment, wound-area recovery in A172-R cells was 39.44%, which was lower than in the untreated group (Figure 4c and d). The attenuation of the wound-healing rate observed in TMZ-treated A172-R cells compared with A172 cells (p<0.001) was considered indicative of acquired TMZ resistance in A172-R cells (Figure 4d). The A172-R cells recovered 31.54% of the wounded area after fisetin-only treatment. In addition, after combined treatment with TMZ and fisetin in A172-R cells, 13.23% of the wound area healed compared with untreated cells (p<0.001). Notably, TMZ+fisetin had a significantly greater suppressive effect on TMZ alone (p<0.001) (Figure 4b-d).

Combination treatment with fisetin reduced TMZ-induced expression of ZEB1 in both intrinsic and acquired TMZ-resistant cells.

TMZ reduced ZEB1 gene expression in T98G cells (p<0.001) (Figure 5a) and A172 cells (p<0.0001) (Figure 5b). In contrast, TMZ induced ZEB1 expression in A172-R cells (p<0.0001) (Figure 5c). Fisetin reduced ZEB1 expression only in T98G cells (p<0.001), had no effect on ZEB1 in A172 cells, and increased ZEB1 expression in A172-R cells (p<0.001) compared with untreated cells. In T98G cells, TMZ-fisetin-induced ZEB1 expression was lower than TMZ-only-induced ZEB1 expression (p<0.001) (Figure 5a). However, compared with TMZ, TMZ+fisetin did not affect ZEB1 levels in A172 or A172-R cells (Figure 5b and c).

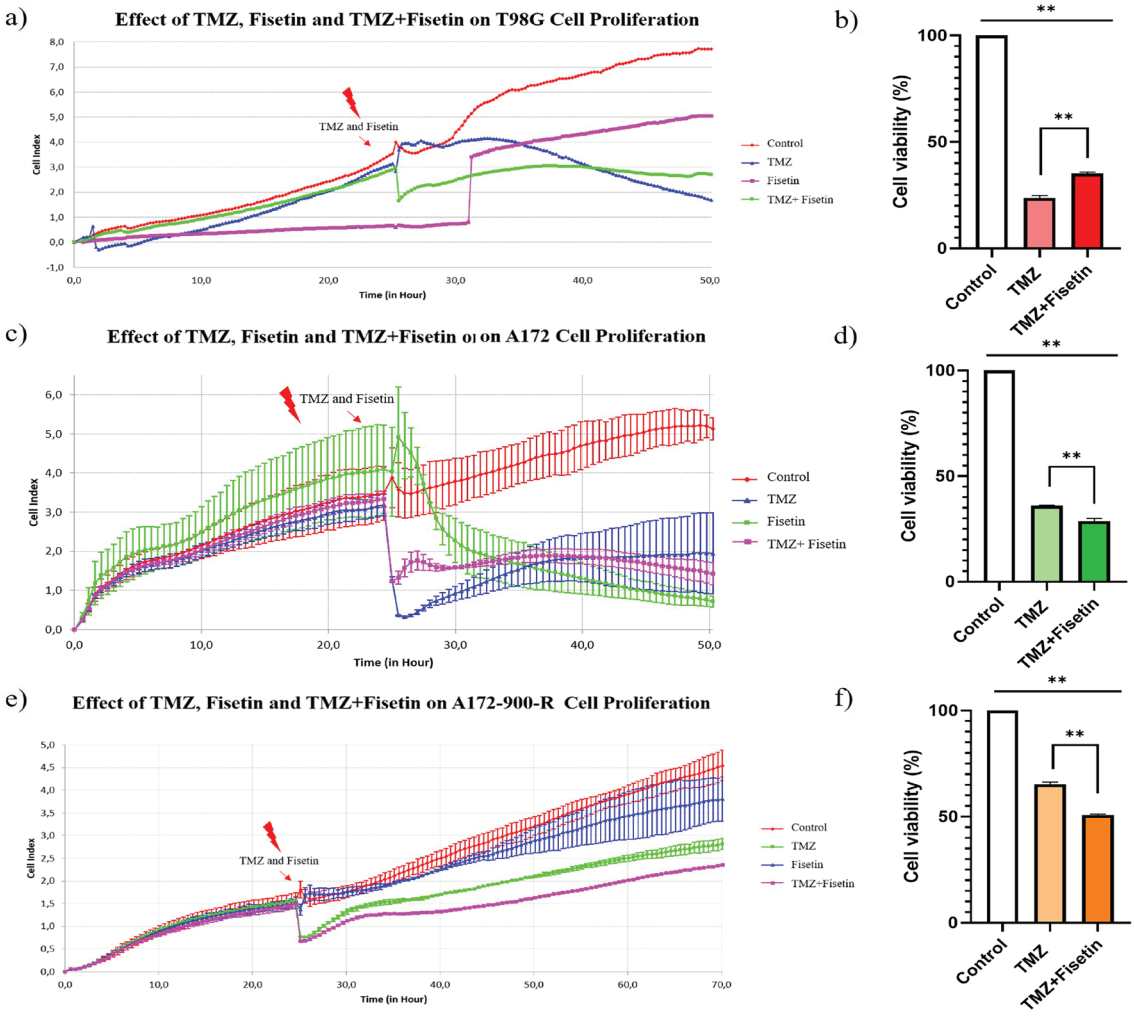

Figure 3. Effect of TMZ, fisetin, and TMZ+fisetin on the proliferation and viability of (a, b) T98G, (c, d) A172, and (e, f) A172-R cells. p values were calculated by one-way ANOVA. All experiments were performed in three biological replicates. *p<0.05, **p<0.0001. Bars represent the mean ± standard deviation

ANOVA: Analysis of variance

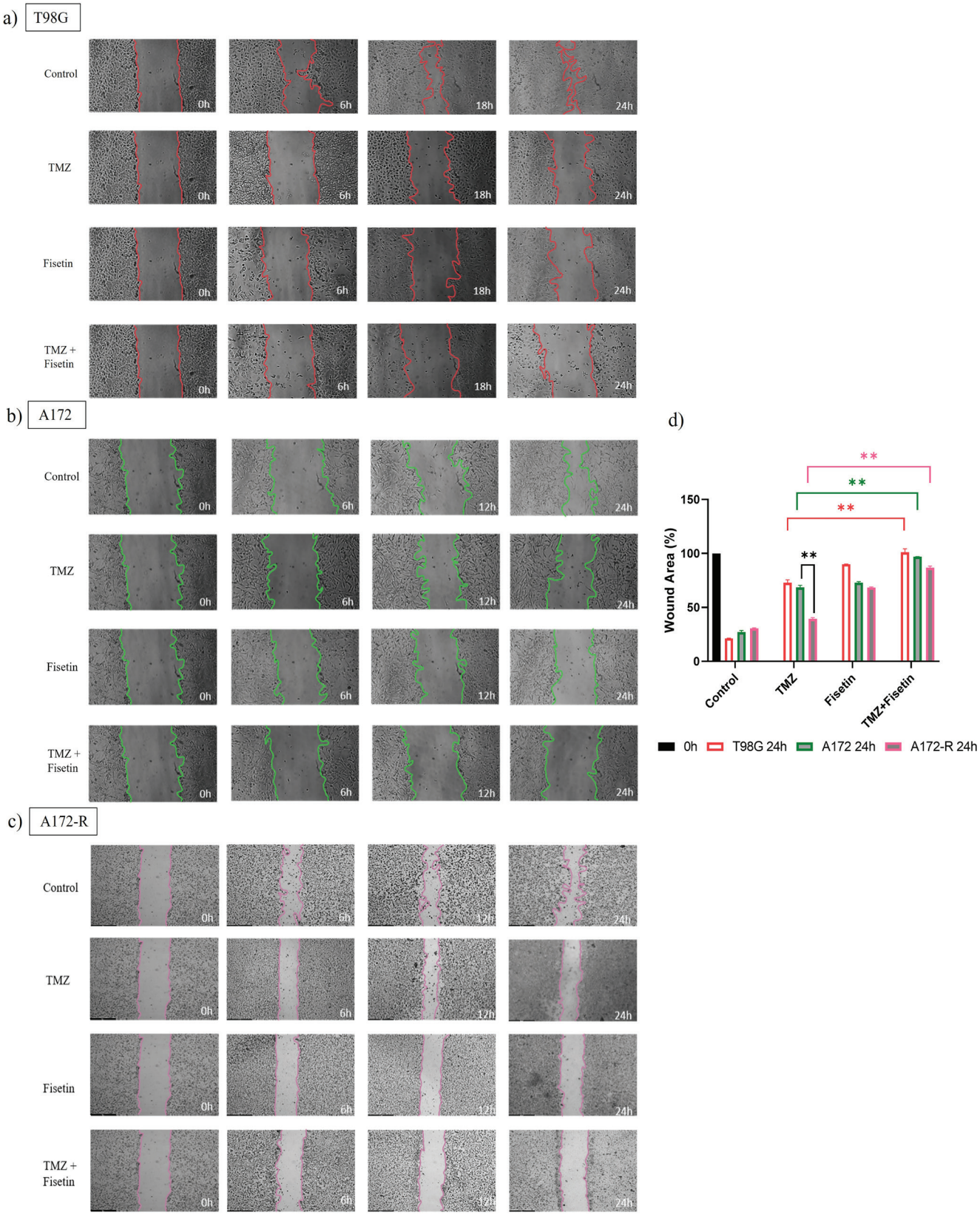

Figure 4. Representative images of the effect of TMZ, fisetin, and TMZ+fisetin on cell migration in the wound healing assay in the (a) T98G, (b) A172, and (c) A172-R cell lines (scale bar: 620 µm). (d) Comparative analysis of cells in terms of treatments. *P* values were calculated using two-way ANOVA. All experiments were performed in three biological replicates. ***p*<0.0001.

TMZ: Temozolomide, ANOVA: Analysis of variance

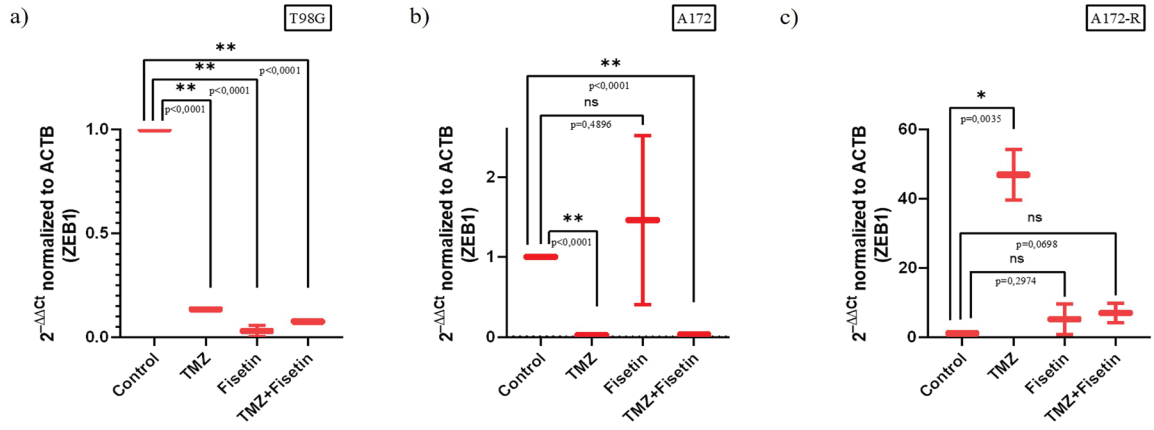

Figure 5. Effect of TMZ, fisetin, and TMZ+fisetin on the RNA expression of ZEB1 in the (a) T98G, (b) A172, and (c) A172-R cell lines. *P* values were calculated using independent-samples t-tests. All experiments were performed in three biological replicates. *$p<0.05$, **$p<0.0001$

TMZ: Temozolomide, ANOVA: Analysis of variance

Breaking of TMZ resistance by fisetin was confirmed in cancer cells collected from primary GB tumors

Three cell lineages derived from primary tumors of GB patients were included in the present study. Patient 1 had a tumor that was intracranially located in the left frontal lobe. Patient 2 was diagnosed with a butterfly GB and multiple primary tumors in the right frontal lobe. Patient 3 experienced postoperative recurrence of the cancer (Figure 6). According to the histopathological analysis, all GB tumors were positive for glial fibrillary acidic protein, Olig-2, NFP, NeuN, and H3K27me3, while they were negative for isocitrate dehydrogenase 1 (IDH-1) and ATRX. In addition, the Ki-67 index was at least 30% in all patients. After surgical tumor resection, all patients received identical postoperative concurrent radiotherapy (40 Gy/15 fx) and chemotherapy (TMZ) as part of the standard care regimen, and they were followed for their clinical status and survival until they died. The disease-free interval of patient 1 was 4 months, while it was 6.5 months and 3.5 months for patients 2 and 3, respectively. These findings indicate differences among the tumor samples included in the study in terms of molecular patterns and aggressiveness.

The effect of TMZ on ZEB1 expression varied across GB tumor-derived cell lineages (Figure 7). While TMZ reduced ZEB1 expression in patients 1 and 2, it was ineffective in patient 3. Conversely, TMZ+fisetin substantially reduced ZEB1 in patients 1 and 3, but was ineffective in patient 2 compared with TMZ alone (Figure 7).

DISCUSSION

GB is a stage IV brain tumor for which even the most aggressive treatments can be rendered ineffective by resistance, and patient survival remains limited to 12.6 months.[4,31] The response to the chemotherapeutic agent TMZ, long established as standard therapy, may vary according to GB characterization. Due to their distinct characteristics, GB patients may exhibit resistance to chemotherapy resulting from genetic and epigenetic changes and from treatment-induced acquired resistance and therefore do not respond well to treatment. Although the methylation status of MGMT was one of the first changes detected in patients and associated with TMZ response, it does not constitute a direct link.[32] The multifaceted mechanisms underlying treatment resistance and the pronounced intratumoral heterogeneity observed in GB suggest that relying on MGMT promoter methylation as a single predictive biomarker for TMZ response is insufficient for guiding clinical decision-making.[33] Supporting this notion, prior studies have demonstrated that, although p53 status may have a greater impact than MGMT on determining TMZ responsiveness, it nonetheless fails to serve as a standalone predictor, underscoring the urgent need to identify additional biomarkers associated with resistance mechanisms.[34] MGMT-methylated GB cells, such as T98G, may still express the MGMT protein and exhibit resistance to TMZ.[23] Conversely, other evidence indicates that resistance may primarily stem from the expression of alternative DNA repair enzymes, such as alkylpurine-DNA-N-glycosylase (APNG), rather than from the expression of MGMT itself. Moreover, certain TMZ-resistant GB cell lines (e.g., CCF-STTG1) do not express MGMT protein, highlighting the existence of MGMT-independent resistance mechanisms.[23] These findings underscore the complexity of TMZ resistance and the limitation of MGMT methylation status in fully capturing tumor behavior. Furthermore, while IDH mutation status plays a pivotal role in the molecular classification of GB—differentiating primary (IDH wild-type) from secondary (IDH mutant) forms—it is clear that MGMT methylation alone is insufficient to predict therapeutic response in this molecularly heterogeneous disease, characterized by genetic and epigenetic alterations.[35]

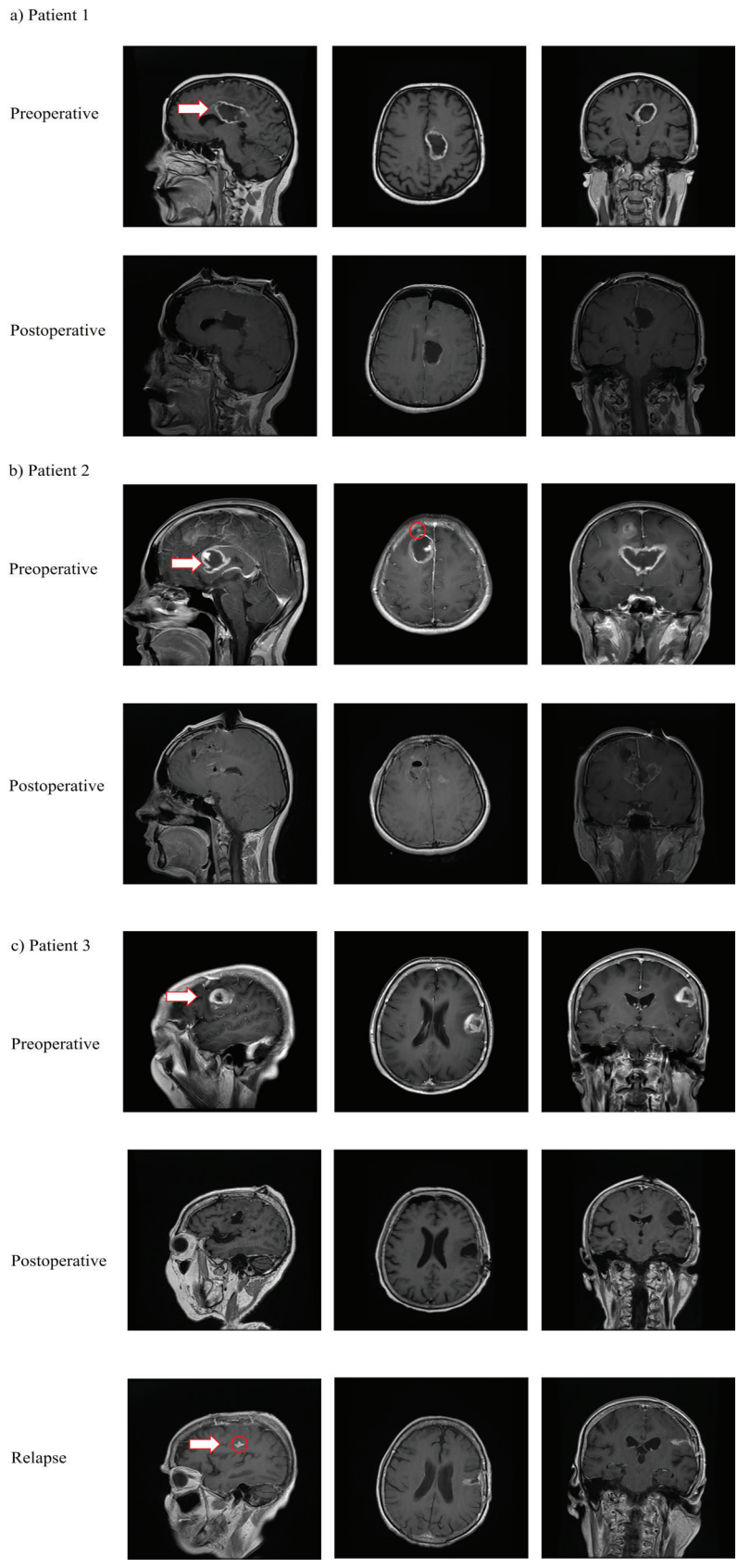

Figure 6. MRI images of GB patient tumors before and after surgery: (a) Patient 1; (b) Patient 2; (c) Patient 3
MRI: Magnetic resonance imaging, GB: Glioblastoma

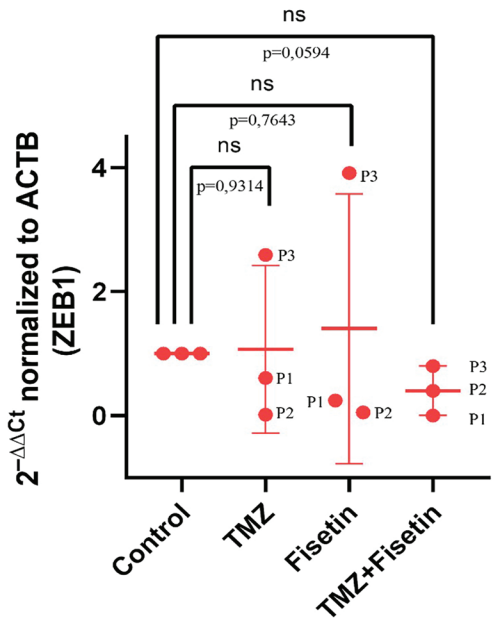

Figure 7. Graphical representation of the effect of the combination of TMZ, fisetin, and TMZ+fisetin on ZEB1 gene expression in the GB patient population. Each round represents a different GB patient (P1 = Patient 1; P2 = Patient 2; P3 = Patient 3)

TMZ: Temozolomide, GB: Glioblastoma, EMT: Epithelial-to-mesenchymal transition, ZEB1: Zinc finger E-box-binding homeobox 1, BBB: Blood-brain barrier, MGMT: O6-methylguanine-DNA methyltransferase, IC_{50}: The time-dependent half-maximal inhibitory concentration, qPCR: Real-time-quantitative polymerase chain reaction, cDNA: Complementary DNA, APNG: Alkylpurine-DNA-N-glycosylase

Indeed, there is an urgent need to evaluate new biomarkers as potential predictive and prognostic markers of patient response and resistance to treatment.[36] Accordingly, this study first investigated invasion and the regulation of the EMT transcription marker ZEB1 in non-resistant, intrinsically resistant, and acquired-resistant GB cell lines and in cancer cells obtained from primary GB tumors. Second, the effects of the natural flavonoid fisetin and its combination with TMZ on cancer cell aggressiveness were analyzed, and ZEB1 expression levels were investigated in these cells *in vitro*.

The selective cytotoxicity of fisetin is a critical finding in the context of GB therapy. Our observations indicate that fisetin possesses a favorable therapeutic window, as it effectively reduces the viability of both TMZ-sensitive and TMZ-resistant GB cells while exhibiting minimal impact on non-tumorigenic fibroblasts. This differential response suggests that fisetin may specifically target tumor-specific signaling pathways, sparing healthy cells even at concentrations well above those required for GB growth inhibition. While fisetin demonstrated a potent anti-proliferative effect independently, its combination with TMZ did not interfere with the known cytotoxic profile of the latter. These findings highlight fisetin's potential as a safe adjunctive agent that suppresses GB cell growth without inducing adverse systemic cytotoxicity, further supporting its role in enhancing the management of drug-resistant brain tumors. Several studies have investigated the safety profile of fisetin in both *in vitro* and *in vivo* models. However, it is important to note that a previous study reported cytotoxic effects in BEAS-2B cells—a normal human bronchial epithelial cell line—only at a significantly higher concentration (270 µmol/L) than its effective dose.[21] This underscores the importance of considering both cell-type-specific responses and concentration-dependent toxicity. *In vivo* studies suggest that fisetin exhibits generally low toxicity in animal models. Guo and Feng[37] reported IC_{50} values ranging from 200 mg/kg (intravenous) to 1700 mg/kg (oral) in rats, depending on the route of administration. Seal et al.[38] further demonstrated that fisetin complexed with metal ions had an IC_{50} of 500 mg/kg in mice, without inducing mortality. While no genotoxic potential was observed, some alterations in hematological and serum biochemical parameters were noted at doses of 400 mg/kg; these alterations may be attributable to enhanced activity resulting from organometallic complex formation. These findings warrant further investigation to clarify the mechanisms underlying such effects. Additionally, fisetin has been shown to possess neuroprotective properties, with minimal toxic symptoms or organ-specific toxicity reported in animal studies.[39] Despite its commercial availability as a dietary supplement, clinical trials remain ongoing to evaluate its therapeutic potential, particularly in cancer treatment (ClinicalTrials.gov.).[40]

Notably, fisetin has been reported to cross the BBB in mice.[41] This pharmacokinetic property enhances the clinical relevance of our findings, which demonstrate its antitumor effects *in vitro* against GB cells. Given its low molecular weight, natural origin, BBB permeability, relatively low toxicity, antitumor activity, and multi-targeted mechanism of action—including modulation of EMT and ZEB1—fisetin emerges as a promising candidate to augment existing therapies such as TMZ. In conclusion, the pharmacokinetic characteristics of fisetin and its demonstrated antitumor effects *in vitro* support further investigation into its potential clinical translation for GB therapy. Nevertheless, practical clinical implementation will require comprehensive pharmacokinetic studies, with a focus on optimizing dosage, improving solubility, and developing effective delivery systems to enhance bioavailability while minimizing off-target effects.

Chemoresistance can be inherited or acquired as a result of long-term exposure to chemotherapeutic drugs.[42] T98G cells are intrinsically resistant to TMZ because of the high expression of MGMT and, particularly, APNG, which are involved in DNA repair.[23] Although A172 cells exhibit a low level of MGMT expression, any of the mismatch repair complex, P-glycoprotein, or the presence of cancer stem cells could promote acquired resistance to TMZ after long-term exposure.[43] Differences in the molecular regulatory patterns of intrinsic and acquired TMZ resistance may lead to different outcomes of therapeutic approaches.[44] For this reason, we compared the effects of fisetin alone and in combination with TMZ on intrinsically TMZ-resistant T98G cells and on acquired TMZ-resistant A172-R cells. Previous studies have shown that a mutated p53 protein fails to reduce ZEB1 expression.[45] Therefore, suppressing

ZEB1 might contribute to the development of an effective therapeutic strategy.[46] T98G cells harbor mutated p53, while A172 cells express wild-type p53.[23] Therefore, TMZ could modestly suppress ZEB1 expression during proliferation in p53-mutant T98G cells. In contrast, TMZ significantly suppressed ZEB1 expression in A172 cells, which express wild-type p53 ($p<0.0001$). Although A172-R cells were more resistant to TMZ than the parental A172 cells, they did not have an inherited p53 mutation, unlike the parental cells. TMZ led to excessive ZEB1 expression in A172-R cells, similar to T98G cells with inherited p53 mutations. Understanding the regulatory mechanism of ZEB1 in GB cells with acquired TMZ resistance requires further investigation. However, fisetin reduced acquired resistance when used in combination with TMZ in both T98G and A172-R cells, regardless of p53 mutation status. Our findings in primary GB cells from patients 1 and 3 confirmed this phenomenon. TMZ increased ZEB1 expression to varying degrees in primary tumor cells from GB patients who developed TMZ resistance across different histopathological patterns. Notably, the tumor of patient 2, a butterfly GB, was not responsive to fisetin. The butterfly GB is known to infiltrate the corpus callosum unilaterally, and it has been associated with more aggressive migratory behavior than other GB subtypes, which have a propensity to invade both hemispheres.[47,48] The lack of ZEB1 suppression by TMZ+fisetin in butterfly GB cells, in contrast to primary GB tumors, may be attributed to intrinsic mesenchymal transition and invasive characteristics of this subtype, which may contribute to resistance to ZEB1 downregulation by the drug combination. Although these patients typically have poor prognoses, the molecular basis of these features remains poorly defined. Given the poorer survival of butterfly GB, these outcomes may be attributable to higher basal ZEB1 expression, consistent with the association between high ZEB1 levels and poor prognosis in GB. Thus, butterfly GB appeared to respond more favorably to aggressive therapeutic approaches.[47] Additionally, the distinct tumor microenvironment and cellular heterogeneity prevalent in butterfly GB may influence the differential response to the therapeutic intervention and highlight the importance of personalized therapy. Therefore, because we were able to enroll only a single butterfly GB primary tumor in the study, our understanding of the basis for fisetin's failure in this tumor is insufficient. However, based on current findings from cell lines and primary GB cells, fisetin may be beneficial for non-butterfly GB tumors.

A previous study reported that fisetin has the potential to overcome cisplatin resistance in lung adenocarcinoma cells.[49] Researchers have shown that this effect of fisetin could be achieved by inducing apoptotic caspase cascades.[49] Our study is the first to demonstrate that fisetin disrupts chemoresistance in both inherited and acquired TMZ-resistant GB cells. One of the key mechanisms that promotes TMZ resistance in GB cells is EMT.[50] The ability of fisetin to simultaneously modulate multiple pathways and exert antitumor effects across various GB cell lines with distinct resistance mechanisms represents a significant therapeutic advantage. Given that ZEB1 is a known target of fisetin and was suppressed by our combined TMZ and fisetin treatment, it is plausible that ZEB1 contributes to TMZ resistance through additional regulatory pathways. Notably, ZEB1 inhibition has been reported to reduce expression of the stemness-related marker SOX2, which has also been implicated in TMZ resistance,[10] particularly in prostate cancer models.[51] One of the upstream signaling pathways influencing ZEB1—and thus TMZ resistance—is the PI3K/Akt pathway.[52] Fisetin has been shown to downregulate the PI3K/Akt pathway in laryngeal cancer[19] and to suppress EMT via modulation of PI3K/Akt signaling in triple-negative breast cancer.[53] In addition, Fisetin was shown to induce p53 protein expression in the human renal cancer cell line Caki.[54] p53 was shown to inhibit the EMT process by reducing expression of the transcription factor ZEB1.[45] In line with this, the decrease in the EMT effect by fisetin, when it was used in combination with sorafenib in BRAF-mutated melanoma cells, was linked to its reduction of ZEB1 expression.[20] Similarly, our findings demonstrated that fisetin reduced ZEB1 expression in both TMZ-resistant T98G and A172-R cell lines. Moreover, fisetin in combination with TMZ decreased TMZ-mediated ZEB1 expression in both non-resistant A172 cells and p53-mutant intrinsically resistant models, suggesting that it may circumvent resistance pathways associated with both p53 dysfunction and ZEB1 overexpression. Interestingly, in acquired TMZ-resistant A172-R cells, treatment with TMZ alone paradoxically increased ZEB1 expression. However, the combination of TMZ and fisetin reversed this effect, leading to a reduction in both ZEB1 expression and resistance. This suppression of ZEB1 by fisetin is likely tied to its ability to inhibit EMT, a process strongly associated with therapy resistance and tumor progression. Therefore, the capacity of fisetin to modulate ZEB1 and EMT pathways may underlie its effectiveness against intrinsic and acquired TMZ resistance in GB.

Furthermore, the decrease in the wound-healing rate of T98G, A172, and A172-R cells confirmed that fisetin inhibits the migratory features of these cells by interrupting the EMT process through suppression of ZEB1. Moreover, fisetin enhanced the inhibitory effect of TMZ on metastasis, which could be explained by the considerable reduction in ZEB1 expression following combined TMZ and fisetin treatment. These findings may provide insight into the effects of fisetin, an anti-migratory agent, on EMT in TMZ-resistant and non-resistant GB cells. Further studies will clarify the exact underlying mechanism.

The efforts to augment the effectiveness of Food and Drug Administration-approved drugs are of paramount importance for GB patients, who continue to face poor survival outcomes. Our current findings suggest that fisetin has the potential to enhance the efficacy of TMZ in GB cells exhibiting both inherent and acquired resistance. New insights have been gained from both established cell-line models and primary patient samples regarding how flavonoids like fisetin—currently under clinical evaluation—can impact adjunctive chemotherapeutic strategies. Specifically, the observed downregulation of ZEB1 and the subsequent inhibition of the EMT process suggest that

fisetin could reduce TMZ resistance by targeting the crosstalk between these molecular pathways. Furthermore, examining dysregulated biomarkers in resistant versus non-resistant lineages is crucial for guiding personalized therapeutic approaches. By considering a patient's specific predisposition to drug resistance, such adjunctive treatments could aid in the selection of more effective, tailored clinical interventions. These results support the clinical relevance of fisetin as a promising candidate for drug development, particularly in the context of personalized therapy.

Study Limitations

However, several limitations must be acknowledged. The study was limited by the small number of patient-derived samples analyzed, which may not fully capture the heterogeneity of GB. Moreover, the scope of resistance mechanisms investigated was relatively narrow. In particular, further research is needed to elucidate the role of fisetin in modulating chemotherapy-related side effects and explore the involvement of ZEB1-associated pathways, such as DNA repair processes, in TMZ resistance. These aspects are critical for a more comprehensive understanding of the therapeutic potential of fisetin and its mechanisms of action.

CONCLUSION

In summary, the dietary flavonoid fisetin inhibits GB cell proliferation and migration, potentially by interrupting EMT by suppressing ZEB1 expression. In addition, fisetin increased the response of GB cells to TMZ regardless of whether chemoresistance was inherited or acquired. Therefore, our findings suggest that fisetin could be a candidate for drug development to support the therapeutic efficacy of TMZ. Understanding the mechanism by which fisetin targets ZEB1 and its biosafety in clinical settings requires further investigation.

Ethics

Ethics Committee Approval: The collection of tumor samples was approved by the Uludağ University Faculty of Medicine Clinical Research Ethics Committee (approval number: 2023-3/43, dated: 14.02.2023).

Informed Consent: Written informed consent was obtained from all patients prior to their inclusion in the study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: A.B., H.K., Concept: S.F., B.T., Design: S.F., M.E., Ç.T., Data Collection or Processing: S.F., M.Ç., M.G., Analysis or Interpretation: S.F., M.Ç., G.T., B.T., Literature Search: S.F., Writing: S.F., G.T., B.T.

Conflict of Interest: The authors declare no conflicts of interest.

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