Sphingosine-1-phosphate analogue FTY720 exhibits a potent anti-proliferative effect on glioblastoma cells

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Abstract. Sphingosine-1-phosphate (S1P) plays a key role in cell survival, growth, migration, and in angiogenesis. In glioma, it triggers the activity of the S1P-receptor 1 and of the sphingosine kinase 1; thus influencing the survival rate of patients. The aim of the present study was to investigate the anti-proliferative effect of the S1P analogue FTY720 (fingolimod) in glioblastoma (GBM) cells. A172, G28, and U87 cells were incubated with micromolar concentrations of FTY720 or temozolomide (TMZ) for 24 to 72 h. Proliferation and half maximal inhibitory concentration (IC_{50}) were determined by using the xCELLigence system. FACS analysis was performed to check the cell cycle distribution of the cells after a 72-h incubation with FTY720. This was then compared to TMZ-incubated and to untreated cells. Gene expression was detected by RT-qPCR in A172, G28, U87 and three primary GBM-derived cell lines. FTY720 was able to reduce the number of viable cells. The IC_{50} value was 4.6 μM in A172 cells, 17.3 μM in G28 cells, and 25.2 μM in U87 cells. FTY720 caused a significant arrest of the cell cycle in all cells and stabilized or over-expressed the level of *AKT1*, *MAPK1*, *PKCE*, *RAC1*, and *ROCK1* transcripts. The *TP53* transcript level remained stable or was downregulated after treatment with FTY720. FTY720 may be a promising target drug for the treatment of GBM, as it has a strong anti-proliferative effect on GBM cells.

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Abbreviations: EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; FTY720, fingolimod; GBM, glioblastoma; MTT, 3-(4,5-diemethytiazol-2-yl)-2,5diphenyl tetrazolium bromide; TMZ, temozolomide; qPCR, real-time quantitative PCR; PCR, polymerase chain reaction; PI3K, phosphoinositide 3-kinase; ROCK1, Rho-associated kinase-1; S1P, sphingosine-1-phosphate; SphK, sphingosine kinase; μM, micromolar

Key words: glioblastoma multiforme, FTY720, temozolomide, cell viability, cell death, tumor-derived cells

Introduction

Sphingosine-1-phosphate (S1P) regulates a variety of critical cellular processes, including apoptosis, proliferation, and cellular motility. Furthermore, it supports the survival of glioblastoma (GBM) stem cells (1-3) and it is also involved in the regulation of neurogenesis and angiogenesis (4). In recent years, S1P has been recognized as an important oncogenic factor in several solid malignancies (5).

The concentration of the bio-active sphingolipid S1P is strongly regulated by the concerted action of synthesizing and catabolizing enzymes (6). Sphingosine kinases 1 and 2 (SphK1 and SphK2) synthesize S1P from membranous sphingosine, whereas the S1P-phosphatases (S1P-phosphatase 1 and 2) dephosphorylate S1P back to sphingosine (6).

Additionally, S1P lyase mediates the irreversible cleavage/division to hexadecenal, phosphoethanolamine and, finally, phosphatidylethanolamine (7). Additionally, the metabolism of ceramide is also a source for sphingolipids and S1P (8). The balance between S1P phosphorylation and degradation is important for the regulation of cell growth, and plays a crucial role in carcinogenesis and other pathological processes (9). Mora *et al* reported that, in glioma cells, sphingosine was mainly used for the synthesis of survival-promoting S1P (10).

Neurons and astrocytes, as well as GBM cells, can synthesize and export S1P (11,12), which acts as a second messenger for the activation of pathways and the crucial regulation of cellular processes (5).

Our previous study reported that SphK1 and all S1P receptors were expressed at different levels in primary, recurrent, and secondary GBM when compared to healthy brain tissue (1). The elevated levels of S1P and SphK1 in GBM tissue were found to be correlated with a shorter survival rate of patients affected by GBM (1). Moreover, S1P was found to stimulate the motility and invasiveness of GBM cells by

triggering S1P-producing enzymes and S1P receptors (13). The S1P G-coupled receptors are responsible for the activation of the Ras/extracellular signal-regulated kinase (ERK), the phosphoinositide 3-kinase (PI3K)/AKT, and the Rho/Ras (ROCK) signaling pathways (14). These intracellular pathways are associated with the growth and survival of GBM cells as well as the mutation of the tumor-suppressor genes p21, p16, phosphatase and tensin homolog (PTEN) and TP53 (15).

Bektas *et al* (16), reported that the inhibition of the S1P pathway induces apoptosis; therefore, new GBM therapy approaches should focus on the sphingosine pathway using S1P-analogues (4,16-18).

FTY720 (fingolimod) is a S1P-analogue which was found to modulate Rho-associated kinase-1 (ROCK1), epithelial-mesenchymal transition (EMT)-related factors, and the PI3K/protein kinase B (AKT)/mammalian target of the rapamycin (mTOR)/p70S6 kinase signaling pathway (19). AKT is active in 70% of GBM patients, particularly in those with PTEN loss (20). The ability of FTY720 to cross the blood-brain barrier and the fact that it is well-tolerated in human patients, makes it an excellent candidate for cancer therapy (7,21-23). FTY720 has also shown an immunomodulating activity by causing immunosuppression in patients affected by glioblastoma (24).

Therefore, the aim of the present study was to investigate the effects of FTY720 on the viability and the proliferation of GBM cells and to compare its cytotoxicity with the effects of temozolomide (TMZ), which is currently used as the standard treatment for patients affected by GBM.

Materials and methods

Cell culture. Human GBM cell lines A172, G28 [obtained from the American Type Culture Collection (ATCC)], U87 (glioblastoma of unknown origin) as well as primary #33, #367, and #391 GBM cells isolated from patient tissues were used for this study. The present study was approved by the local ethics committee (Ethics Committee of Medicine Faculty, Justus Liebig University Giessen), (application no. AZ 07/09). All patients provided signed consent for the collection of tumor tissue. All patients were female and were surgically treated at Department of Neurosurgery in Justus Liebig University Giessen between 2013-2020. Their ages were 67 (#33), 45 (#391) and 68 years (#367). The diagnosis of GBM was established at the Department of Neuropathology in Justus Liebig University Giessen.

For the primary cell cultures, tissue specimens were freshly collected from surgery and immediately minced in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) with a sterile disposable scalpel and trypsinized with trypsin/EDTA 0.05% for several minutes. Trypsinization was stopped with 10 ml DMEM and the mince was passed through a cell strainer (60 μm, Sigma Aldrich; Merck KGa) to obtain a single cell suspension. Cells were washed with PBS and the pellet was resuspended with DMEM and transferred to a 25 cm^2 culture flask (Greiner, Bio-One) and grown at 37˚C and 5% CO_2 saturation. Cells were passaged 2-3 times and expanded to 175 cm^2 flasks (Greiner, Bio-One) before washing in PBS, resuspended with DMSO (Merck KGa) and snap frozen in liquid nitrogen. For this study, only cell cultures from patients with confirmed diagnosis of glioblastoma at our Department of Neuropathology, Justus Liebig University Giessen were chosen.

For our experiments cells were defrosted in a 37˚C water bath and cultured as an adherent monolayer in 25 cm^2 flasks as previously described (25).

All cells were cultured in DMEM (Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; Biochrom AG) and 1% penicillin-streptomycin (Biochrom AG) at 37˚C and 5% CO_2 saturation. The cells were washed with phosphate-buffered saline (PBS; Biochrom AG) and trypsinized with trypsin/EDTA 0.05% (Biochrom AG) after reaching 80% confluence. PBS and trypsin/EDTA were pre-heated to 37˚C.

Drugs and treatment. FTY720 (Cayman Chemical Co.) and TMZ were dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich; Merck KGa) at a concentration of 1 mM. FTY720 stock solution (1 mM) was further diluted in culture medium (DMEM) to yield the concentrations of 1, 10, 25, 50, and 100 μM. Aliquots of the FTY720 stock and the final diluted concentrations were stored at -20˚C.

Cells were treated for 24, 48, or 72 h with FTY720. The control samples refer to cells incubated with DMSO only.

xCELLigence. Impedance-based measurements of cell proliferation and measurements of half maximal inhibitory concentration (IC_{50}) were performed with the xCELLigence Real-Time Cell Analyzer (RTCA) in E-plates (Roche Applied Science) under standard culture conditions as described above. The readout recorded by the RTCA is a dimensionless cell index (CI) value that correlates with cell number/quantity/amount. The cells were continuously monitored every 15 min and allowed to adhere and proliferate for 24 h in a drug-free medium. For the treatment, the medium was completely replaced with a FTY720-containing medium in serial concentrations of 5, 10 and 25 μM. For the controls, a drug-free, DMSO-containing medium was used. Readings were performed for at least 72 h after the treatment. The samples were analyzed at the minimum in triplicate. The RTCA-software v1.2.2 (Roche Applied Science) was used to analyze the data. Curves were normalized to the time-point just prior to adding the drug, to which a CI value of 1 was assigned.

MTT assay. To assess cell proliferation and survival, a MTT assay was performed with varying doses of FTY720 and TMZ. Briefly/firstly, 10^4 cells per well were seeded into 96-well plates. The cell lines were treated with 5, 10, 25, and 50 μM of FTY720. Positive control cells were treated with TMZ at concentrations of 25, 50, 75, and 100 μM, while negative control cells were treated with 0.05% DMSO. Measurements were performed after 24, 48, and 72 h. Cells were incubated for 4 h with 10 μl of MTT solution (Roche Applied Science). Finally, 100 μl of solution containing 10% sodium dodecyl sulfate (SDS) in a 0.01 μM hydrochloride solution was added. The cells were then incubated overnight at 37˚C in an atmosphere with 5% CO_2 saturation. Absorbance at a wavelength of 550 nm was measured, with 650 nm serving as a reference wavelength. IC_{50} values for FTY720 were calculated at the 72 h observation time point and compared to the effects of TMZ.

Flow cytometry (FACS). Cell lines were cultured in 6-well plates and prepared in triplicates. Cells were left to adhere to the bottom of the culture flask overnight and subsequently treated with FTY720 in the previously-mentioned concentrations for 24, 48, and 72 h, respectively. Negative controls were prepared with DMSO in equal concentrations. Thereafter, the cells were washed with PBS, trypsinized and centrifuged for 5 min at 252 x g. The pellet was resuspended in 1.5 ml of ice cold PBS. Cells were fixed with 3.5 ml of ethanol (Carl Roth GmbH & Co. KG) while being carefully vortexed, and subsequently, stored at -20˚C for 24 h.

Before measurement, the cells were centrifuged for 10 min at 252 x g and the pellet was then resuspended in 1 ml of PBS. Ten microliters of an RNAse solution (concentration 100 μg/ml) was added and the solution was incubated at 37˚C on a thermocycler at 18 x g for 10 min. Fifty microliters of a propidium iodide (PI) solution (concentration 50 μg/ml) was then added. Incubation took place in darkness for 5 min at room temperature, and analysis was performed by using a BD FACSCalibur™ flow cytometer. The data was analyzed with CellQuest™ Pro software (BD Biosciences). The rate of apoptosis (sub-G1 phase), and the G1 and G2 phases were determined using histogram analysis.

RNA isolation, cDNA synthesis, and quantitative real-time PCR (qPCR). RNA isolation was performed using the peqGOLD Total RNA kit (PeqLab, VWR) following the manufacturer's instructions. The obtained RNA concentrations were measured photometrically using the NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using the QuantiTect® Reverse Transcription kit (Qiagen GmbH) following the manufacturer's instructions.

qPCR was performed using the TaqMan™ Gene Expression Master Mix (Biosystems, Darmstadt, Germany). The following commercially available primers were used (all purchased from Thermo Fisher Scientific, Inc.): Actin β (Hs99999903_m1), Akt1 (Hs00920512_m1), MAPK1 (Hs01046828_m1), Rac1 (Hs01588892_g1), Roc1 (Hs01127701_m1), PKCE (Hs00942879_m1), and TP53 (Hs01034249_m1). The oligos were previously tested by the manufacturer for their specific binding. Sequences are not provided.

Analysis was performed in quadruplicate. StepOne™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the StepOne™ software v2.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for the amplification and data analysis.

Statistical analysis. Statistical analysis was performed by the Student's t-test, Man-Whitney U, and Wilcoxon tests. A two-sided P<0.05 was considered to indicate a statistically significant difference. The analysis was performed using Excel 2010 (Microsoft®), Graphpad Prism v6 software (GraphPad Software Inc.), and SPSS® statistics v24 (IBM Corp.). Significant differences between the effect of treatment with FTY720 and TMZ were determined statistically by performing the Student's t-test. The effect of FTY720 on the cell cycle was analyzed by performing the Mann-Whitney U. The overexpression and downregulation of cell cycle regulator genes after treatment with FTY720 compared to untreated cells and time-dependent changes were investigated in all the genes and analyzed by ANOVA (post hoc test: Bonferroni).

Results

Effect of FTY720 on glioblastoma cell proliferation. The treatment with FTY720 caused cell growth inhibition, with an IC_{50} value of 4.6 μM in A172 cells, 17.3 μM in G28 cells and 25.2 μM in U87 cells (Fig. 1).

A172 showed a decrease in cell proliferation after 6 h of incubation with 5 μM of FTY720. The cell viability remained below 50% over 72 h of treatment with 10 μM of FTY720. Cell growth arrest was observed after treatment with 25 μM of FTY720. A similar effect was observed in U87 cells, where 5 μM of FTY720 produced a decrease in cell viability as well. FTY720 at 10 μM achieved a stable reduction of viability below 50% over 72 h. Again, 25 μM of FTY720 caused cell growth arrest in G28 cells.

Comparison between FTY720 and temozolomide. A reduction of 50% in cell viability was observed in A172 cells after 24 h of treatment with 10 μM of FTY720. A cell proliferation rate lower than 50% was observed after 72 h of treatment with all concentrations of FTY720. The effect of FTY720 was stronger than the effect of TMZ after 24 h (P=0.011), 48 h (P<0.001), and 72 h (P<0.001). While G28 cell proliferation increased after treatment with TMZ, it was strongly suppressed by treatment with FTY720, analogous to A172 cells. FTY720 at 25 μM caused a reduction of cell viability below 50% after 24 h, and below 5% after 48 h of treatment. Reduction of cell viability (below 2.5%) could be induced by the administration of 50 μM of FTY720. In G28 cells, FTY720 had a significantly greater decreasing effect than TMZ (P<0.001). FTY720 at 50 μM caused a significant reduction of cell viability (over 50%). Prolonged treatment (72 h) with 25 μM of FTY720 was sufficient to decrease cell viability below 50%. Contrarily, TMZ caused an increase in cell viability after 24 h and only a slight reduction after 48 and 72 h.

Overall, A172, G28, and U87 cell lines showed a significant dose-dependent decrease in cells after 72 h of treatment with FTY720 compared to the TMZ-treated cells (P=0.001; Fig. 2A-C).

Cell cycle distribution of GBM cells treated with FTY720. A172 cells, treated with FTY720, showed a significant decrease in G0/G1-phase events (P=0.0015). An increase in the percentage of events detected at the S phase (18.1%) and the G2/M phase (15.6%) was observed. G28 cells showed significant variation in the distribution of the events in all cell cycle phases compared to the control cells. In the sub-G1 phase, there was an increase of 26% (P<0.001) and in the S phase, an increase of 6% (P<0.001). A decrease of 28% was observed in the G0/G1 phase. The strongest effect of FTY720 was detected in U87 cells. The number of events detected in the sub-G1 phase increased up to 50% after treatment with FTY720 (P<0.001) and the G0/G1 events simultaneously decreased to 21.3% (P<0.001; Fig. 3).

Expression of cell cycle regulator genes after treatment with FTY720. A172 cells showed a significant increase in *AKT1* (2.1-fold, P=0.003), *MAPK1* (2.6-fold, P<0.001) and

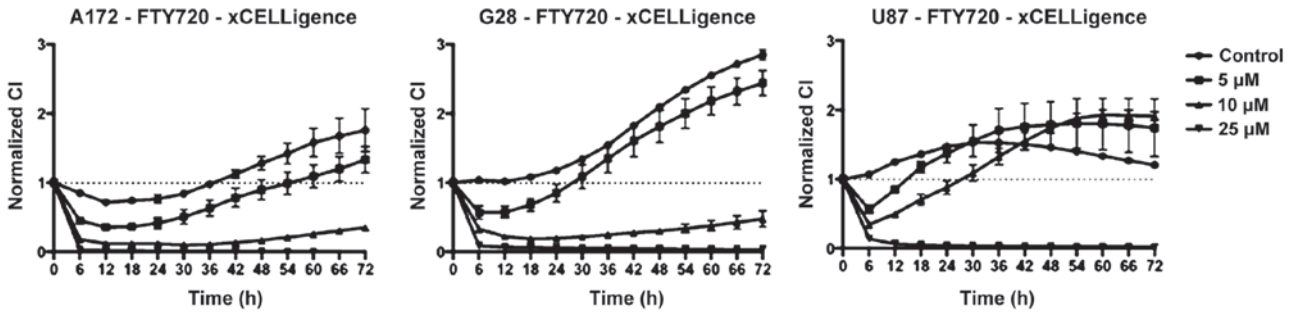

Figure 1. Measurement of cell viability with xCELLigence. Analysis of cell viability of three glioblastoma cell lines (A172, G28 and U87) compared to untreated cells. Shown are means ± standard deviations (SD) of three independent experiments performed in triplicates. CI, cell index; FTY720, fingolimod.

PKCE (4-fold, P<0.001) transcripts after 24 h of treatment with FTY720; *RAC1* and *ROCK1* transcripts were stable in comparison to the untreated cells. Longer time exposure to FTY720 (72 h) caused a significant downregulation of *ROCK1* (0.4-fold, P=0.007) and a further significant overexpression of *PKCE* (18.2-fold, P<0.001) transcript. All other analyzed transcripts were stable (Fig. 4).

G28 cells showed a significant overexpression of *MAPK1* (3.4-fold, P<0.001), *PKCE* (3-fold, P<0.001), *RAC1* (2.4-fold, P=0.003) and *ROCK1* (2.4-fold, P<0.001) transcripts and a stable expression of the *AKT1* transcript after 24 h of treatment with FTY720. The exposure to FTY720 for 48 and 72 h did not alter the expression of the transcripts, which remained stable.

U87 cells expressed a significant upregulation of the *AKT1* transcript (P<0.001) at all time points of exposure to FTY720 and a significant overexpression of *MAPK1* after 48 and 72 h of treatment with FTY720 (P<0.001). *PKCE*, *RAC1* and *ROCK1* were stably expressed.

The GBM-derived cells #33 and #367 showed a stable expression of *AKT1*, *MAPK1* and *PKCE* transcripts after treatment with FTY720. Interestingly, the transcription levels of *RAC1* and *ROCK1* were significantly downregulated (P<0.001) in the #367 cells, whereas they remained stable in #33 cells after treatment with FTY720. In contrast, the cells #391 showed a significant overexpression (P<0.001) of *MAPK1, PKCE, RAC1* and *ROCK1* transcripts after 24 and 48 h of treatment with FTY720. Longer exposure to FTY720 (72 h) caused no variation in the expression of these markers in comparison to the untreated cells. The level of *AKT1* transcript was stable at all treatment time points (Fig. 4).

Expression of the tumor-suppressor gene *TP53* was detected by RT-qPCR in A172, G28, U87, and the three GBM-derived cells (#33, #367 and #391) after 24, 48, and 72 h of treatment with FTY720. The A172 cells showed no significant change in *TP53* transcript level. U87 cells showed a significant downregulation (P<0.001) of the *TP53* transcription after 24, 48 and 72 h of treatment with FTY720. G28 cells were characterized by a significant downregulation of *TP53* (0.3-fold, P<0.001) after short time exposure (24 h) to FTY720, whereas its transcript level remained stable after 48 and 72 h of exposure. The #33 cells showed a stable expression of *TP53* transcript after treatment with FTY720. The #367 cells were characterized by a significant downregulation of the *TP53* transcript (0.1-fold, P<0.001) after 24 h of administration of FTY720 and a stable expression after longer time exposure (48 and 72 h). The #391 cells showed a significant overexpression of the *TP53* transcript after 24 h (6.3-fold, P<0.001), a downregulation of the *TP53* transcript after 48 h and a stable expression after 72 h of treatment with FTY720 (Fig. 4).

Discussion

The discovery of factors that correlate with glioblastoma (GBM) resistance to conventional chemotherapy is still ongoing. It would be valuable to select and analyze the patients who are potentially resistant to temozolomide (TMZ) and in need of development of novel therapeutic strategies for the treatment of GBM (22,26).

The S1P pathway, sphingosine kinase 1 and S1P-receptor 1 are involved in cell survival, growth, migration, angiogenesis and affect the survival of the patients affected by glioblastoma (1,27).

The present study demonstrated that treatment with fingolimod (FTY720) induced a significant reduction in cell viability in A172, G28, and U87 cells. Treatment with 10 μM of FTY720 reduced the percentage of viable A172 cells to less than 5%. Similar results were obtained for G28 and U87 cells, reaching a viability percentage below 2% after 72 h of incubation with 50 μM of FTY720; thus, supporting the previous study of Zhang and colleagues (21). Conversely, the reduction in viable cells caused by the administration of TMZ, even at 50 μM concentration, was not comparable to that of FTY720; inducing only 40% of a decrease in cell viability in all cell lines used in the study.

Synergy has already been observed between FTY720 and other substances, including doxorubicin, etoposide, and cetuximab for the treatment of colorectal cancer; or in combination with cisplatin for the treatment of melanoma and hepatocellular carcinoma (27-30). Interestingly, the combination of FTY720 and cisplatin has been responsible for the inhibition of autophagy, resulting in an additive cytotoxic effect (29,30).

Previous studies have shown that FTY720 can induce apoptosis, and inhibit the migration and invasion of GBM cells by inhibiting the activity of matrix metalloproteases (21,31). Rac1, RhoA, Ras, and Cdc42 are members of the Rho family of GTPases involved in cell migration. Rac1 and RhoA are both sufficient for the generation of cell polarity. Inhibition of ROCK restored the migration of GBM cells, but S1P2-dependent inhibition of Rac1 activity was not involved in the S1P-mediated

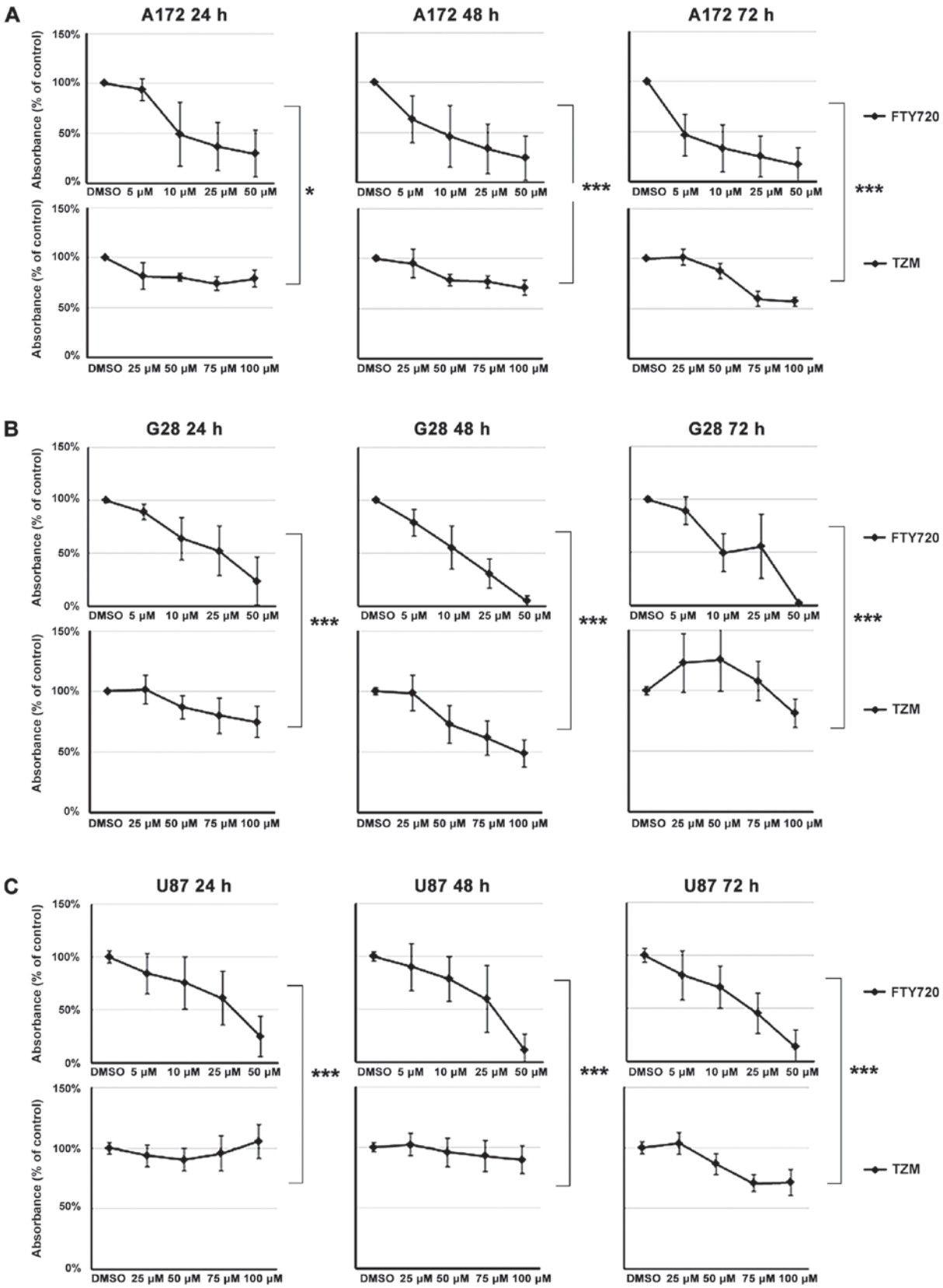

Figure 2. MTT assays of (A) A172, (B) G28 and (C) U87 cells treated with serial concentrations of FTY720 and TMZ. Shown are means ± standard deviations (SD) of experiments performed in triplicate. FTY720-treated cells vs untreated cells analyzed by Student's t-test. FTY720 fingolimod; TMZ, temozolomide. *P<0.05, ***P<0.001.

inhibition of migration in human GBM (32). In the present study, we confirmed that *RAC1, ROCK1,* and *MAPK1* transcripts were modulated by treatment with FTY720.

A decreased level of the *RAC1* transcript was observed in #367 cells after treatment with FTY720. Therefore, its inhibition could further impede the epithelial-mesenchymal

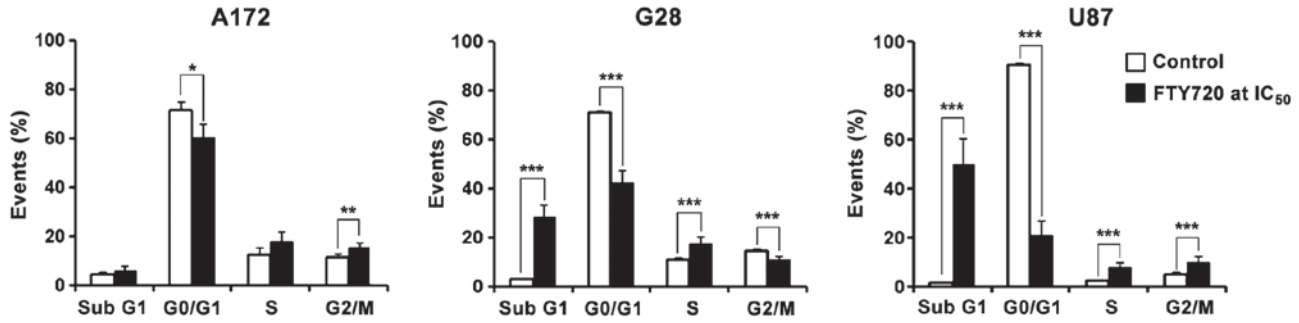

Figure 3. FACS analysis of A172, G28 and U87 cell lines. Shown are means ± standard deviation (SD) of three independent experiments performed in triplicates. The effect of FTY720 on the cell cycle was analyzed using Mann-Whitney U test. FTY720, fingolimod; $IC_{50,}$ half maximal inhibitory concentration. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

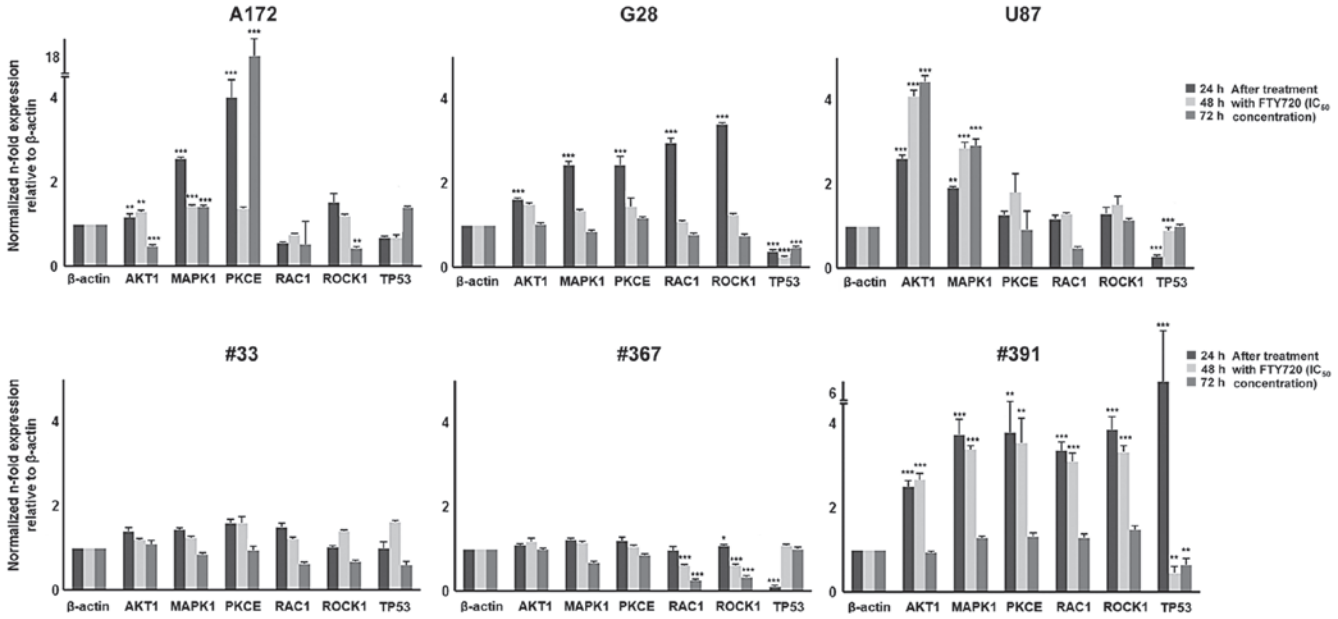

Figure 4. qPCR analysis of cell cycle regulators *AKT1, MAPK1, PKCE, RAC1, ROCK1* and *TP53* in A172, G28, U87, #33, #367 an #391 cells after 24, 48 and 72 h of treatment with FTY720 (IC_{50} concentration). The values were normalized in n-fold expression relative to β-actin. Measured dose- and time-dependent changes were investigated in all the genes and analyzed by ANOVA (post hoc test: Bonferroni). P-values show the significant overexpression or downregulation after 24, 48 and 72 h of treatment with FTY720 compared to untreated cells. Shown are means ± SEM of three independent experiments performed in triplicates. FTY720, fingolimod; $IC_{50,}$ half maximal inhibitory concentration. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with β-actin.

transition of GBM cells; thus, blocking tumor growth and metastasis (33,34).

Because of the interaction between Ras and the abundant S1P, an oncogenic process in cancer cells could be inhibited by the downregulation of S1P (13). However, FTY720 induced, in the majority of the cells included in this study, a stable or overexpressed level of the transcripts of the genes implicated in cell cycle progression which include *AKT1, MAPK1, PKCE, RAC1* and *ROCK1*.

A172 cells bear wild-type *TP53*, while mutated *TP53* is present in the G28, #33, and #391 cells (35). Interestingly, *TP53* expression, after incubation with FTY720, was stable or significantly downregulated in all cell lines involved in the study. Only the #391 cells showed a significant overexpression of the *TP53* transcript after short time exposure to FTY720; thereby showing that *TP53*, independently of its status, could not be triggered by treatment with FTY720, with the only exception represented by the #391 cells. Future investigations at the protein level are needed to identify the status of p53 and its cellular localization after treatment with FTY720.

Drug resistance to chemotherapy is still an unsolved issue for the treatment of GBM. Many patients relapse and develop resistance to TMZ-based chemotherapy (36). Consequently, a combination of TMZ with other anticancer mediators, such as FTY720, which can overcome drug resistance, could be one of the main approaches for achieving a better outcome for GBM patients.

Estrada-Bernal *et al* showed that FTY720, in combination with TMZ, appeared to decrease the invasiveness of brain tumor stem cells in xenograft models (3). Furthermore, mice (with brain tumor stem cells derived from human glioblastoma tissue intracranial) treated with both FTY720 and TMZ showed increased survival compared to those treated with a single compound (3).

FTY270 has also shown immunomodulatory effects and could lead, at a high dosage, to the immunosuppression of patients affected by GBM (37). FTY720 was found to prevent

peripheral lymphocytes from migrating into the central nervous system (CNS) and directly inhibited the activation of microglia and astrocytes in the brain (38-40). Inhibition of innate and adaptive immune/inflammation responses is probably triggered by the treatment with FTY720.

In conclusion, FTY720 exerted a cytotoxic effect in GBM cells that significantly surpasses that of TMZ. It was responsible for the reduction in cell viability, cell cycle arrest and cell death of GBM cells, even after the overexpression of the genes indicated in the regulation of cell cycle process. The lack of the protein level of the factors involved in the mechanisms modulated by FTY720 was a limitation of the study that needs to be further expanded in order to better clarify the effect of FTY720 on the molecular pathways responsible for proliferation and survival of glioblastoma cells. In addition, it is essential to consider phosphorylated AKT, which was not investigated in our study. These findings suggest that FTY720 could be included in further investigations, either in combination with TMZ or alone, for patients with TMZ drug resistance.

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Availability of data and materials

The data and materials that support the findings are available from the corresponding author upon reasonable request.

Authors' contributions

MAK was responsible for the study and manuscript design, analysis and review. BAB carried out the data collection and analysis. JN carried out the statistical analysis. MAW and EU reviewed the manuscript. FU carried out data analysis and review. PDF carried out data analysis, review and statistical analysis. FPS and MS carried out the study design and review. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the local ethics committee (Ethics Committee of Medicine Faculty, Justus Liebig University Giessen), (application no. AZ 07/09) and the performed research followed international and national regulations in accordance with the Declaration of Helsinki. Patients consent was obtained before treatment and is available from the corresponding author, MAK, upon reasonable request.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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