Ependymoma stem cells are highly sensitive to temozolomide in vitro and in orthotopic models

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Background. Ependymoma management remains challenging because of the inherent chemoresistance of this tumor. To determine whether ependymoma stem cells (SCs) might contribute to therapy resistance, we investigated the sensitivity of ependymoma SCs to temozolomide and etoposide.

Methods. The efficacies of the two DNA damaging agents were explored in two ependymoma SC lines in vitro and in vivo models.

Results. Ependymoma SC lines were highly sensitive to temozolomide and etoposide in vitro, but only temozolomide impaired tumor-initiation properties. Consistently, temozolomide but not etoposide showed significant antitumoral activity on ependymoma SC-driven subcutaneous and orthotopic xenografts by reducing the mitotic fraction. In vitro temozolomide at the EC50 (10 μM) induced accumulation of cells in the G2/M phase that was unexpectedly accompanied by downregulation of p27 and p21 without modulation of full-length p53 (FLp53). Differentiation-committed ependymoma SCs acquired resistance to temozolomide. Inhibition of proliferation was partly due to apoptosis, that occurred earlier in differentiated cells as compared to neurospheres. The activation of apoptosis correlated with an increase in p53β/γ isoforms without modulation of FLp53 under both serum-free and differentiation-promoting media. Incubation of cells in both conditions with temozolomide resulted in increased glioneuronal differentiation exhibiting elevated glial fibrillary acidic protein, galactosylceramidase, and βIII-tubulin expression compared to untreated controls. O6-methylguanine DNA methyltransferase (MGMT) transcript levels were very low in SCs, and were increased by treatment and, epigenetically, by differentiation through MGMT promoter unmethylation.

Conclusion. Ependymoma growth might be impaired by temozolomide through preferential depletion of a less differentiated, more tumorigenic, MGMT-negative cell population with stem-like properties.

Keywords: differentiation, ependymoma stem cells, MGMT, orthotopic models, temozolomide.
defining criteria have been isolated from ependymoma and identified in a restricted subpopulation with a molecular signature resembling that of radial glial cells.14,15

Temozolomide (TMZ) and etoposide (VP16) are two DNA damaging agents that are used in the treatment of ependymoma.16,17 Clinical trials are ongoing to optimize schedules and dosing of these drugs (http://www.clinicaltrials.gov/).

The effects of TMZ and VP16 on ependymoma SCs have been addressed only in vitro studies, that found resistance or minor sensitivity of neurosphere-derived cells to both agents.18–20 We have previously established orthotopic ependymoma xenografts driven by cells with stem-like properties.21 This resource afforded us to explore for the first time the effects of TMZ and VP16 on tumorigenicity of ependymoma stem cells (SCs) and the antitumoral activity of both agents on subcutaneous and intracranial ependymoma SC-driven xenografts. Because only TMZ proved to be effective in vivo, we further investigated the cellular and molecular mechanisms underlying the response to TMZ in ependymoma SCs.

Materials and methods

Cell cultures

We used the ependymoma lines EPP and EPV that we established from two recurrent infratentorial pediatric ependymoma by using neural SC permissive conditions.21 Cells were grown in NeuroCult medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with epidermal growth factor (EGF; 20 ng/mL; Sigma-Aldrich–Aldrich, Dorset, UK) and basic fibroblast growth factor (bFGF; 10 ng/mL; Peprotech, Rocky-Hill, NJ).

To induce differentiation, cells were seeded onto vessels coated with poly-L-ornithine and laminin (Invitrogen, Carlsbad, CA) in Neurobasal (Invitrogen) supplemented with EGF and bFGF.15,21 After 24 h, medium was changed to 10% fetal bovine serum (FBS, Invitrogen)/Neurobasal without mitogens and cells were allowed to differentiate for up to 14 days.

Cell proliferation assay

The antiproliferative effects of TMZ and VP16 were determined in undifferentiated and differentiated cells. TMZ was purchased from Sigma-Aldrich and diluted in dimethyl sulfoxide to a stock solution of 100 mM, whereas VP16 was that for clinical use (Teva Pharma B. V., Utrecht, NL). Vehicle or serial concentrations of each drug were added to the medium and cells were cultured for up to 7 days, with medium changed on day 3 and day 5. Cell number and viability were assessed by an automated cell counter (NucleoCounter 100TM, ChemoMetec, Allerød, Denmark), and expressed as a percentage of the control. The half maximal effective concentration (EC50) was calculated using the GraphPad Prism software package version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Flow cytometry

For CD133 analysis, cells were exposed to serial concentrations of TMZ and VP16 for 3 and 5 days. Cells were then collected and labeled with monoclonal CD133/1-phycocerythrin-conjugated antibody or isotype control antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at 4°C. After washing, cells were resuspended in 0.5 mL culture medium and analyzed by flow cytometry (CyAn Flow Cytometer, Beckman Coulter, Orange County, CA).

For cell cycle analysis, ependymoma cells were incubated for 24, 48, and 72 h in the presence of TMZ (10 μM and 100 μM) or vehicle and then fixed in 70% ethanol. Cell pellets were treated with 0.5 mg/mL RNase (Sigma-Aldrich) in phosphate buffer saline (PBS) plus 0.1% saponin (Sigma-Aldrich), incubated at 37°C for 30 min before staining with 20 μg/mL propidium iodide (Sigma-Aldrich) for 30 min at 4°C. Cell were then analyzed for DNA content by means of FACSCalibur flow cytometer (Becton Dickinson, San José, CA).

Subcutaneous and intracranial transplantations

All experimental animal investigations complied with the guidelines of the “Istituto Superiore di Sanità” (National Institute of Health, Rome, Italy) and the Ethical Committee of Catholic University and all animal care was in accordance with local institutional guidelines.

For subcutaneous transplantations, 3 × 106 cells/100 μL 0.9% sodium chloride solution were inoculated together with an equal volume of Matrigel (Becton Dickinson, Franklin Lakes, NJ) in both flanks of five-week-old nude male CD1 nu/nu mice (Charles River, Calco, Italy). Tumor volume (TV) and body weight were monitored twice per week throughout the study. TVs were calculated by the formula: TV = d2 × D/2, where d and D are the shortest and longest diameters, respectively.

For the orthotopic model, 3 × 105 cells/10 μL PBS were implanted into the third ventricle of nude mice using a stereotaxic injection frame (Kopf Instrument, Better Hospital Equipment Corp, Miami Lakes, FL), after administration of general anesthesia (80 mg/kg ketamine + 10 mg/kg xylazine, Sigma-Aldrich). The injection coordinates were: lateral to right, 1 mm from bregma; dorsoventral −3.5 mm. The animals were monitored daily until signs of neurologic deficit developed, at which time they were euthanized and their brains removed for histopathologic analysis.

For the orthotopic transplantation, mice were randomized in separate groups (8–10 mice/group), each group receiving one of the following treatments: oral vehicle (control), TMZ (35 mg/kg for five times per week for 10 weeks, oral gavage), VP16 (15 mg/kg in one daily injection on days 1–3 of treatment, intraperitoneal), combination of the two drugs at the indicated doses and schedules. VP16 and TMZ were experimentally prepared as previously described.22,23 The dose of TMZ that mimics a metronomic administration in mice was extrapolated from literature data.23–25

All statistical analyses were performed using the GraphPad Prism software package version 6.0 (GraphPad Software Inc). The survival days of animals were determined using the Kaplan-Meier plots and compared by the log-rank test. P values <.05 were considered to be significant.

Tissue processing and immunohistochemistry

Xenograft specimens were fixed with 4% paraformaldehyde, paraffin-embedded and cut into 3–μm sections. Immunohistochemical analysis of monoclonal mouse anti-human Ki67...
(Novocastra Laboratories, Newcastle, UK) was carried out according to a standard protocol previously described.21

Western blot analysis
Cells were exposed to 10 μM or 100 μM TMZ for time periods ranging from 3 h up to 7 days. At the end of incubation, cells were immediately processed in lysis buffer.21 Total lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to Hybond nitrocellulose membrane (Amersham Pharmacia, Buckinghamshire, UK), and probed with antibodies to p53, p21, caspase 3, Bcl2, anti-poly(ADP-ribose) polymerase (PARP), and actin, all from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Vector, Burlingame, CA) and the immunoblots were visualized using the ECL detection system (Amersham Pharmacia).

Real-Time Quantitative Reverse Transcriptase (RT) PCR
DNA and total RNA were extracted from cells using the AllPrep DNA/RNA Kit (Qiagen GmbH, Hilden, D). RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. TaqMan gene expression assays for CD133, nestin, Olig2, Sox2, glioblastoma acidic protein (GFAP), βIII-tubulin, O6-methylguanine-DNA methyltransferase (MGMT), galactosylceramidase (GalC), as well as for the reference normalization gene (hyoxanthine guanine phosphoribosyltransferase, HPRT) were obtained from Applied Biosystems. Each amplification reaction was performed in triplicate on a 7500 Real-Time PCR System (Applied Biosystems). The amount of each target mRNA was normalized to that of HPRT as previously reported.21

Methylation-specific PCR (MSP)
The methylation status of MGMT promoter was determined by a two-stage methylation-specific PCR (MSP) approach with minor modifications.26–28 Genomic DNA (2 μg) was treated with sodium bisulphite using the Epitech Kit (Qiagen) according to the manufacturer’s instructions. Stage-1 PCR was performed to amplify a 289-bp fragment of the MGMT gene by using primers that recognize the bisulphite-modified template but do not discriminate between methylated and unmethylated alleles.27,28 In the stage-2 PCR, the primer combinations allowed for the amplification of a 122-bp fragment from methylated DNA or a 129-bp fragment from unmethylated DNA.26 Each PCR product was separated on 2% agarose gels.

Results
TMZ, but not VP16, shows significant antitumoral activity in subcutaneous and intracranial ependymoma xenografts
We next investigated the antitumoral effects of the two drugs administrated either as single agents or in combination in subcutaneous tumor models (Fig. 2A). TMZ alone induced complete resorption of tumors and, importantly, no regrowth was observed after interruption of treatment up to the end of the experiment in all of the animals (8/8 animals, ~120 d from the last administration). VP16 administrated alone was ineffective. The combination of the two drugs did not exert any superior antitumoral efficacy as compared to TMZ alone. The dose and schedule of VP16 that we used had previously been shown to prolong the growth delay of human small cell lung carcinoma xenografts.22

In parallel experiments in orthotopic models, TMZ caused a significant prolongation of survival (log-rank, P = .0021), whereas VP16 and TMZ + VP16 did not (log-rank, P = .88, and P = .343, respectively) (Fig. 2B, and data not shown). One mouse out of 13 in the TMZ-group was potentially cured. TMZ strongly reduced the proliferating fraction in orthotopic xenografts, as evidenced by staining of samples for the expression of Ki-67 (Fig. 2C).

Weight loss was ~12% in all of the treated groups and mice completely recovered after drug administration was stopped (data not shown). By comparing TMZ antitumoral activity in the two models, the efficacy was lower in orthotopic than in subcutaneous xenografts.
and this underlies the importance of models that more faithfully mirror the environment where human tumor develops for preclinical drug testing.

**TMZ induces dose-dependent perturbations of cell cycle in ependymoma SCs**

Because we observed that only TMZ inhibited the growth of ependymoma SCs-driven xenografts, we focused on determining the cellular mechanisms underlying the response to this agent in cell-based assays.

To address whether decrease in cell number was due to impaired proliferation, we analyzed the perturbations of cell cycle after exposure to TMZ (10 μM and 100 μM) for 24, 48 and 72 h. Fluorescence-activated cell sorting analysis showed that 10 μM TMZ induced an increase in the G₂/M phase at 72 h, that was preceded by a time-dependent accumulation of cells in the S phase at the expense of the G₀/G₁ population (Fig. 3A).
contrast, 100 μM TMZ determined an accumulation in the G0/G1 phase with a concomitant G2/M peak loss at 72 h. The cytokinetic effects of TMZ were similar in the two cell lines.

We next determined the expression of p53, p27, and p21, that are involved in cell cycle arrest and DNA damage response induced by genotoxic agents, including TMZ. A 72-h treatment caused no modulation of full-length p53 (Flp53), and, unexpectedly, a loss of the checkpoint controls p27 and p21, that was evident at both 10 μM and 100 μM TMZ, and was not related to the phase of the cell cycle arrest (G2/M vs G0/G1) (Fig. 4B). A time course analysis in the first 24 h of drug exposure confirmed a dose-dependent downregulation of both p21 and p27, with p21 decreasing already after 6 h (100 μM TMZ) and p27 after 24 h (Fig. 3B).

**Differentiated ependymoma SCs acquire resistance to TMZ**

Because SCs represent only a minor component of the tumor bulk, we next evaluated whether the differentiation status of ependymoma SCs could affect the response to TMZ. To this end, stem-like and differentiation-committed cells were exposed to the agent for 3 and 7 days. Sensitivity to TMZ was strongly reduced in differentiated cells, since a dose as high as 100 μM was not even able to reduce cell proliferation by 50% after 3 days of treatment, whereas the EC50 in controls was around 10 μM (Figs. 1A and 4A). However, at 100 μM the relative contribution of cell death in the inhibition of proliferation was higher in differentiated cells, where it accounted for ~30% versus ~5% in controls. Cell loss was partly due to the activation of the apoptotic pathway, as evidenced by parallel western blot analyses that showed a dose-dependent upregulation of cleaved PARP and caspase 3 only in differentiating condition (Fig. 4B). Flp53 was not modulated. A band of the apparent molecular weight of ~48 kD was detected along with Flp53, whose expression was dose-dependently induced by treatment only in the differentiated lines, correlating with the induction of apoptotic markers. We identified this band as the carboxy-terminal truncated p53 isoforms, because we used the mouse monoclonal antibody DO-1 that specifically recognizes these two variants together with Flp53.

After 7 days, the effect of TMZ was evident only in stem-like cells, where ~80% growth inhibition was reached with 10 μM TMZ (Fig. 4C). The percentage of dead cells increased dose-dependently, being the relative contribution to the inhibition of proliferation ~30%. PARP and caspase3 were dose-dependently cleaved in undifferentiated EPV cells (Fig. 4D), whereas in EPP cells the activation of caspase 3 was detected only at 100 μM TMZ, because it was likely counteracted by the induction of the antiapoptotic protein Bcl2. The pattern of the modulation of p27 and p21 was similar to that observed after 3 days of treatment in both pairs of lines. Altogether these data indicate that differentiated ependymoma cells, although less responsive to TMZ, activate the apoptotic pathway earlier than the more sensitive SCs.

**TMZ treatment increases the expression of lineage specific markers in undifferentiated and differentiated ependymoma cells**

We next examined whether TMZ determines a loss of stemness features and/or acquisition of differentiation markers. EPP and EPV cells were treated with 10 μM TMZ for 7 days. In both lines, there was no decrease in the expression of the stemness markers CD133, nestin, and Sox2, whereas Olig2 declined only in EPV cells (Fig. 5A). However, treatment upregulated βIII-tubulin (neuronal lineage) in EPP cells, GFAP (astrocytic lineage) and GalC (oligodendroglial lineage) in EPV cells.

Differentiation-inducing capability of TMZ was observed also in differentiated cells. Mitogen deprivation caused dramatic morphological changes that were enhanced by TMZ, as evidenced by an increase in number and length of neurite-like processes (Fig. 5B). At a biochemical level, differentiation was accompanied by upregulation of GFAP, βIII-tubulin, and GalC (Fig. 5C). TMZ
increased further the expression of these markers, mostly in EPP, where there was a 4-fold increase in GFAP, and approximately a 2-fold increase in GalC and βIII-tubulin.

To address whether treatment could modulate differentiation markers in vivo, we stained sections for GFAP expression, which shows the highest expression in vitro. However, no obvious difference was observed between control and treated animals (data not shown).

MGMT is induced by TMZ treatment and differentiation

The most cytotoxic effects of alkylating agents such as TMZ are due to methylation of guanine at position O6. The DNA repair enzyme MGMT removes the methyl group from O6-methylguanine, thus protecting cells against DNA damage and cell death. MGMT expression is the best characterized mechanism of TMZ resistance. Loss of MGMT expression and diminished DNA-repair activity have been related to epigenetic silencing of the MGMT gene mediated by promoter methylation.

Ependymoma SCs are expected to not express MGMT because of their high responsiveness to TMZ. Indeed, levels of MGMT mRNA were very low in both lines, and dose-dependently induced by TMZ treatment (~5-fold after 100 μM TMZ) (Fig. 6A). Interestingly, differentiation highly increased MGMT expression in both lines.

We next assessed MGMT promoter methylation by MSP in stem-like conditions and after differentiation. We used the T98G line (which expresses high level of the protein) and peripheral blood lymphocytes as a control for unmethylated MGMT and the U87 line as a control for methylated MGMT (Fig. 6B). EPP and EPV SCs showed hypermethylation of the MGMT promoter, whereas unmethylated MGMT was present in EPP cells and totally absent in EPV cells (Fig. 6C). Unmethylated MGMT increased with the differentiation, consistently with the increase in mRNA expression. TMZ treatment did not determine any change in the level of MGMT promoter methylation (data not shown).

Discussion

In this study we found that ependymoma SCs are highly sensitive to TMZ at clinically achievable concentrations both in vitro and in vivo, whereas differentiated cells acquire resistance to the agent. TMZ preferentially depletes cells with stemness characteristics, because treatment results in a decrease in tumor-initiation properties and induction of differentiation markers. Ependymoma SCs are specifically sensitive to TMZ, because VP16 (present work) and the dual EGF receptor/HER2 inhibitor AEE788 do not determine a statistical significant survival difference of mice bearing...
ependymoma SC-driven orthotopic xenografts in ex vivo and in vivo models.

TMZ increased the expression of the lineage-specific markers βIII-tubulin, GalC and GFAP in ependymoma cells in both stem-like and differentiating conditions. This might be a consequence of the selective depletion of cells with a more immature phenotype or of the inherent differentiation-inducing capability of TMZ. Eritroid differentiation of leukemia cells after TMZ has been ascribed to either treatment-induced hypomethylation of differentiation-related genes or cell cycle arrest consequent to DNA damage. In glioblastoma SCs, TMZ upregulates the expression of βIII-tubulin, microtubule-associated protein 2, and GFAP, and potentiates BMP2-induced differentiation, that is accompanied by a parallel decrease in MGMT expression and sensitization of cells to TMZ through a dramatic increase in apoptosis. In our ependymoma cells, some induction of apoptosis was observed with a different onset in the two experimental conditions, occurring earlier in the slow-dividing differentiated cells, and at a later time point in the fast-dividing SCs. However, the inhibition of proliferation was mainly due to a p53-independent arrest in G2/M or G0/G1 phase after exposure to a low or high concentration of TMZ, respectively. TMZ arrests cells in both G0/G1 and G2/M depending on the specific cellular context and tumor type, p53 status, and DNA mismatch repair system. Multiple effects usually occur in the cell cycle after treatment with a drug, as the cells flow through G1, S and G2/M checkpoints (blocking activity, death rate, delay and recycling), each one with its own kinetics and concentration thresholds. The activation of proteins involved in cell cycle checkpoints after exposure to TMZ is dose- and time-dependent. It is likely that ependymoma cells...
exposed to a low concentration are still able to progress slowly through S phase to eventually block in G2/M. After a high dose, cells might “freeze” in G0/G1 – where the majority of cells reside – because of high levels of DNA damage, that lead to an irreversible and immediate block.

Unexpectedly, cell cycle perturbations were accompanied by a decrease in the expression of p21 and p27, that, so far, remains elusive. In glioblastoma, TMZ triggers a canonical p53-dependent pathway with upregulation of p21 leading to cell cycle arrest and apoptosis, but similar effects have been described also in cells carrying a mutated p53.4232 p21 and p27 function as inhibitors of cyclin dependent kinases and regulate cell cycle progression and differentiation.42 However, some evidence suggests additional functions for these proteins, including pro-survival properties.44 If this hypothesis holds true, their downregulation might facilitate the apoptotic process. Indeed, we found that the levels of p21 and p27 were inversely related to the levels of apoptotic markers.

The presence of the alternative spliced variants p53β/γ is a novel finding in ependymoma. Several isoforms of p53 have been found to be differentially expressed in cancer, although their functional role is not fully understood. Both p53β and p53γ are suggested to influence carcinogenesis and drug-sensitivity in a tumor-dependent manner by modulating cell cycle progression, senescence, and apoptosis.34 In glioblastoma cells the p53β/γ isoforms seem to have functional significance in that they were modulated by TMZ unlike FLp53, and this paralleled upregulation of apoptotic markers. Of note, the expression levels of p53β/γ isoforms were higher in ependymoma neurospheres than in their differentiated counterparts, similarly to what was previously found in melanoma cells.33 Therefore, p53β/γ isoforms might play a role in modulating stemness/differentiation pathways.

TMZ is the first-line therapy in glioblastoma and the persistence of chemoresistant glioblastoma SCs may account for frequent treatment failure. In vitro, TMZ enriches the fraction of glioblastoma cells with stem-like properties and elevated MGMT expression, suggesting that MGMT is a key determinant of intrinsic or acquired resistance of glioblastoma SCs to the agent.46 – 48 In other works TMZ preferentially depletes the glioblastoma SC compartment, being the sensitivity inversely related to the expression of the MGMT protein.26,49

Because of the lack of ependymoma models, no study has so far investigated MGMT expression and promoter methylation status in SCs from this tumor. In ependymal tumors positive MGMT methylation status is not frequent, this data correlating with the poor response to TMZ treatment in ependymoma clinical trials.50 We found that the two ependymoma SC lines displayed high methylation status of MGMT promoter and lack of MGMT expression, in keeping with their high susceptibility to TMZ. Of note, prolonged treatment increased MGMT expression. On the whole, our data suggest that in ependymoma MGMT might be a mechanism of expression of GFAP, GalC and βIII-tubulin in ependymoma neurospheres (Undiff) and differentiated cells (Diff) treated with vehicle (Control) or 10 μM TMZ for 7 days. Level of each target gene was normalized to the level of the reference gene HPRT in each sample. Average values from two independent amplifications each in triplicate are shown.

Fig. 5. Differentiation-inducing capability of TMZ on ependymoma cells. (A) Real time quantitative RT-PCR analysis of expression of CD133, Olig2, Sox2, nestin, GFAP, GalC and βIII-tubulin. Ependymoma SCs were treated with 10 μM TMZ for 7 days. mRNA level of each target gene was normalized to the level of the reference gene HPRT in each sample. Means ± SD relative to untreated controls, which were used as calibrators (1 = no change). (B) Phase-bright photomicrographs of differentiated EPP cells treated with vehicle (Control) or 10 μM TMZ for 7 days. Original magnification, both × 20. (C) Real-time quantitative RT-PCR analysis of GFAP, GalC and βIII-tubulin in ependymoma neurospheres (Undiff) and differentiated cells (Diff) treated with vehicle (Control) or 10 μM TMZ for 7 days. Level of each target gene was normalized to the level of the reference gene HPRT in each sample. Average values from two independent amplifications each in triplicate are shown.
of acquired resistance to TMZ through drug-mediated selection of an inherently resistant SC subpopulation that expresses MGMT or epigenetic induction of MGMT by promoter hypomethylation. Alternatively, the increase in MGMT expression might be related to the pro-differentiation properties of TMZ, in agreement with the increase in unmethylated MGMT promoter and MGMT expression that we found in differentiated ependymoma lines.

Epigenetic changes mediated by DNA methylation appear to be critical in the pathogenesis of tumors, including ependymoma. A dynamic transition between active and repressive state of gene transcription could provide survival advantages to the CSC subpopulation. Because MGMT is a mechanism of defense against mutagenic alkylation lesions, MGMT-silenced cancer cells, such as our ependymoma SCs, might acquire a mutator phenotype that predisposes them to subsequent genetic and epigenetic changes essential in tumorigenesis. In this view, the acquisition of MGMT might be a differentiated function to protect cells against genotoxic insults. Interestingly, MGMT appears to be developmentally regulated in human brain, increasing with gestation age and later in life.

No clinical nor preclinical studies have so far addressed the efficacy of TMZ given at low doses on continuous (metronomic) administration in ependymoma. We found that TMZ on a metronomic schedule significantly reduces tumor growth rate in both subcutaneous and orthotopic models. Our in vitro experiments indicate that prolonged exposure to a low dose of TMZ is required to induce apoptosis in ependymoma SCs. On the other hand, exposure to TMZ increases the expression of differentiation markers and of MGMT in both stem-like and differentiation-committed cells, both mechanisms being associated with resistance to TMZ, which suggests that even daily administration could spare differentiated cells. However, a metronomic schedule could yet increase the therapeutic efficacy of TMZ, because protracted administration inactivates MGMT more effectively than short treatment, thus counteracting the emergence of MGMT-expressing clones. Indeed, metronomic treatment is effective in glioblastoma with unmethylated MGMT status. Extended dosing schedule offers other potential advantages, such as enhanced pro-apoptotic activity, antiangiogenic effects, sensitization of tumor cells to radiotherapy, and, because of higher tolerability, association with other chemotherapeutics. Finally, drug-induced differentiation in itself could contribute to the therapeutic activity of TMZ, by reducing the “stemness” features of cells, hence tumorigenicity. We previously found that ex vivo differentiation of ependymoma SCs significantly prolonged the survival of xenografted mice.

In conclusion, ependymoma SCs appear to be resistant to VP16 and specifically sensitive to TMZ, that exerts antiproliferative, pro-apoptotic, and pro-differentiation effects on an MGMT-negative cell population with stem-like properties. Optimized TMZ dosing schemes and schedules might increase the therapeutic potential of this agent in the treatment of patients with ependymoma.

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