Long-term *In Vitro* Treatment of Human Glioblastoma Cells with Temozolomide Increases Resistance *In Vivo* through Up-regulation of GLUT Transporter and Aldo-Keto Reductase Enzyme AKR1C Expression

**Abstract**

Glioblastoma (GBM) is the most frequent malignant glioma. Treatment of GBM patients is multimodal with maximum surgical resection, followed by concurrent radiation and chemotherapy with the alkylating drug temozolomide (TMZ). The present study aims to identify genes implicated in the acquired resistance of two human GBM cells of astrocytic origin, T98G and U373, to TMZ. Resistance to TMZ was induced by culturing these cells *in vitro* for months with incremental TMZ concentrations up to 1 mM. Only partial resistance to TMZ has been achieved and was demonstrated *in vivo* in immunocompromised mice bearing orthotopic U373 and T98G xenografts. Our data show that long-term treatment of human astroglioma cells with TMZ induces increased expression of facilitative glucose transporter/solute carrier GLUT/SLC2A family members, mainly GLUT-3, and of the AKR1C family of proteins. The latter proteins are phase 1 drug-metabolizing enzymes involved in the maintenance of steroid homeostasis, prostaglandin metabolism, and metabolic activation of polycyclic aromatic hydrocarbons. GLUT-3 has been previously suggested to exert roles in GBM neovascularization processes, and TMZ was found to exert antiangiogenic effects in experimental gliomas. AKR1C1 was previously shown to be associated with oncogenic potential, with proproliferative effects similar to AKR1C3 in the latter case. Both AKR1C1 and AKR1C2 proteins are involved in cancer pro-proliferative cell chemoresistance. Selective targeting of GLUT-3 in GBM and/or AKR1C proteins (by means of jasmonates, for example) could thus delay the acquisition of resistance to TMZ of astroglioma cells in the context of prolonged treatment with this drug.
Introduction

Malignant gliomas represent the most common type of primary brain tumor and constitute a spectrum of clinicopathologic entities from low- to high-grade malignancies. Nearly all low-grade tumors (except grade 1) eventually progress to high-grade malignancies [1]. Glioblastoma (GBM) is the most frequent and malignant glioma [2]. Treatment of GBM patients is multimodal with maximum surgical resection, followed by concurrent radiation and chemotherapy with the alkylating drug temozolomide (TMZ) and/or with nitrosourea [3–7]. However, whereas these treatments prolong GBM patient survival, they do not affect the overall dismal prognosis of this disease. No GBM patient has been cured to date [3–7] because of the diffuse nature of GBM cell invasion into the brain parenchyma and the natural resistance of GBM-migrating cells to apoptosis and thus to chemotherapy and radiotherapy [6]. One of the most beneficial treatments for GBM patients is surgery followed by radiotherapy with concomitant TMZ administration [3–5]. Moreover, TMZ increases GBM sensitivity to radiotherapy [3–5] most effectively in O6-methylguanine-DNA methyltransferase–negative GBMs by increasing the degree of radiation-induced double-strand DNA damage [8]. TMZ, can also prevent irradiation-induced glioma cell invasion [9] through caspase-mediated prevention of irradiation-induced FAK activation [10]. TMZ also displays antiangiogenic effects in experimental gliomas [11]. TMZ has been shown to first induce proapoptotic defenses in GBM cells [12,13], a feature that leads to late apoptosis [14]. Thus, although TMZ does not cure GBM patients, it significantly improves GBM patient survival and quality of life. Stupp et al. [5] assigned 573 GBM patients to treatment; 278 (97%) of the 286 patients in the radiotherapy-alone group and 254 (89%) of 287 in the combined treatment radiotherapy and TMZ group died during the 5 years of follow-up. Overall survival rates were 27% at 2 years, 16% at 3 years, 12% at 4 years, and 10% at 5 years with TMZ versus 11%, 4%, 3%, and 2% with radiotherapy alone, respectively [5]. A benefit of combined therapy was recorded in all clinical prognostic subgroups, including patients aged 60 to 70 years [5].

However, GBMs can present innate resistance to TMZ or develop acquired resistance during treatment [4,6]. Innate resistance of GBM cells to TMZ includes various events, such as loss of the phosphatase and tensin homolog, leading to the activation of the phosphoinositide-3-kinase/Akt pathway [6,15], unmethylation of O6-methylguanine-DNA methyltransferase [16], robust base excision repair [17], high activity of the DNA repair protein O6-alkylguanine-DNA alkyltransferase [18], and deficiency in the DNA mismatch repair system [19]. Acquired resistance of GBM cells to TMZ during treatment includes loss of the mismatch repair protein MSH6 [20] and the selection of less-differentiated preexisting resistant cells in the parental tumor [21].

The present study aimed to identify genes implicated in acquired resistance of two human GBM cells of astrocytic origin, T98G and U373 [22,23]. Resistance to TMZ was induced in these cells by culturing them in vitro over months with incremental TMZ concentrations up to 1 mM. Only partial resistance to TMZ has been achieved in vitro, and this demonstrated in immunocompromised mice bearing orthotopic U373 and T98G xenografts. Whole-genome analyses (Affymetrix, High Wycombe, UK), along with proteomic validations (Western blot analysis and immunoﬂuorescence), were performed in the U373 and T98G GBM cells that were treated for months with TMZ and in the untreated control cells. Stem cell markers were also analyzed in the various GBM cell variants because GBM response to TMZ can be inﬂuenced by the proportion and/or nature of GBM stem cells [24–28].

Materials and Methods

Cell Lines, Media, and Compounds

Established cell lines. The human U373 (ATCC code HTB-17) GBM, T98G (ATCC code CRL1690) GBM, and the human HT29 colon cancer (ATCC code HTB-38) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in our laboratory as detailed previously [21,22].

Compounds. TMZ was obtained from Schering Plough (Brussels, Belgium).

Long-term Treatment of U373 and T98G GBM Cell Lines with TMZ

U373 and T98G GBM cell lines were treated as follows: (a) 0.1 μM TMZ two times per week (Monday and Thursday) for 4 weeks (with fresh medium change at each TMZ treatment), (b) 1 μM TMZ two times per week for 5 weeks, (c) 10 μM TMZ two times per week for 12 weeks with 1 week of washout after each week of TMZ treatment, (d) 500 μM TMZ once a week for 3 weeks with a 1 week of washout between the 2 weeks of TMZ treatment, (e) 500 μM TMZ two times per week for 8 weeks with 1 week of washout between 2 weeks of TMZ treatment, and (f) 1 mM TMZ two times per week for 4 weeks with 1 week of washout between the weeks of TMZ treatment. The cells were analyzed for genomic and proteomic purposes after 4 weeks of washout after the treatment described in (f). U373 and T98G GBM cells remained permanently cultured in the presence of 150 μM TMZ for the experiments performed in the current study.

Animal Models

In vivo orthotopic xenografts of human U373 and T98G cells left untreated and thus sensitive ("TMZ-S" phenotype) versus TMZ long-term treated ("TMZ-LTT" phenotype) GBM cells were obtained as described previously [21,22]. All mice (6-week-old female nu/nu mice of 21-23 g; Janvier, Le Genest-Saint-Isle, France) had GBM TMZ-S (11 mice per group/experimental model) or TMZ-LTT (11 mice per group/experimental model) cells stereotactically implanted into their brains on the same day. All of the in vivo experiments described in the present study were performed based on authorization no. LA1230509 of the Animal Ethics Committee of the Federal Department of Health, Nutritional Safety and the Environment (Belgium).

Analyses of GLUT Transporter and AKR1C Enzyme Genomic Expression in a Clinical Series of 159 Gliomas and 23 Normal Brain Samples from the Henry Ford Hospital

The genomic expression of four GLUT transporters (GLUT-1, -3, -5, and -10) and three AKR1C enzymes (AKR1C1, AKR1C2, and AKR1C3) was analyzed in a series of 179 human brain samples that included 23 normal brain tissues, 10 grade 2 astrocytomas, 19 grade 3 astrocytomas, 77 GBMs, 38 grade 2 oligodendrogliomas, and 12 grade 3 oligodendrogliomas. Microarray data were generated by the Henry Ford Hospital (Detroit, MD) from the Affymetrix Array Series GSE4290, and these are available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4290.

Reverse Transcription–Polymerase Chain Reaction Analyses

Total RNA was extracted using the TRIzol isolation reagent (Life Technologies, Inc, Merelbeke, Belgium) according to the manufacturer's instructions. The extracted RNA was treated with DNase I (Life
Genomic Analyses

Whole genomic analyses were performed on U373 and T98G TMZ-S versus TMZ-LTT GBM cells at the VIB MicroArray Facility (UZ Gasthuisberg, Catholic University of Leuven, Leuven, Belgium) using the Affymetrix Human Genome U133 set Plus 2.0. We are expressing the genomic-related data as medians and median absolute deviations (MADs). Data analysis was carried out as described previously [29,30].

Proteomic Analyses

Western blot analyses. Proteins were electrophoresed on a 10% SDS-PAGE using a Bio-Rad electrophoresis unit at 100 V for 1 hour and transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% fat-free milk or 5% bovine serum albumin for 1 hour. Membranes were incubated overnight with primary antibodies at different concentrations: monoclonal mouse anti-AKR1C3 (1:500; Sigma, Bornem, Belgium), polyclonal rabbit anti-AKR1C2 (1:500; Abnova, Taipei, Taiwan), polyclonal rabbit anti-Glut1 (1:500; Abcam, Cambridge, UK), and polyclonal rabbit anti-Glut3 (1:150; Abcam). Proteins expression was detected by incubating the membrane with the appropriate secondary antibodies. Immunoreactive protein bands were detected using the Pierce Superni§al Chemiluminescence system (Thermo Fisher Scientific, Erembodegem, Belgium).

Immunofluorescence analyses. Cells were cultured on coverslips and fixed with 4% formaldehyde in PBS for 20 minutes at 4°C. Fixed cells were permeabilized by adding 0.2% (vol/vol) Triton X-100 and 10% (wt/vol) bovine serum albumin for a total of 20 minutes. Cells were washed twice with PBS and blocked in PBS containing 0.1% of bovine serum albumin for 1 hour at room temperature. Cells were then stained for 1 hour at room temperature with a polyclonal antibody against GLUT-10 and GLUT-3 (Abcam). Antigens were detected using antirabbit secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen). Coverslips were mounted on microscope slides with 10 μl of Moviol agent (Calbiochem, VWR, Heverlee, Belgium). Pictures were obtained using a 40x microscope oil immersion objective (Zeiss observer.Z1; Zeiss, Oberkochen, Germany) and an Axiocam HRm Zeiss camera controlled by software. Pictures were converted to stacks and navigated using the Axiosvision Rel 4.6 software. Three coverslips were analyzed, and three pictures were taken for each coverslip (with the same exposure time) for each condition (TMZ-S vs TMZ-LTT cell lines). The most representative data are shown.

Transient Knock-down of AKR1C Expression by Means of an Anti-AKR1C Small Interfering RNA in Human T98G and U373 GBM Cells

We have tested three different sequences of anti-AKR1C3 small interfering RNA (siRNA), and we have chosen the most efficient one for carrying out the silencing process. The sequence of this anti-AKR1C3 siRNA (Eurogentec, Seraing, Belgium) is 5’-UUGCAGACUUUC-CACCA-3’ for the sense sequence and 5’-UUGGUGAAGUGG-CUCACC-3’ for the antisense sequence. A siRNA-negative control (scramble; Eurogentec) was used as a process control. The sense and the antisense strands were annealed by the manufacturer in 50 mM Tris, pH 7.5 to 8.0, in 100 mM diethylpyrocarbonate-treated water. The final concentration of the siRNA duplex was 100 μM.

U373 and T98G TMZ-LTT and TMZ-S cell lines were transfected with DiC14-amidine/siRNA lipoplexes. DiC14-amidine (3-tetradecylamino-N-tert-butyl-N-tetradecylpropionamidine) was synthesized as previously described [31], and liposomes were prepared as previously [32]. Briefly, after having dissolved DiC14-amidine in chloroform, the solvent was evaporated under a steam of N2. The resulting lipid film was further dried under vacuum overnight then hydrated with prewarmed buffer (10 nM HEPES, 150 mM NaCl at pH 7.3) to reach a concentration of 10 mM and vortexed for 1 minute. Liposomes were then extruded seven times through a 0.4-μm polycarbonate filter (GE Omnisics, Herentals, Belgium) at 55°C and stored at 4°C in a 4-ml polystyrene tube (Falcon, VWR, Heverlee, Belgium). For experiments, liposomes were heated for 2 hours at 55°C before use.

To form the DiC14-amidine/siRNA lipoplexes, a volume of siRNA at 0.4 μM (usually 200 μl) was added into an equal volume of liposomes (20 μg of DiC14-amidine/ml) in RPMI (Invitrogen) while gently shaking the tube. The liposome/siRNA mixture was allowed to stand for 20 minutes at room temperature before use. Under these conditions, the lipoplex has a cationic lipid/siRNA ratio of 7.5:1 (wt/wt), and the charge ratio is calculated to be 4.54 positive charges for 1 negative charge. The liposome/siRNA mixture (at a final concentration of 0.032 μM
in siRNA) was added to U373 and T98G TMZ-LTT and TMZ-S cells for 2 hours. On day 2, each group of cells was pooled and replated for subsequent experiments. On days 3, 5, 7, and 9, glioma cells were lysed directly in Cellytic solution (Sigma). The efficiency of the AKR1C3 siRNA was evaluated by means of Western blot analysis.

Computer-Assisted Phase-Contrast Video Microscopy

For human U373 and T98G GBM cell lines, the effects of long-term treatment with TMZ and AKR1C3 siRNA (vs scramble siRNA) on cell viability, growth, and division were characterized in vitro by the use of computer-assisted phase-contrast video microscopy, as described elsewhere [33]. Cells were monitored for 72 hours. Films were compiled on the obtained time-lapse image sequences, which enabled a rapid screening of cell viability.

Cell count–based determination of global growth ratio. In each (control or treated) condition, the cell growth level was evaluated by the ratio between the numbers of cells counted in the last and first frames of the image sequences. The global growth ratio (GGR) was defined by the ratio between the two growth levels obtained in the treated and control conditions. All of the cell counts were performed in triplicate using an interactive computer tool [33,34]. The GGRs were computed at the 24th, the 48th and the 72nd hour of quantitative video microscopy analyses by dividing the cell growth level in TMZ-LTT GBM cell populations by the cell growth level at the same experimental times in the corresponding TMZ-S GBM cell populations. In the AKR1C3 siRNA experiments, we followed the number of cells for 6 days (2 days after transfection). For a quantitative measure of proliferation on AKR1C3 siRNA, the GGRs were computed between siRNA and scramble conditions at 3, 5, 7, and 9 days after transfection.

Specific analysis of cell division rate and duration. Cells undergoing division exhibit very bright patterns compared with nondividing cells. On the basis of this observation, we developed a custom division detection system capable of identifying cells undergoing division in time-lapse sequences. This detection method is based on an automatic event detection completed by an interactive validation/correction procedure as previously described [33,34]. At the end of the sequence analysis, all events are linked to different cell divisions, making available the number of cell divisions as well as their durations [33,34]. We computed the cell division numbers normalized by the number of cells that were counted in the first frame.

Determination of In Vitro Cell Cycle Kinetics

TMZ-S and TMZ-LTT GBM cells were incubated for 72 hours in culture medium. Cells were collected by trypsinization and were neutralized with cell medium. Cell suspensions were centrifuged for 10 minutes at 1000 rpm at room temperature and then washed two times with PBS before being fixed in 70% ethanol. Cells were stored at −20°C for 24 hours. Flow cytometry using propidium iodide was performed. Briefly, fixed samples were washed with PBS and resuspended in propidium iodide (1 mg/ml) and RNase (200 μg/ml) for at least 30 minutes at 37°C in the dark. Stained cells were analyzed on a cell laboratory Quanta SC flow cytometer (Beckman Coulter, Suelée, Belgium).

We analyzed the percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle and took into account the percentage of polyploid cells (as detailed elsewhere [35,36]) that were removed from the cell cycle kinetic determination.

Statistical Analyses

Data obtained from independent groups were compared by the nonparametric Kruskal-Wallis (more than two groups) or Mann-Whitney U tests (two groups). The standard survival time analyses were carried out with the log-rank test. The statistical analysis was performed using Statistica software (Statsoft, Tulsa, OK).

Results

Chronic In Vivo Treatment of TMZ-S and TMZ-LTT U373 and T98G Orthotopic GBM Xenografts with TMZ

Figure 1 A shows that without additional TMZ treatment, the U373 TMZ-LTT xenografts in vivo displayed more aggressive behavior in vivo than U373 TMZ-S xenografts in survival (P < .001). This feature has not been observed with the T98G model (Figure 1 B). The aggressive nature of U373 TMZ-LTT xenografts when compared with U373 TMZ-S cells seems to be partially associated with the higher invasive pattern for U373 TMZ-LTT xenografts, as illustrated in Figure 1 C. For all tested models, per os TMZ treatment strongly and significantly improved mouse survival (Figure 1, A and B). It should be noted that we killed all mice after 287 days to avoid contamination. To characterize the resistance acquisition in the TMZ-LTT groups, we computed survival gains as being the differences of the median survival times between each treated group and its respective control. For the U373 TMZ-S group, the median survival was 116 days for the control and up to 287 days for the treated group, whereas the median survival was 51 days for the control and 203 days for the TMZ-LTT–treated group (Figure 1 A). The survival gains were 152 days for the TMZ-LTT group and up to 171 days in the TMZ-S group. We performed similar studies for the T98G cell lines. The survival gains were 124 days for the TMZ-LTT and up to 222 days for the TMZ-S groups (Figure 1 B). The diminution in survival gain for the TMZ-LTT group compared with the TMZ-S group suggests that long-term treatment with TMZ induces the development of a certain level of resistance to this chemotherapeutic agent, whereas full resistance has not been reached.

The TMZ IC50 (inhibitory concentration 50%) in vitro growth-inhibitory concentrations ranged between 150 and 200 μM for TMZ-S groups in U373 and T98G cell lines (as revealed by the MTT colorimetric assay; data not shown), whereas TMZ-LTT U373 and T98G cells were adapted to grow up to 1 mM.

Characterization of Cancer Stem Cell Profiles in TMZ-LTT versus TMZ-S U373 and T98G GBM Cells

Beier et al. [28] recently demonstrated that TMZ induces a dose- and time-dependent decline of the stem cell subpopulation (i.e., CD133+ cells) in various experimental glioma models. We thus analyzed the expression of CD133 and other stem cell markers such as CD44, nestin, and MELK [24,37] in TMZ-LTT U373 and T98G GBM cells and compared the expression to the levels found in TMZ-S cells. The RT-PCR analyses (Figure 1 D) did not reveal any difference in the patterns of expression of stem cell markers in U373 and T98G TMZ-S versus TMZ-LTT GBM cells [22,23]. Indeed, in our current study, long-term TMZ treatment did not eliminate CD133+ cells in the U373 and T98G GBM cell lines (Figure 1 D) [22,23], whereas we had previously shown that this occurred with Hs683 malignant oligodendroglioma cells [30]. It has been established that highly malignant gliomas contain various proportions of astrocytic versus oligodendroglial tumor cells [38,39]. Thus, the possibility remains that TMZ preferentially eliminates CD133+ cells in malignant gliomas with an oligodendroglial component.
It must be noted that U373 GBM cells express GFAP depending on the substratum on which the cells are cultured [22]. In the current study, the GBM cells were cultured on plastic. The human HT29 colon cancer cells were used as a control cell population to illustrate the absence of vimentin and the presence of cytokeratin CK20 expression in these cells (Figure 1D).

Cell Population Growth Dynamics and Cell Cycle Kinetics

Table 1 reveals that genes involved in cell proliferation enabled the best discrimination between U373 and T98G TMZ-S and TMZ-LTT cells. We therefore made use of quantitative video microscopy and flow cytometry to analyze the growth dynamics and cell cycle kinetics of the U373 and T98G TMZ-S versus TMZ-LTT GBM cell populations. Figure 2A shows that U373 TMZ-LTT GBM cells display higher growth kinetic rates over time when compared with U373 TMZ-S GBM cells, as shown by the GGR index. Indeed, a GGR of about 1.4 at 24 hours for U373 LTT cell indicates that the growth level was 1.4 times higher in the U373 TMZ-LTT GBM cell population at the 24th hour of the quantitative video microscopy analysis when compared with the growth rate of U373 TMZ-S cells. This increase in cell grow rate consistent with the in vivo data illustrated in Figure 1A, 1Ca, and 1Cb clearly shows higher biological aggressiveness for U373 TMZ-LTT GBM cells compared with U373 TMZ-S GBM cells. In contrast, T98G TMZ-LTT GBM cells seem to grow slower than T98G TMZ-S cells (Figure 2A). This feature has been validated by the in vitro experiments reported below and could, at least partly, relate...
to acquired dependence to the chemotherapeutic as already demonstrated by Martello et al. [40].

We performed flow cytometry analysis to investigate the cell cycle kinetics in U373 and T98G TMZ-LTT versus TMZ-S cell populations (Figure 2B). No significant differences were found in terms of cell cycle kinetics between these various GBM cell populations (Figure 2B). We made use of T98G GBM TMZ-S cells treated for 72 hours with 150 μM TMZ as a positive control (Ct+) for the flow cytometry analysis.

The data in Figure 2C show that the higher growth kinetics (as revealed by the GGR assessment in Figure 2A) observed in U373 TMZ-LTT GBM cells when compared with TMZ-S counterparts (Figure 2A) could be partially attributed to an increased mitotic rate, with the fraction in G1, S, and G2 phases remaining unaffected between TMZ-LTT and TMZ-S GBM cells (Figure 2B). Similarly, the lower growth kinetics observed in T98G TMZ-LTT GBM cells when compared with TMZ-S counterparts (Figure 2A) could be partially attributed to a weaker mitotic rate (Figure 2C). The higher mitotic rate observed in U373 TMZ-LTT GBM cells when compared with the TMZ-S counterparts (Figure 2C) seems to translate into shortened (P < .001) mitosis duration times (data not shown). In a similar fashion, the decrease in mitotic rates observed in T98G TMZ-LTT GBM cells when compared with the TMZ-S counterparts (Figure 2C) seems to translate to increased (P < .001) mitosis duration times (data not shown).

**Whole Human Genome Analyses in GBM TMZ-S versus GBM TMZ-LTT Models**

Table 1 details the gene categories whose expression profiles were significantly altered in human U373 and T98G GBM cell lines treated for long periods with increasing concentrations of TMZ up to 1 mM. The genes that were most significantly altered both in the U373 and T98G GBM models included the AKRIC (AKR1C1, AKR1C2, and AKR1C3) and GLUT transporter (mainly GLUT-1, GLUT-3, GLUT-5, and GLUT-10) families of genes. We therefore focused our attention on the proteomic expressions of these genes in the U373 and T98G TMZ-S and TMZ-LTT cell lines.

Figure 2D illustrates which were the three genes whose expression were the most increased during long-term TMZ treatment. For example, “SCRG1 x13” indicates that the expression of the SCRG1 gene was increased by 13 times under prolonged treatment with TMZ. It is interesting to note that SCRG1 is a potential marker of autophagy [41], knowing that TMZ indeed induces marked proautophagic effects in GBM cells [12,13].

**The GLUT genes.** Figure 3 shows that GLUT-1 was already highly expressed in the U373 and T98G TMZ-S cells and that treating these cells for long periods with increasing concentrations of TMZ only slightly increased GLUT-1 expression in the U373 and T98G TMZ-LTT cells. The data obtained from clinical samples confirm the high levels of GLUT-1 expression in clinical samples of gliomas as well as in normal brain tissues, with no differences in expression between the various histopathologic groups.

Sustained treatment of U373 and T98G TMZ-S GBM cells with TMZ increased GLUT-3 expression in both cell lines. We therefore decided to investigate GLUT-3 expression at the proteomic level in these cell lines. Figure 4, A and B, shows that we failed to detect an increase in GLUT-3 and GLUT-10 expressions using Western blot analysis. We thus proceeded with immunofluorescence analysis and

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*The system of categorizing genes (e.g., “GO (gene ontology) Biological System”) in the databases provided by the EASE software package [29,30].
†The specific category of genes within the system.
‡The probability value characterizing the change of proportion between the two ratios: population hits/population total and list_hits/list_total. It constitutes the upper band of the distribution of leave-1-out Fisher exact probabilities computed on these two ratios.
§An iteratively running overrepresentation analysis on random gene lists to determine true probability more accurately.
*The number of genes in the list of differentially expressed genes (list_diff resulting from the microarray data analysis) that belong to the Gene Category.
**The number of genes in list_diff that belong to any gene category within the system.
"**The number of genes in list_tot that belong to any gene category within the system.
observed variations in the expression of the two glucose transporters, which may partly explain the apparent discrepancies between the Western blot and immunofluorescence analyses. The latter show that GLUT-10 protein expression is indeed more important in U373 TMZ-LTT (Figure 4Cb) than in U373 TMZ-S (Figure 4Ca) GBM cells. We obtained similar data with T98G TMZ-S versus TMZ-LTT GBM cells (data not shown). In fact, the expression of GLUT-10 is localized to a perinuclear body, the nature of which has been already demonstrated by Coucke et al. [42]. Figure 4Cd highlights the GLUT-10 perinuclear localization, with the corresponding bright field in Figure 4Cc.

We also observed an increase in GLUT-3 perinuclear protein expression in U373 TMZ-LTT (Figure 4Db) and T98G TMZ-LTT (data not shown) compared with U373 TMZ-S (Figure 4Da) and T98G TMZ-S (data not shown) GBM cells.

Sustained TMZ treatment increased GLUT-5 messenger RNA (mRNA) levels in U373 GBM cells (GLUT-5 seems not to be expressed in T98G GBM cells) and GLUT-10 mRNA levels in T98G GBM cells, with significant levels of GLUT-10 mRNA already present in U373 TMZ-S GBM cells (Figure 3). When analyzing the glioma clinical samples, GLUT-10 mRNA expression levels were maximal in GBMs, whereas GLUT-3 and GLUT-5 mRNA levels displayed heterogeneous patterns among the various histopathologic groups under study.

The AKR1C genes. Figure 5 shows that sustained treatment of U373 and T98G GBM cells with TMZ for long periods (several months) led to marked increases in AKR1C1, AKR1C2, and AKR1C3 mRNA levels, a feature that has been confirmed at the proteomic level for AKR1C2 and AKR1C3, as illustrated at the bottom of Figure 5. We also investigated the patterns of AKR1C1 expression in U373 and T98G TMZ-S versus TMZ-LTT at the proteomic level but could not confirm the overexpression of AKR1C1 observed at the mRNA level (data not shown).

As detailed in the Discussion, AKR1C gene products are implicated in chemoresistance and cell proliferation processes. Reducing AKR1C3 expression in U373 and T98G GBM cells affected cell behavior on two levels: 1) the growth dynamics of U373 and T98G GBM cell populations (by means of quantitative video microscopy) and 2) the sensitivity of U373 and T98G GBM cells to TMZ (by means of the MTT colorimetric assay). We focused on AKR1C3 because we were able to design selective and efficient silencing for AKR1C3. We have not investigated...
the silencing of AKR1C1 and AKR1C2 because the homology of the two genes is high, preventing us from obtaining selective AKR1C1 versus AKR1C2 siRNA.

The data revealed that transient silencing of AKR1C3 in U373 TMZ-LTT GBM cells significantly decreased their global growth rate (as assessed by the GGR index in Figure 6). Western blot analysis revealed the efficiency of knockdown using the AKR1C3 siRNA (Figure 6). Silencing AKR1C3 in U373 and T98G TMZ-LTT GBM cells did not alter their sensitivity to a variety of cytotoxic drugs (data not shown). Altogether, these data suggest that TMZ-induced increases in AKR1C3 expression did not modify GBM cell sensitivity to cytotoxic drugs, whereas U373 GBM cells defended themselves in

Figure 3. Left panels: Determination of GLUT-1, -3, -5, and -10 mRNA levels (by means of an Affymetrix whole genome analysis; see Materials and Methods) in human T98G and U373 TMZ-S (open bars) and TMZ-LTT (gray bars) GBM cells. The data are illustrated as medians and MADs (error bars). Right panels: Determination of GLUT-1, -3, -5, and -10 mRNA levels in a clinical series of 23 normal brain samples (NB) and 159 gliomas (O-II = oligodendrogloma grade 2 [n = 38], O-III = oligodendrogloma grade 3 [n = 12], A-II = astrocytoma grade 2 [n = 10], A-III = astrocytoma grade 3 [n = 19], and GBM = GBM grade 4 [n = 77]). Data were extracted from microarray experiments performed at the Henry Ford Hospital GSE4290), as described in the Materials and Methods section. Data are reported as individual tissue measurements (1 tissue measurement = 1 black dot) and median values (bars). Statistical comparisons have been performed between each tumor group and the normal brain tissue (control) group.
increasing their proliferation rates (Figure 2), a feature that translated in vivo by an increased biological aggressiveness (Figure 1). AKR1C3 thus seems implicated in this process observed in U373 GBM cells.

In contrast, T98G GBM cells became dependent on TMZ for their growth as detailed previously (Figure 2).

Discussion

Gliomas account for more than 50% of all primary brain tumors [1–6]. The worst prognosis is associated with gliomas of astrocytic origin, whereas gliomas with an oligodendroglial origin offer a higher sensitivity to chemotherapy, especially when oligodendroglia cells display 1p19q deletions [1,4]. TMZ provides therapeutic benefits and is commonly used with radiotherapy in highly malignant astrocytic tumors, including GBMs [1–7]. However, all GBMs become resistant to TMZ [43]. Nevertheless, it seems that glioma cell sensitivity to TMZ may differ in whether they arose from oligodendroglial versus astroglial progenitors. Indeed, we recently reported increased TMZ sensitivity of Hs683 orthotopic malignant oligodendrogliomas that were previously treated in vitro with increasing TMZ concentrations before being xenografted into the brains of immunocompromised mice [30]. Whole genome and proteomic analysis revealed that this increased TMZ sensitivity could be explained, at least partly, by a TMZ-induced p38-dependent dormancy state, which in turn resulted in changes in amino acid metabolism balance, delay in growth, and a decrease in Hs683 oligodendroglioma cell–invasive properties [30].

In the current study, we made use of two GBM cell lines of astrocytic origin, namely, U373 and T98G [22,23], and observed that TMZ resistance acquired by these two cells lines translated into overexpression of several glucose transporters (GLUT) and of three members of the aldo-keto reductase (AKR) family, namely, AKR1C1, AKR1C2, and AKR1C3.
As emphasized by Gatenby and Gillies [44], a near-universal property of primary and metastatic cancers is up-regulation of glycolysis, resulting in increased glucose consumption, a feature that was elucidated in 1956 by Warburg [45]. Persistent metabolism of glucose to lactate even in aerobic conditions seems to be an adaptation to intermittent hypoxia [46]. HIF-1α, a hypoxia-activated transcription factor, is implicated in the overexpression of several important enzymes implicated in the conversion of glucose into lactate [46].

Figure 5. Left panels: Determination of AKR1C1, AKR1C2, and AKR1C3 mRNA levels of expression (by means of an Affymetrix whole genome analysis; see Materials and Methods) in human T98G and U373 TMZ-S (open bars) and TMZ-LTT (gray bars) GBM cells. Data are illustrated as medians and MADs (error bars). Right panels: Determination of AKR1C1, AKR1C2, and AKR1C3 mRNA expression levels in a clinical series of 23 normal brain samples (NB) and 159 gliomas (O-II = oligodendroglioma grade 2 [n = 38], O-III = oligodendroglioma grade 3 [n = 12], A-II = astrocytoma grade 2 [n = 10], A-III = astrocytoma grade 3 [n = 19], and GBM = GBM, grade 4 [n = 77]). Data were extracted from microarray experiments performed at the Henry Ford Hospital as described in Materials and Methods section. Data are reported as individual tissue measurements (1 tissue measurement = 1 black dot) and median values (bars). Statistical comparisons have been performed between each tumor group and the normal brain tissue (control) group. Bottom: Western blot analysis of AKR1C2 and AKR1C3 protein expression in T98G and U373 TMZ-S and TMZ-LTT GBM cells.
GLUT-3
AKR1C2
versus to prostaglandin J
AKR1C3
AKR1C2
siRNA (Figures 3 and 4).

GLUT-1, and GLUT-10
AKR1C2
and [48], and alter angiogenesis [42]. Overexpression of
scramble transfected cells. All experiments were performed in triplicate. Bottom panel: Methodological control (Western blot analysis) of AKR1C3 siRNA versus scramble siRNA efficiency in U373 TMZ-LTT GBM cells. The Western blot (WB) bands have been digitized, and quantitative data below the WB bands represent the number of pixels of the band multiplied by pixel intensity, the final result of which has been normalized to the corresponding tubulin band.

Our data reveal that long-term treatment of astroglioma U373 and T98G cells leads to overexpression of GLUT-1, -3, and -10. Hypoxia increases the expression of GLUT-1 [47,48], GLUT-3 [48], and GLUT-10 [47]. Therefore, it seems that astroglioma cells facing adverse TMZ-induced events react by overexpressing GLUT transporters and subsequently increasing glucose intake to partly counteract the TMZ-induced angiogenic effects [11]. Indeed, glycolytic breakdown of glucose is preceded by the transport of glucose across the cell membrane, a rate-limiting process mediated by facilitative glucose transporter proteins belonging to the facilitative glucose transporter/solute carrier GLUT/SLC2A family [44,48]. Mutations in GLUT-10 alter angiogenesis [42]. Overexpression of GLUT-3 mRNA is correlated with tumor grade, and protein expression of this glucose transporter has been demonstrated in GBM but not in other grade [49]. Nishio et al. [50] have suggested that the increased expression of GLUT-3 may be closely related to the malignant progression of astrocytomas to GBMs and related to the aberrant neovascularization that accompanies GBMs. GLUT-3 indeed seems to be a GLUT transporter that correlates directly with glioma aggressiveness [49,51]. Furthermore, our current data clearly show that long-term treatment of both U373 and T98G GBM cells translates into overexpression of GLUT-3 (Figures 3 and 4).

Other studies have reported associations between increased GLUT expression and increased proliferative indices [48,52]. It is well known that astrocytes play a role in brain metabolism, being a key element in the capture of energetic compounds from the circulation and delivering these compounds to active neurons [53]. Glioma cells display higher proliferation rates than normal astrocytes, and this proliferative dysfunction is associated with changes in gap junction communication [53]. Indeed, the inhibition of gap junction communication is associated with an increase in glucose uptake because of a rapid change in the localization of both GLUT-1 and type 1 hexokinase; this effect persists from the up-regulation of GLUT-1 and type 1 hexokinase and to the induction of GLUT-3 and type 2 hexokinase [53]. In addition, cyclins D1 and D3 have been found to act as sensors of the inhibition of gap junctions and have been proposed to play the role of mediators in the observed mitogenic effect [53]. All of these features may partly explain the data we report here. The data suggest potential relationships between GLUT expression, cell proliferation, and partial acquisition of resistance to TMZ, at least in U373 GBM cells, which diffusely invade the brain parenchyma [54] and display higher biological aggressiveness compared with T98G GBM cells [22,23].

GLUT-5 is a particular glucose transporter because it displays a high affinity for fructose and a low affinity for glucose [55]. The data from the present study show that GLUT-5 is expressed at very low basal levels in U373 GBM cells but is not expressed in T98G GBM cells and that long-term TMZ treatment augments its expression in U373 GBM cells (Figure 3).

AKR1C1, AKR1C2, and AKR1C3 are members of the AKR superfamily [56]. The AKR1 family of proteins are phase 1 drug-metabolizing enzymes involved in the maintenance of steroid homeostasis, prostaglandin metabolism, and metabolic activation of polycyclic aromatic hydrocarbons [56,57]. Increased expression of AKR1C proteins directly correlates with poor prognosis in breast and prostate cancers [56] and with chemoresistant features in non–small cell lung [58] and colon [59] cancer. The data in the current study reveal that the expression of AKR1C1, AKR1C2, and AKR1C3 was significantly increased in both U373 and T98G GBM cells (at both the mRNA and protein levels) after long-term TMZ treatment (Figure 5). Transient silencing of AKR1C3 protein in both U373 TMZ-LTT GBM cells induced a decrease in proliferation rates (Figure 6) but an increase in chemosensitivity of AKR1C3-deficient U373 and T98G TMZ-LTT cells when compared with naïve (wild-type) U373 and T98G TMZ-LTT cells. Chien et al. [57] established mouse NIH3T3 mouse cell lines ectopically and stably expressing human AKR1C1, AKR1C2, or AKR1C3 and showed that ectopic expression of human AKR1C1 and AKR1C2, but not AKR1C3, significantly enhanced foci formation. After subcutaneous injection of these stable cell lines into nude mice, fibrosarcomas formed from all three cell lines [57]. However, the number and size of tumors formed by the AKR1C3-expressing cell line was fewer and smaller than those formed by AKR1C1- and AKR1C2-expressing cells [57]. Davies et al. [60] identified AKR1C isoforms as novel targets of jasmonates in cancer cells. Furthermore, these authors provided further evidence of the promise of these compounds, or derivatives thereof, as adjunctive therapies in the treatment of cancer.

Increased levels of expression of AKR1C1 paralled increased cell proliferation activity in human colon cancer cells [59]. It has been known that AKR1C3 can catalyze the conversion of prostaglandin D2 to prostaglandin F2α, which is an activator of cell proliferation [61]. This reaction inhibits the natural conversion of prostaglandin D2 to prostaglandin J2, which is known to be a ligand of peroxysome proliferator-activated receptor gamma (PPARγ) [61]. The activation of PPARγ by a natural agonist (such prostaglandin J2) usually increases the expression of GLUT-3, as demonstrated by Garcia-Bueno et al. [62]. Thus, although we observed an overexpression of AKR1C3, we should...
detect a decrease in cellular GLUT-3 expression. However, the reverse feature has been observed, and it is already known that HIF-1α activates transcription of GLUT-1 and -3 [45]. Thus, altogether, these data suggest that GLUT-3 overexpression is controlled by HIF-1α rather than by the PPARγ signaling pathways.

In conclusion, our data show that long-term treatment of human astrogliaoma cells with TMZ induces increased expression of facilitative glucose transporter/solute carrier GLUT/SLC2A family members, primarily GLUT-3, and of the AKR1C family of proteins that are phase 1 drug-metabolizing enzymes involved in the maintenance of steroid homeostasis, prostaglandin metabolism, and metabolic activation of polycyclic aromatic hydrocarbons. GLUT-3 has previously been suggested to exert roles in GBM neovascularization processes, whereas TMZ has been shown to exert antiangiogenic effects in experimental gliomas. AKR1C1 has previously been shown to be associated with oncogenic potential and proproliferative effects, as has AKR1C3 in this latter case. Both AKR1C1 and AKR1C2 proteins are involved in cancer cell chemoresistance. Selective targeting of GLUT-3 and/or AKR1C proteins (by means of jasmonates, in the case of AKR1C) in GBM could delay the acquisition of resistance to TMZ by astrogliaoma cells when treated for long periods with this drug.

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