Temozolomide Modifies Caveolin-1 Expression in Experimental Malignant Gliomas *In Vitro* and *In Vivo*[1]

Céline Bruyère*, Laurence Abeloos[†], Delphine Lamoral-Theys[‡], Rebecca Senetta[§], Véronique Mathieu*[,2], Marie Le Mercier*[,3], Richard E. Kast[¶], Paola Cassoni[§], Guy Vandenbussche[#], Robert Kiss*[,2] and Florence Lefranc*[,†,2]

*Laboratoire de Toxicologie, Faculté de Pharmacie, Université Libre de Bruxelles, Brussels, Belgium; [†]Service de Neurochirurgie, Hôpital Erasme, Brussels, Belgium; [‡]Laboratoire de Chimie Bioanalytique, Toxicologie et Chimie Physique Appliquée, Faculté de Pharmacie, Université Libre de Bruxelles, Brussels, Belgium; [§]Department of Biomedical Sciences and Human Oncology, University of Turin, Turin, Italy; [¶]Department of Psychiatry, University of Vermont, Burlington, VT, USA; [#]Laboratory for the Structure and Function of Biological Membranes, Faculty of Sciences, Université Libre de Bruxelles, Brussels, Belgium

Abstract

BACKGROUND: Caveolin-1 is a protein that displays promotive *versus* preventive roles in cancer progression according to circumstances. Temozolomide (TMZ) is the standard chemotherapeutic to treat glioma patients. The present work aims to characterize TMZ-induced effects on caveolin-1 expression in glioma cells. *METHODS:* Human astroglioma (U373 and T98G) and oligodendroglioma (Hs683) cell lines were used *in vitro* as well as *in vivo* orthotopic xenografts (Hs683 and U373) into the brains of immunocompromised mice. *In vitro* TMZ-induced effects on protein expression and cellular localization were determined by Western blot analysis and on the actin cytoskeleton organization by means of immunofluorescence approaches. *In vivo* TMZ-induced effects in caveolin-1 expression in human glioma xenografts were monitored by means of immunohistochemistry. *RESULTS:* TMZ modified caveolin-1 expression and localization *in vitro* and *in vivo* after an administration schedule that slightly, if at all, impaired cell growth characteristics *in vitro*. Caveolin-1 by itself (at a 100-ng/ml concentration) was able to significantly reduce invasiveness (Boyden chambers) of the three human glioma cell lines. The TMZ-induced modification in caveolin-1 expression in flotation/raft compartments was paralleled by altered Cyr61 and β_1 integrin expression, two elements that have already been reported to collaborate with caveolin-1 in regulating glioma cell biology, and all these features led to profound reorganization of the actin cytoskeleton. An experimental Src kinase inhibitor, AZD0530, almost completely antagonized the TMZ-induced modulation in caveolin-1 expression. *CONCLUSION:* TMZ modifies caveolin-1 expression *in vitro* and *in vivo* in glioma cells, a feature that directly affects glioma cell migration properties.

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Introduction

The combination of temozolomide (TMZ) with radiotherapy has been shown to improve survival in glioblastoma (GBM) patients after surgical resection [1,2]. However, the prognosis associated with GBM patients remains dismal because i) GBMs cannot be completely removed surgically because of the highly infiltrative nature of these tumors into the brain parenchyma [1,3] and ii) GBM cells defend themselves against TMZ during chronic treatments, with the emergence of TMZ-resistant tumors observed at both the experimental [4] and the clinical [1,2] levels.

The therapeutic benefits contributed by TMZ relates to the fact that it induces double-strand DNA breaks through generation of methylguanosine [5] concomitantly with sustained autophagy-related processes [6,7], all these features ending with apoptosis in GBM cells [8]. TMZ also displays antiangiogenic effects [9]. In contrast, as mentioned above, TMZ treatment of GBMs can lead to the emergence of TMZ-resistant tumors. Fisher et al. [10] showed that treatment of human malignant glioma cells with TMZ activate stress mechanisms that include up-regulation of angiogenesis-inducing proteins, notably hypoxia-inducible factor-1α and vascular endothelial growth factor. We recently demonstrated that TMZ activates galectin-1 (a hypoxia-regulated protein [11]) expression in malignant glioma cells, a process that leads to marked proangiogenic effects [12] and the activation of chemoresistance [13]. The fact of decreasing galectin-1 expression in experimental malignant gliomas [12,13] and melanomas [14] leads in turn to increased therapeutic benefits contributed by TMZ.

Numerous hypoxia-regulated proteins are implicated in glioma biology [15,16] among which caveolin-1 [17], and we were therefore wondering whether TMZ could modify caveolin-1 expression and/or cellular localization in GBM cells.

Caveolin-1 is a 21- to 24-kDa scaffold protein and an essential constituent of caveolae, which are flask-shaped invaginations of the plasma membrane [18]. In caveolae, caveolin-1 directly interacts with several signaling molecules, including growth factor receptors (e.g., the epidermal growth factor receptors [EGFRs]), kinases (including Src), G proteins, and adhesion molecules [19,20]. Caveolin-1 contributes to the regulation of multiple cancer-associated processes, including cellular transformation, tumor growth, cell migration and metastasis, cell death and survival, multiple drug resistance, and angiogenesis [21–23]. However, both cancer growth-enhancing and growth-inhibiting effects have been reported for caveolin-1 under different conditions [21–23]. Caveolin-1 is expressed in normal and malignant glial cells [24–27] as well as in brain blood vessels [28].

The present work thus aims to i) investigate as whether TMZ is able to modify caveolin-1 expression and/or cellular localization in glioma cells, ii) characterize caveolin-1–mediated effects on glioma cell invasion, and iii) identify some signaling elements by which TMZ modifies caveolin-1 expression in detergent-insoluble floating complexes of glioma cells.

We used three GBM models. The U373 and T98G models are of astrocytic origin [29,30]. U373 GBM cells display resistance to proapoptotic stimuli [30,31] but actual sensitivity to sustained proautophagic stimuli, leading to massive cell death [32]. The Hs683 GBM model is of oligodendroglial origin [29,33] and displays sensitivity to proapoptotic drugs [30,31]. Hs683 GBM cells contain only one *Notch2* gene copy per diploid genome as do oligodendrogliomas, whereas U373 GBM cells contain two copies as do astrogliomas [34]. The HS683 model might correspond to the few GBMs displaying an oligodendroglial origin [35] and/or component [36]. These three models markedly and diffusely invade the brain parenchyma when orthotopically grafted into the brains of immunocompromised mice [4,9,12,30,37].

Materials and Methods

Cell Cultures and Compounds

Hs683 (ATCC code HTB-138), U373 (ATCC code HTB-17), and T98G (ATCC code CRL-1690) human GBM cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in our laboratory, as detailed previously [9,12,13,32,33,38,39].

TMZ was obtained from Schering Plough (Brussels, Belgium). Caveolin-1 recombinant protein purified on glutathione sepharose 4 Fast Flow was obtained from Abnova (Taipei, Taiwan), Nycodenz (5-(N-2,3-dihydroxypropylacetamido)-2,4,6-tri-iodo-N-N′-bis(2,3 dihydroxypropyl) isophthalamide), a nonionic iodinated gradient medium was purchased from Gentaur (Brussels, Belgium), and the EGFR kinase inhibitor (erlotinib) and Src tyrosine kinase inhibitor (AZD0530) were obtained from Selleck Chemicals Co (Shanghai, China).

Protein Extraction for Raft Resident Caveolin-1

Caveolin-1 is a part of caveolae that are Triton-insoluble complexes and are difficult to solubilize using conventional detergents found in typical lysis buffers. Therefore, flotation of detergent-insoluble proteins was performed as described by Naslavsky et al. [40].

Briefly, in all experiments, confluent cells growing in three 75-mm dishes were pelleted and lysed with 750 μl of lysis buffer (1% of TX-100 in TNE (150 mM NaCl, 25 mM Tris-HCl, and 5 mM EDTA, pH 7.5)) on ice for 30 minutes. Lysates were spun at low speed (3000 rpm, 4°C, 5 minutes) to generate a postnuclear supernatant. All subsequent steps were performed on ice. Lysates were adjusted to 35% Nycodenz by adding an equal volume of ice-cold 70% Nycodenz dissolved in TNE. One-and-a-half milliliters of the samples was loaded at the bottom of 4-ml tubes (Beckman Coulter, Analis, Suarlee, Belgium). An 8% to 25% Nycodenz linear step gradient in TNE was then overlaid above the lysate (350 μl each of 25%, 22.5%, 20%, 18%, 15%, 12%, and 8% Nycodenz). The tubes were spun at 55,000 rpm for 4 hours at 4°C in a Beckman Optima LE-80K with a SW 60 Ti rotor (Beckman Coulter). Twelve 330-μl fractions were collected from the bottom of the tube.

Protein Expression Measurements

Western blot analyses were performed to measure protein expression, as detailed previously [9,12,13,38,39]. The primary antibody incubation step was omitted as a negative control experiment. Integrity and quantity of the extracts were assessed by tubulin immunoblot analysis. Proteins were detected by Western blot analysis using the following primary antibodies: anti–caveolin-1 (1:250 dilution; BD Transduction Laboratories, Erembodegem, Belgium), antitubulin (1:3000 dilution; Abcam, Cambridge, UK), anti-Cyr61 (1:1000 dilution; Abcam, Cambridge, UK), and anti–β_1 integrin (1:500 dilution; BD Transduction Laboratories). Secondary antibodies were purchased from Pierce (PerbioScience, Erembodegem, Belgium). Western blots were developed using the Pierce SuperSignal Chemiluminescence System.

Cytology

The effect of TMZ (100 μM) on actin cytoskeleton organization of GBM cells was investigated using fluorescent probes, as detailed

elsewhere [39,41]. The green fluorochrome Alexa Fluor 488–conjugated phallacidin (Molecular Probes, Inc, Invitrogen, Merelbeke, Belgium) was used to label fibrillary actin, whereas the red fluorochrome Fluor 594–conjugated DNAseI (Molecular Probes, Inc) was used to label globular actin.

Invasion Assay

Invasive features of T98G, U373, and Hs683 GBM cells were assessed *in vitro* using the Boyden transwell invasion system (BD BioCoat Matrigel invasion chambers; BD Biosciences Discovery Labware, Bedford, MA), as detailed elsewhere [33]. We studied the effects of caveolin-1 on malignant glioma cell migration when applied to the top as opposed to the bottom of the Boyden chambers.

Immunohistochemical Procedures for Human Glioma Xenografts

We used histologic slides for caveolin-1 immunohistochemical staining in Hs683 and U373 GBM xenografts from a previous *in vivo* experiment, in which we demonstrated TMZ-induced antiangiogenic effects and improvements in survival [9]. In this previous experiment, immunocompromised mice bearing orthotopic Hs683 or U373 xenografts were treated (or left untreated as control) three times a week (Monday, Wednesday, and Friday) for three and five consecutive weeks, respectively, with TMZ (40 mg/kg, intravenously) [9].

The mouse brains were fixed in 4% buffered formaldehyde, routinely processed, and paraffin-embedded. Three-micrometer sections were prepared and routinely stained with hematoxylin and eosin. Two additional sections, collected on SuperFrost Plus Slides, were used for immunohistochemical analysis. Caveolin-1 immunoreactivity was studied in all cases, and the immunohistochemical reaction with the anti–caveolin-1 antibody (rabbit polyclonal antibody, diluted 1:350; Santa Cruz Biotechnology, Santa Cruz, CA) was performed in an automated immunostainer (Ventana BenchMark Auto-Stainer; Ventana Medical Systems, Tucson, AZ).

Results

Temozolomide Modulates Raft Resident Caveolin-1 Expression in Human GBM Cell Lines In Vitro

We previously determined the 50% reduction in *in vitro* growth (IC$_{50}$) concentrations for TMZ on after 3 days of glioma cell culture with the drug, and we obtained 118 ± 3 µM for U373, 634 ± 23 µM for T98G, and 719 ± 12 µM for Hs683 GBM cells [9]. In the current experiment, U373, T98G, and Hs683 GBM cells were chronically treated *in vitro* with 100 µM TMZ, which was added to the culture medium each day for 7 hours (after which fresh medium replaced the medium with drugs for 17 hours) for five consecutive days. Protein analyses were performed at 3, 5, and 7 days after the fifth TMZ addition to the GBM culture medium (Figures 1 and 2). We previously
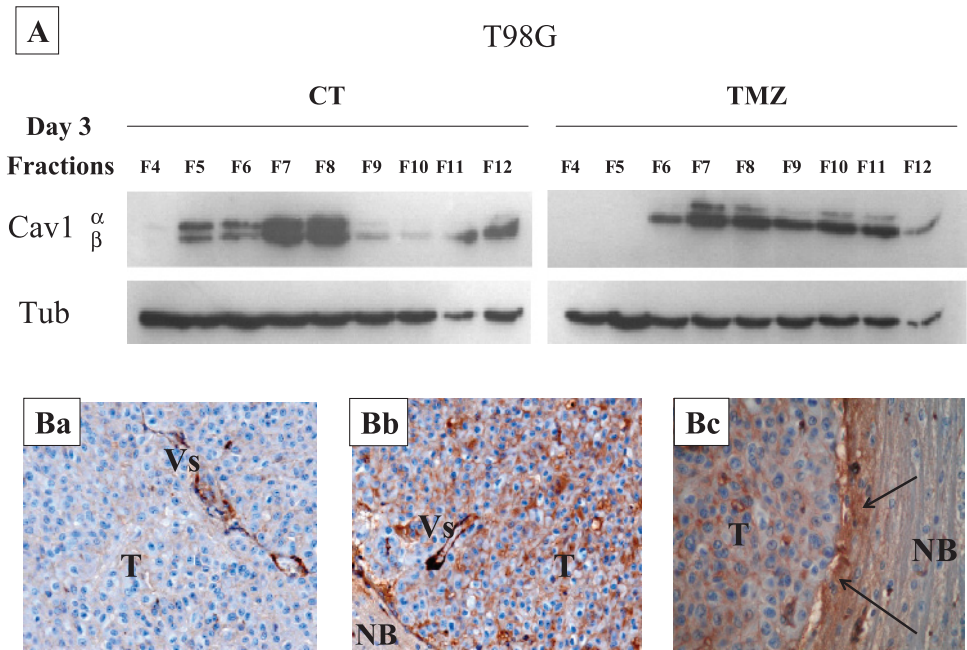
Figure 1. Temozolomide modulates raft resident caveolin-1 expression *in vitro* and increases caveolin-1 expression *in vivo* in experimental GBMs. (A) Western blot analysis of the expression of raft resident caveolin-1 in the different flotation fractions (F4-F12) obtained from T98G GBM cells left untreated (CT) or treated with 100 μM TMZ for 7 h/d for five consecutive days. Raft resident caveolin-1 expression was measured on the third day (day 3) after the end of the TMZ treatment. (B) Caveolin-1 immunoreactivity in Hs683 GBM tumors implanted into brains of immunodeficient mice and treated (Bb and Bc) or not treated (Ba) with TMZ (40 mg/kg per os, three times per week for three consecutive weeks, with immunohistochemical analyses performed 1 week after the end of the treatment). Caveolin-1 expression was absent from the Hs683 tumor (T) left untreated (Ba), in which only the vessels (Vs) were immunoreactive for caveolin-1. Caveolin-1 expression was marked in Hs683 tumors (T) after TMZ treatment (Bb). Caveolin-1 expression in the tumor bulk (T) was associated with accumulation of caveolin-1 in the extracellular matrix around the invasive part of the tumor (black arrows in Bc). The normal brain (NB) expressed weak levels of caveolin-1 (Bc).
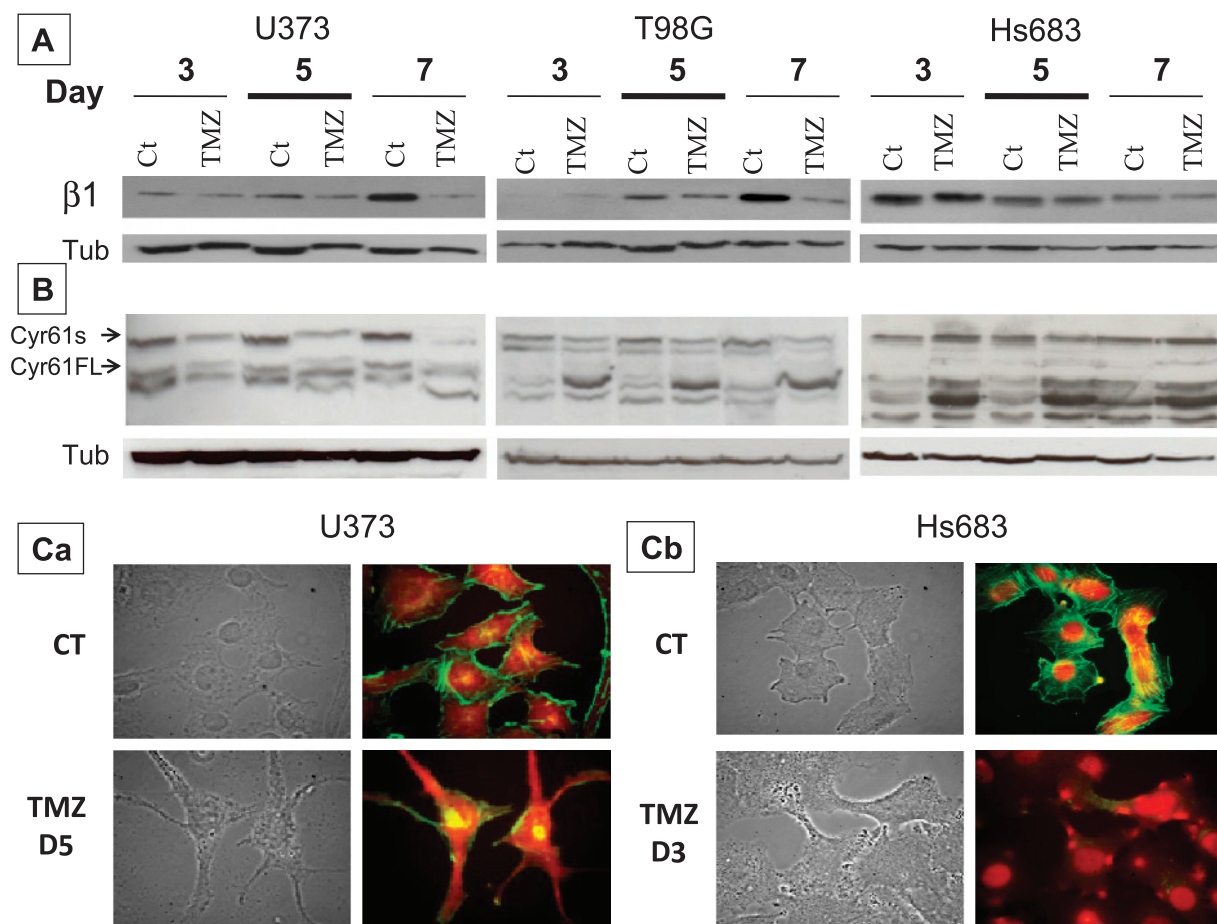
Figure 2. Temozolomide decreases β_1 integrin and modulates Cyr61 and actin cytoskeleton organization in GBM cell lines. (A) Western blot analysis of the expression of β_1 integrin in U373, T98G, and Hs683 GBM cells left untreated (CT) or treated with 100 μM TMZ for 7 h/d for five consecutive days. The β_1 integrin expression was measured on the third (day 3), fifth (day 5), and seventh (day 7) days after the end of the TMZ treatment. (B) Western blot analyses of the expression of Cyr61 in U373, T98G, and Hs683 GBM cell lines left untreated (CT) or treated with 100 μM TMZ using a treatment schedule identical to that described above for β_1 integrin. (C) Fluorescence microscopic visualization of the actin cytoskeleton (red fluorescence shows globular (nonpolymerized) actin and green fluorescence shows fibrillary (polymerized) actin) under control conditions (CT) and 3 (TMZ D3) and 5 (TMZ D5) days after termination of TMZ (100 μM) treatment in U373 (Ca) and Hs683 (Cb) glioma cells according to a treatment schedule identical to that described above for β_1 integrin.

showed that 5 × 100 μM TMZ treatment (with observation 7 days after the fifth TMZ administration) did not impair T98G cell population growth, slightly impaired Hs683 cell population growth, and moderately impaired U373 cell population growth [9]. Thus, the *in vitro* 5 × 100 μM treatment adopted here for three distinct human GBM cell lines slightly, if at all, impaired cell growth characteristics.

Raft resident caveolin-1 was expressed in flotation fractions 5 to 12 of the T98G GBM cell line with the largest expression in fractions 5 to 8 (Figure 1*A*). TMZ treatment, 3 days after the fifth *in vitro* administration into the culture medium, changed the expression and therefore the localization of raft resident caveolin-1 into the different flotation compartments: caveolin-1 disappeared from fraction 5, decreased in fractions 6 to 8, and increased in fractions 9 to 11 with TMZ treatment (Figure 1*A*). We obtained similar results with the two other GBM cell lines (data not shown). Raft resident caveolin-1 was not expressed in fractions 1 to 4 of the three GBM cell lines (data not shown).

By contrast, using a classic extraction method, the three GBM cell lines did not express soluble caveolin-1 under control conditions, whereas they displayed increased intracellular soluble caveolin-1 expres-

sion 3 to 5 days after the fifth *in vitro* administration of TMZ into the culture medium (Figure 4*A*).

Temozolomide Increases Caveolin-1 Expression in Human GBM Xenografts In Vivo

Chronic TMZ treatment modified raft resident caveolin-1 expression *in vitro* in T98G GBM cells (Figure 1*A*) and in Hs683 and U373 (data not shown) and *in vivo* in Hs683 (Figure 1*B*) and in U373 (data not shown) GBM xenografts. Immunohistochemical analyses further revealed that the highest TMZ-induced increases in caveolin-1 expression was observed in the extracellular matrix, particularly around the invasive areas (the *black arrows* in Figure 1*Bc*) of the tumor (*T* in Figure 1*Bc*) compared with normal brain parenchyma (*NB* in Figure 1*Bc*).

Temozolomide Decreases β_1 Integrin Expression and Modulates Cyr61 Signaling and Actin Cytoskeleton Polymerization in Human GBM Cell Lines In Vitro

Caveolin-1 is part of the signaling pathways controlling integrin function and expression, as previously demonstrated for $\alpha_5\beta_1$ integrins

[42]. Caveolin-1 has also been identified as a component in cysteine-rich 61 (Cyr61) integrin signaling [43]. Cyr61 is a matricellular protein, and expression of this protein is known to correlate with poor prognosis in GBM patients [44]. TMZ-induced effects on $\alpha_5\beta_1$ integrin and Cyr61 expression were investigated in Hs683, U373, and T98G GBM cells *in vitro* (Figure 2). We again adopted a regimen of daily *in vitro* TMZ (100 μM) treatment for 7 h/d over five consecutive days.

TMZ did not modify α_5 integrin expression patterns in these three GBM cell lines (data not shown). In contrast, we found that TMZ significantly decreased expression levels of β_1 integrin in the GBM cell lines of astroglial origin (i.e., U373 and T98G) 5 or 7 days after the fifth *in vitro* administration of TMZ (Figure 2A). In addition, β_1 integrin expression levels progressively increased over time in these two GBM models of astroglial origin (Figure 2A). In contrast, β_1 integrin expression levels progressively decreased over time in the Hs683 GBM model of oligodendroglial origin, and TMZ failed to further decrease levels over control (Figure 2A). We previously demonstrated marked differences in β integrin expression levels between astrogliomas and oligodendrogliomas, and between U373 and T98G *versus* Hs683 GBM cells [29]. Whereas U373 and T98G GBM cells display marked β_1 expression, as do astrogliomas [29,45], Hs683 GBM cells display high levels of β_4 integrin, as do oligodendrogliomas [29,45]. The differences in β integrin expression between U373 and T98G GBM cells with an astroglioma origin *versus* Hs683 GBM cells with an oligodendroglioma origin [29] could explain, at least partly, why TMZ did not induce similar patterns of β_1 integrin expression in U373, T98G, and Hs683 GBM cells (Figure 2A).

The same features have been observed with respect to Cyr61. Indeed, chronic *in vitro* TMZ (100 μM) treatment for 7 h/d for five consecutive days significantly decreased expression levels of the secreted form of Cyr61 in U373 and T98G GBM cell lines of astrocytic origin, whereas this treatment had no effect or even slightly increased expression of the Cyr61 secreted form in the Hs683 GBM cell line of oligodendroglial origin (Figure 2B). The reasons for the differences in the full-length Cyr61 observed with TMZ treatment are not yet understood for the moment (Figure 2B).

Malignant glioma adhesion depends on various integrins [29,45–47], and modifications in integrin signaling and/or expression can modify actin cytoskeleton organization [48]. Figure 2C reveals that TMZ treatment significantly depolymerized actin stress fibers not only in U373 and T98G cells (data not shown) but also in Hs683 GBM cells. This effect was sustained even 5 days after termination of TMZ treatment (Figure 2Ca). The finding that TMZ depolymerized the actin cytoskeleton in Hs683 oligodendroglioma cells but did not change β_1 integrin expression may indicate the involvement of other β integrins [29,45–47], expression of which may be modified by TMZ treatment.

Extracellular Caveolin-1 Significantly Decreases U373, T98G, and Hs683 GBM Cell Invasiveness In Vitro

Caveolin-1 is secreted by androgen-insensitive prostate cancer cells and is detected in the serum of patients with prostate cancer [49,50]. The highest reported serum level of caveolin-1 was approximately 100 ng/ml [49]. We therefore used 10 and 100 ng/ml caveolin-1 to investigate whether caveolin-1 can modify invasiveness of glioma cells cultured in Boyden chambers *in vitro*. As shown in Figure 3, 100 ng/ml caveolin-1 applied to the top of the Boyden chambers significantly decreased the invasiveness levels in the two GBM models of astrocytic

origin (i.e., U373 and T98G) but not in the Hs683 GBM model of oligodendroglial origin. In contrast, caveolin-1 added to the bottom of the Boyden chamber reduced invasiveness in the three models, suggesting that caveolin-1 exerted repulsive, or nonpermissive, effects on glioma cell migration and invasion. Lower concentrations (10 ng/ml) of caveolin-1 did not modify glioma cell invasiveness (data not shown). Altogether, these data suggest that part of the therapeutic benefits
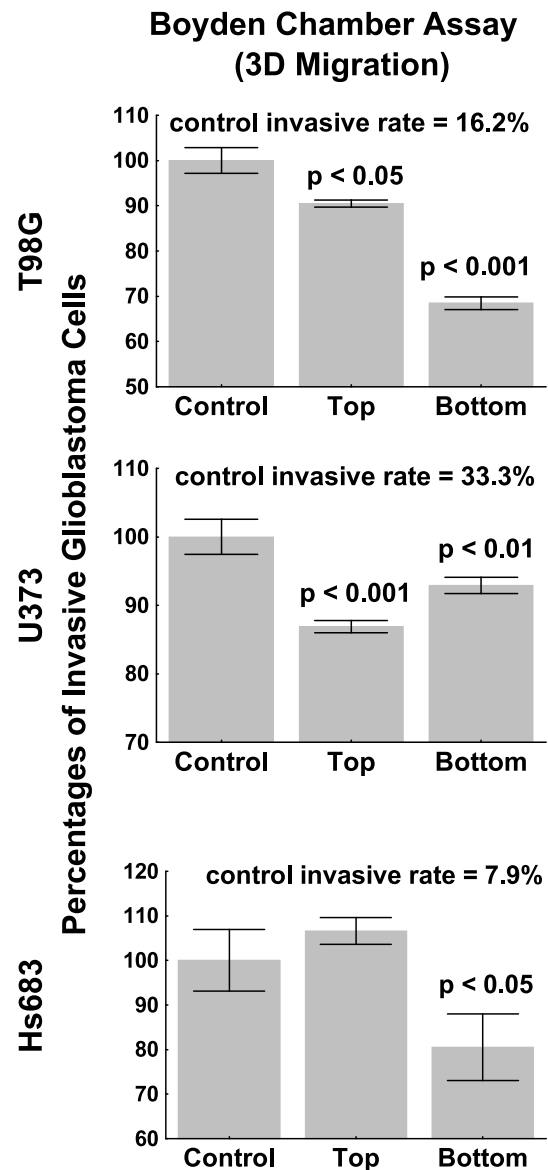
Boyden Chamber Assay (3D Migration)

Figure 3. Extracellular caveolin-1 decreases GBM cell line invasion. Invasion was evaluated using Matrigel Boyden chambers. The number of invasive cells was counted in 10 fields per chamber (the chamber surface includes 30 fields), and the experiment was conducted in triplicate. The mean of the 10 values was used to calculate the percentage of invasive cells in the population seeded, and the control invasion rate shown on each graph was reported to be 100%. The effects of 100 ng/ml recombinant caveolin-1 were examined both when the protein was added in the upper ("Top"; promigratory or antimigratory factor) or the lower ("Bottom"; chemoattractant *vs* chemorepulsive factor) compartments of the chamber. Results are expressed as mean of the triplicate ± SE.
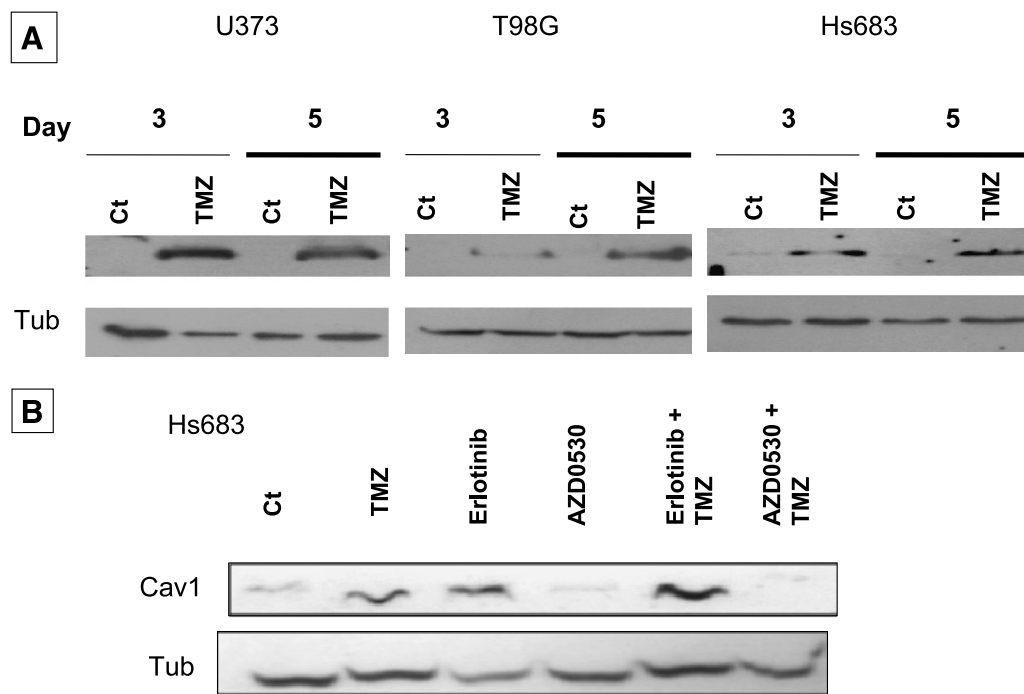
Figure 4. The TMZ-induced caveolin-1 modulation is Src-dependent in Hs683 GBM cells. (A) Western blot analysis of the expression of soluble caveolin-1 in U373, T98G, and Hs683 GBM cells left untreated (CT) or treated with 100 μM TMZ for 7 h/d for five consecutive days. Soluble caveolin-1 expression was measured on the third (day 3) and fifth (day 5) days after the end of the TMZ treatment. (B) Western blot analyses of soluble caveolin-1 expression in Hs683 glioma cells treated with TMZ (100 μM) four times per week (day 1-4) for 7 h/d, the EGFR inhibitor (10 μM) (erlotinib; day 1), the Src inhibitor AZD0530 (10 μM) (day 1), and combination of the inhibitors and TMZ (+TMZ) compared with control untreated cells (Ct). Soluble caveolin-1 expression was measured on day 5.

contributed by TMZ treatment in GBM patients could relate to TMZ-induced modification in caveolin-1 expression, a feature that in turn seems to lower GBM cell invasiveness.

Temozolomide-Induced Caveolin-1 Modulation in GBM Cells Is at Least Partly Mediated through Src Signaling

Caveolin-1 actively interacts with Src and EGFR [19,20], and several Src and EGFR inhibitors are currently assayed in clinical trials as potential treatments for recurrent GBM patients (clinicaltrial.gov) [51]. We therefore investigated the effects of inhibitors of EGFR kinase (erlotinib) and Src (AZD0530) on TMZ-induced intracellular soluble caveolin-1 expression in Hs683 GBM cells. Figure 4 shows that AZD0530 prevented TMZ-induced soluble caveolin-1 expression but did not change basal levels of soluble caveolin-1 in Hs683 GBM cells. In contrast, erlotinib increased soluble caveolin-1 expression to a similar degree as TMZ in these Hs683 GBM cells (Figure 4). These data suggest that Src could be a signaling element for TMZ when increasing intracellular soluble caveolin-1 expression in GBM cells. In contrast, EGFR seems to negatively regulate soluble caveolin-1 expression in GBM cells and its inhibition leads to increased soluble caveolin-1 expression, a feature that becomes additive to the TMZ-induced effects on soluble caveolin-1 expression in these Hs683 GBM cells (Figure 4). Reconciling the data from Figure 3 with those from Figure 4 suggests that a treatment combining an anti-EGFR compound with TMZ would reinforce an increase in soluble caveolin-1 expression (Figure 4), which in turn would decrease GBM cell invasiveness (Figure 3).

Discussion

GBM cells develop TMZ resistance during long-term treatment, and this resistance is partly mediated by O(6)-methylguanine-DNA methyltransferase (MGMT) [5,52]. The MGMT promoter methylation status [53], but not anti-MGMT immunohistochemistry [54], can be used to predict responses to TMZ treatment in GBM patients. Identification of additional biomarkers that would predict distinct clinical responses at the single patient level within MGMT-positive and -negative groups of GBM patients will help in tailoring a drug regimen to the specific biochemistry and particular vulnerabilities of each patient's tumor, thereby increasing effectiveness and avoiding unnecessary toxicities [32,51]. We already suggested such a potential role for galectin-1 [55], and the current study investigated such a potential role for caveolin-1. Data from the present study show that TMZ-treated GBM cells differently express raft resident and soluble caveolin-1 compared with untreated GBM cells.

Evidence for a role of caveolin-1 in cancer biology remains controversial [56–61]. Even within the specific group of gliomas, immunohistochemical caveolin-1 expression varies among glioma subgroups [25,26].

Caveolin-1 is upregulated in cancer cells facing adverse events. Specifically, caveolin-1 induction in tumor cells and tumor endothelial cells after radiation was previously shown to be responsible for the acquisition of a radio-resistant phenotype [62,63]. Conversely, HDAC inhibitors, proteasome inhibitors, and vascular endothelial growth factor receptor inhibitors, which are in clinical trials for GBM [51], were shown to reduce caveolin-1 levels. Moreover, Palozza et al. [64] recently demonstrated that β-carotene acts as a growth-inhibitory agent in caveolin-1–positive human colon and prostate cancer cells but not

in caveolin-1–negative cells. This finding suggests that modifications in caveolin-1 expression in glioma cells treated with TMZ could also modify the response of cells to future treatments.

The fact remains that as long as cancer cell biology is taken into account, caveolin-1–related roles seem to be tightly dependent on the cancer cell type may be because various cancer cell types express various activated signaling pathways. Data from the present study clearly evidence opposite roles for EGFR and Src on TMZ-induced modulation in caveolin-1 expression (Figure 4).

Accumulating experimental evidences indicate that caveolin-1 regulates signal transduction–associated proteins in caveolae [19,20,22]. For example, caveolin-1 modifies expression levels of Src, EGFR, endothelial nitric oxide synthase, G protein α and subunits, and H-Ras [65]. Several groups have provided rationale for interaction between caveolin-1 and β_1 integrin [62,66,67]. Caveolin-1 promotes Fyn-dependent shc phosphorylation and MAPK activation in response to integrin ligation through association with β_1 integrins and the Src-related kinase Fyn [66]. This would strengthen the rationale to explore these pathways in gliomas. Conversely, Src and other kinases phosphorylate caveolin-1 on Tyr14 in response to various stimuli, and this phosphorylation is crucial for caveolin-1 functions [48]. The interaction at Tyr14 contributes to modifications in the actin cytoskeleton organization [48,68].

By manipulating the levels of caveolin-1 in a GBM cell line (U87MG), Martin et al. [42] showed that reduced levels of caveolin-1 shifted cells toward a more aggressive phenotype, whereas forced expression of caveolin-1 slowed proliferation, clonogenicity and invasion. The current data perfectly fit in with those data reported by Martin et al. [42] because we show here that caveolin-1 reduces GBM cell invasion (Figure 3).

In addition, it is already well documented that GBM microenvironment directly affects GBM cell biology [15,16,69]. Our current data suggest that GBM microenvironment may also be a major determinant of caveolin-1–mediated roles in cancer cell biology according to the fact that TMZ-mediated increased caveolin-1 expression GBM xenografts mainly occur in the glioma microenvironment (Figure 1*Bc*).

In conclusion, TMZ modified caveolin-1 expression *in vitro* and *in vivo* in human GBM models of astrocytic *versus* oligodendroglial origin, and this effect seemed to be mediated partly through activation of Src signaling but not of EGFR signaling. In contrast, blocking EGFR also leads to increases in soluble caveolin-1 expression in GBM cells. TMZ-induced modification in caveolin-1 expression in GBM cells was paralleled by the decrease in β_1 integrin expression and disorganization of the actin cytoskeleton. Caveolin-1 decreases GBM cell invasion.

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