Cancer Biology and Signal Transduction

Temozolomide Induces the Production of Epidermal Growth Factor to Regulate MDR1 Expression in Glioblastoma Cells

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Abstract
Glioblastoma multiforme (GBM) commonly resists the frontline chemotherapy treatment temozolomide. The multidrug resistance gene (MDR1) and its protein, P-glycoprotein (P-gp), are associated with chemoresistance. This study investigated the mechanisms underlying MDR1-mediated resistance by GBM to temozolomide. P-gp trafficking was studied by flow cytometry and Western blot analysis. MDR1 expression was analyzed by real-time PCR and reporter gene assays. AP-1 interaction with MDR1 was studied by chromatin immunoprecipitation assay. EGF production was analyzed by ELISA, EGFR signaling was determined by Western blot analysis, and in vivo response to erlotinib and/or temozolomide was studied in nude mice. During the early phase of temozolomide treatment, intracellular P-gp was trafficked to the cell membrane, followed by conformational change into active P-gp. At the later phase, gene transcription of MDR1 was induced by temozolomide-mediated production of EGF. EGF activated ERK1/2-JNK-AP-1 cofactors (c-jun and c-fos). An inhibitor of EGFR kinase (erlotinib) given to nude mice with GBM prevented temozolomide-induced resistance. The results identified an essential role for activated EGFR in the resistance of GBM to temozolomide. Temozolomide resistance occurred through a biphasic response; first, by a conformational change in P-gp into the active form and, second, by releasing EGF, which caused autocrine stimulation of GBM cells to induce MDR1. Pharmacologic inhibition of EGFR kinase blunted the ability of GBM cells to resist temozolomide. These findings may explain reports on the common occurrence of mutant EGFR (EGFRvIII) and EGFR expansion in the resistance of GBM cells. Mol Cancer Ther; 13(10); 2399–411. ©2014 AACR.

Introduction
Resistance to chemotherapeutic agents is a common cause for antineoplastic treatment failure, which leads to oncogenic relapse and metastasis (1). The DNA alkylating agent temozolomide is currently the frontline treatment for glioblastoma multiforme (GBM; ref. 2). GBM is a World Health Organization grade IV astrocytoma and the most common adult primary brain tumor. There are several reports that explain temozolomide resistance, such as upregulation of O-6-methylguanine transferase (MGMT; ref. 3). An understanding of the molecular mechanisms underlying temozolomide resistance is essential for the development of combination therapy that could sensitize GBM to temozolomide.

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Molecular Therapeutics

The multiple drug resistance-1 (MDR1) gene and its product, P-glycoprotein (P-gp), were the first identified xenobiotic drug ATP-dependent efflux pump (4). Subsequently, >10 other efflux pumps have been identified, collectively referred to as the ATP-binding cassette (ABC) protein family. P-gp is a 170-kDa transmembrane glycoprotein. The MDR1 gene is regulated upstream by a TATAA-less promoter (5). A functional Inr (Initiator) sequence +1 of the transcriptional initiation site (TIS) has been identified (6). In addition, a number of cis-acting elements have been described, including a GC box, an inverted CCAAT box, and an AP-1 consensus/CCAAT-like box (7). The transcriptional activation of the MDR1 gene has been studied in response to different stressors, including chemotherapy (8). In metastatic sarcomas, the MDR1 gene was shown to be induced by doxorubicin, a P-gp target (9).

A number of parallels exist between human developmental pathways and oncogenic transformation/maintenance (10). The EGFR signaling pathway represents such an overlap. Activated EGFR can form the Grb2/SOS complex to activate the p21 RAS pathway and MAPK/ERK-AP-1 activation (11). EGFR is amplified or mutated in approximately 60% of primary GBM tumors (12). About 40% to 60% of GBMs show constitutive activation of mutant EGFR (EGFRvIII), resulting in poor clinical prognosis (13). The EGFR has tyrosine kinase functions and serves as an essential signal transduction molecule in the development of such cancers. EGFRvIII also shows resistance to erlotinib (14). The EGFR is amplified in approximately 60% of GBMs (15). EGFRvIII shows resistance to erlotinib (14). The EGFR is amplified in approximately 60% of GBMs (15).
as an attractive target to pharmacologically inhibit as a treatment target for GBM. Recent clinical trials with tyrosine kinase inhibitors have shown some improvement in GBM outcome (14). This study reports on the mechanism by which temozolomide induced its own resistance by regulating the expression of MDR1 at transcription and posttranscription. This study also reports on the autocrine stimulation of EGFR to regulate the expression of MDR1. This study adds to the report on posttranscriptional regulation by miR9 involvement in temozolomide resistance (15).

Materials and Methods

Cell lines

U87 and T98G WHO grade IV GBM cells were purchased from the ATCC and then expanded as per the manufacturer’s instructions. The cell lines were characterized by the ATCC. Upon arrival, the GBM cells were expanded and only used with three passages.

To prepare temozolomide-resistant cells, we considered differences in the expressions of the epigenetic silencing of the DNA repair gene MGMT in the two cell lines (3). Unlike T98G, U87 cells do not express MGMT. However, the IC50 of temozolomide was similar for both cell lines (100–500 μmol/L). Other authors reported an IC50 of temozolomide as 125 μmol/L (16). This study determined the IC50 of temozolomide for T98G and U87 as 150 to 200 μmol/L. We therefore used 200 μmol/L temozolomide to select the resistant GBM cells. Temozolomide resistance was established by treating U87 and T98G with 200 μmol/L (IC50 = 200–400 μmol/L) of temozolomide for 3 months. During this period, temozolomide-containing media were replaced at 3-day intervals.

Reagents and antibodies

All tissue culture media were purchased from Gibco and fetal calf serum was from Hyclone Laboratories. Temozolomide was purchased from Sigma Aldrich. MDR1-targeting siRNA was purchased from Thermo Scientific and transfected using Lipofectamine RNAiMax (Invitrogen).

Murine anti-human P-gp (UIC2 clone)-PE was purchased from Biolegend and mouse anti-P-gp (F4 clone) from Thermo Scientific. Rabbit anti-c-Jun, phospho-c-Jun, c-Fos, phospho-c-Fos, mouse anti-β-actin mAb, HRP-anti-rabbit, and HRP-anti-mouse IgG were purchased from Cell Signaling Technology. Rabbit polyclonal anti-EGFR was purchased from Biovision. FITC-conjugated anti-EGFR was purchased from BD Biosciences. ChIP-Grade Protein G Agarose Beads were purchased from Cell Signaling Technology.

Pharmacologic inhibitors

The Golgi transport inhibitor Brefeldin A (2 μmol/L; IC50 = 2 μmol/L) and the RNA pol II inhibitor α-Amanitin (30 μmol/L; IC50 = 30 μmol/L) were purchased from Sigma Aldrich.

EGFR signaling inhibition was achieved using the following inhibitors: erlotinib (10 μmol/L; IC50 = 5–20 μmol/L), SP600125 (25 μmol/L; IC50 = 10–50 μmol/L), PD98059 (5 μmol/L; IC50 = 5–10 μmol/L), and U0126 (70 μmol/L; IC50 = 50–70 μmol/L). SP600125, PD98059, and U0126 were purchased from Sigma Aldrich, and erlotinib was purchased from Santa Cruz Biotechnology.

Vectors

The MDR1 reporter gene constructs containing the varying fragments upstream of the human MDR1 gene have been previously described (17). The pGL2.luciferase vector (Promega) was used a negative control.

Analyses of the 5′ regulatory region of MDR1

The MDR1 promoter was analyzed using the Genematrix platform. The 5′ regulatory region of the MDR1 gene was identified from the NCBI database (NM_000927) as −1000 base pairs, relative to the TIS. This region was analyzed for the identification of potential transcriptional binding regions.

Real-time RT-PCR

RNA was extracted with TRIzol reagent (Invitrogen). Reverse transcription with 200 ng of cDNA was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), in accordance with the manufacturer’s recommendation. Real-time PCR was performed on the 7300 Real-Time PCR System (Applied Biosystems) as follows: an initial incubation at 50°C for 2 minutes followed by 95°C for 10 minutes. After this, the cycling conditions were as follows: 95°C for 15 seconds and 60°C for 60 seconds, for 40 cycles. The following primer sequences were used for MDR1 and β-actin: MDR1, (F) 5′-TCA GGT GGC TCT GGA TAA-3′ and (R) 5′-CTG CTG TCT GCA TTG TGA-3′; β-actin, (F) 5′-TGC CCT GAG GCA CTC TTC-3′ and (R) 5′-GTG CCA CCA GGG CAG TGA TCT-3′. Primers were purchased from Sigma. The relative expression was calculated using the 2−ΔΔCt, as previously described (18).

Western blot analysis

GBM cells were either treated with 200 μmol/L of temozolomide or an equivalent amount of vehicle. At different times, whole-cell extracts were isolated with Mammalian Protein Extraction Reagent (M-PER) or the cytoplasmic/nuclear (NE)-PER reagent (Thermo Scientific). The extracts (3–7 μg) were analyzed by Western blots on 12% SDS-PAGE gels (Bio-Rad), as described (19). Proteins were transferred onto polyvinylidene difluoride membranes (Perkin Elmer). The membranes were incubated overnight with primary antibodies at a final dilution of 1:500–1,000. Primary antibodies were detected during a 2-hour incubation period with horseradish peroxidase (HRP)-conjugated IgG at a 1:2,000 final dilution. HRP activity was detected by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Membranes were stripped with...
Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis for transcription factor binding to the MDR1 5'-regulatory region was performed using the ChIP-IT Enzymatic Kit (Active Motif). U87 and T98G cells were treated with vehicle (DMSO) or 200 μmol/L temozolomide for 72 hours and then the cells were fixed with 3.7% paraformaldehyde. Immunoprecipitation was performed with a ChIP-validated anti-c-Jun from Active Motif. Samples were analyzed on 1% agarose (Invitrogen) after 30 cycles of PCR amplification with the following primers spanning the AP-1 site: (F) 5'-CCT CCT GGA AAT TCA ACC TG-3', (R) 5'-GAA GAG CCG CTA CTC GAA TG-3'.

Flow cytometry

The membrane expression of P-gp was studied by flow cytometry as follows: the active conformation was detected with the conformational-dependent murine UIC2 mAb conjugated to either FITC or phycoerythrin (PE). Basal P-gp expression was analyzed with the conformational-independent F4 clone mouse mAb.

Cell-surface EGFR was analyzed by labeling with a mouse anti-human EGFR-FITC–conjugated antibody. The cells were immediately analyzed on a FACSCalibur II (BD Biosciences) and the data were analyzed with the FlowJo software (BD Biosciences).

Transfection and reporter gene assay

U87 and T98G cells were transfected with the MDRI reporter constructs with Effectene Transfection Reagent (Qiagen). After 48 hours, protein lysates were obtained using M-PER (Thermo Scientific). The extracts were analyzed for total protein using a kit from Bio-Rad. Luciferase activity was normalized to total protein and calculated as relative light units (RLU). The relative expression was calculated by normalizing to control (untreated) RLU.

Preparation of EGF-free media

The EGF-free media were prepared by immunoprecipitating the EGF with anti-EGF and then capturing the immune complexes with ChIP-Grade Protein G Agarose Beads.

ELISA

EGF, secreted and cell-associated, was quantitated with the human EGF ELISA Kit (EHEGF, Thermo Scientific). All assays were performed according to the manufacturer’s specific instructions. The unknown EGF was calculated from a standard curve developed with serial dilutions of known EGF levels at optical density at 450 nm. The background was subtracted from the data obtained at 550 nm. The standard curves were calculated using a linear regression algorithm to provide the best standard curve fit.

Cell viability assay

Cell viability was assayed studied with GBM cells exposed to temozolomide for 72 hours. The viability studies used the Cell Titer Blue Cell Viability Assay (Promega). The method depended on the integrity of the cell membrane and was independent of cell-cycle status and was similarly used by others to assess temozolomide resistance (20). The cells were seeded at 1.5 × 10⁴ per well in triplicates in the assay.

In vivo studies

The use of mice was approved by the Institutional Animal Care and Use Committee. Female NCr nude BALB/c mice (6 weeks) were obtained from Taconic Farms and then housed in the Association for assessment and accreditation of laboratory animal care international (AAALAC)-accredited barrier facility at New Jersey Medical School (Newark, NJ). U87 and T98G cells (5 × 10⁶ cells in 0.1 mL PBS) were added to equal volume of BD Matrigel (BD Biosciences). The total volume (0.2 mL) was injected subcutaneously in the dorsal flank of the mice. After 5 days, the tumors were measured with a caliper and then the treatment was initiated as follows: erlotinib, at 6 mg/kg, was injected as a single dose, intraperitoneally in 0.5 mL volume (21). The next day, the mice were treated with temozolomide with the maximum tolerated dose (10 mg/kg in 0.5 mL). The temozolomide was injected by intraperitoneal route (22). The injections were repeated four additional times at 2-day intervals. The tumors were measured daily and the volumes (mm³) were calculated as follows: \( V \) (volume) = \( 4/3\pi w l \) in which \( w \) = width (mm) and \( l \) = length (mm). The results are shown for the volumes of the tumors at day 0 and at 5 days after treatment. Groups of mice were kept up to 3 months after which the mice were euthanized with overdosed CO₂.

Statistical analyses

Data were analyzed using the paired t test for two comparable groups (control vs. experimental). A P value of <0.05 was considered significant.

Results

Temozolomide induced the active form of P-gp in GBM cells

The experimental and clinical literature suggested a key role for P-gp in GBM resistance to temozolomide (23). GBM cells were reported to express both the active and inactive forms of membrane P-gp (24). To discriminate between active and total P-gp in GBM cells, we performed flow cytometry with antibodies detected active (UIC2-PE) and total (F4-FITC) P-gp. Untreated (baseline) analyses indicated both total and active P-gp in the GBM cells (Fig. 1A, left and middle graphs). We next treated the GBM cells with temozolomide for 2 hours and then repeated the flow cytometry studies.
The mean fluorescence intensity (MFI) for active P-gp was increased by 2-fold for temozolomide-treated GBM cells (Fig. 1A, right vs. middle).

The studies shown in Fig. 1A used short-term exposure of GBM cells to temozolomide. We therefore asked whether chronic exposure to temozolomide could sustain the active form of P-gp. U87 and T98G cells were treated with 200 μmol/L temozolomide for 90 days. The media were changed with fresh temozolomide-containing media at 3-day intervals. The long-term temozolomide-resistant GBM cells showed morphologic changes similar to mesenchymal phenotype.

Figure 1. Induction and time-dependent trafficking of active P-gp in temozolomide (TMZ)-treated GBM cells. A, flow cytometry was used to study the basal and active form of P-gp in untreated and temozolomide (200 μmol/L)-treated (3 days) U87 and T98G cells. The MFIs of the histograms are inserted in each panel. The solid histograms represent isotype control, whereas the open histogram represents the tested antibody, which is accompanied by the MFI in each panel. B, morphology of long-term (90 days)-treated U87 and T98G cells. Temozolomide-containing media were replaced at 3-day intervals. C, flow cytometry for active P-gp in the long-term temozolomide-treated U87 and T98G cells. D, Western blots with anti-P-gp using whole-cell extracts from T98G and U87 cells, treated or untreated with 200 μmol/L temozolomide for different times. The membranes were stripped and reprobed with anti-β-actin. The band density is shown in Supplementary Fig. S1. E, T98G and U87 were treated with 200 μmol/L temozolomide or vehicle. At different times, the cells were studied by dual-color immunofluorescence for intracellular active P-gp (red) and DAPI (blue). The images were acquired with the EVOS FL at × 200 magnification.
Flow cytometry for active P-gp, using the PE-UIC2 antibody, indicated 2- to 3-fold increase in the MFI of the chronically treated GBM cells as compared with the short-term treated cells (Fig. 1A, right graphs vs. Fig. 1C).

**Undetectable intracellular P-gp and increased membrane P-gp by temozolomide**

The synthesis and trafficking of P-gp are essential for drug efflux (25). In this set of studies, we asked whether temozolomide can induce traffic of P-gp to the cell membrane of GBM cells. First, we asked whether there is a timeline increase in total P-gp in temozolomide-treated U87 and T98G cells. The cells were treated with 200 μmol/L temozolomide. At different times, Western blots were performed with whole-cell lysates with the F4 antibody, which detected total P-gp. We observed a time-dependent increase in P-gp protein (Fig. 1D). The band densities are shown in Supplementary Figs. S1 and S2.

We next studied cellular trafficking of active P-gp to the cell membrane. Paraformaldehyde-fixed GBM cells were studied for intracellular active P-gp by immunocytochemistry. The studies used the UIC2 antibody, which detected the active form. Vehicle treatment showed P-gp in the subcellular compartments with sparse membrane localization (Fig. 1E). In contrast, temozolomide treatment (24–72 hours) reduced subcellular active P-gp while showing concomitant increase of active P-gp in the cell membrane. Although the findings were semiquantitative, these findings suggested that temozolomide facilitated the traffic of active P-gp to the cell membrane.

**Temozolomide induced a biphasic response in the trafficking of functional P-gp**

To study the intracellular method by how active P-gp is transported to the cell membrane, we treated GBM cells with Golgi (Brefeldin A) and RNA polymerase II inhibitors (α-Amanitin) and then performed flow cytometry for active P-gp. We established the baseline membrane expression of P-gp with GBM cells treated for 2 hours with 200 μmol/L temozolomide (Fig. 2A, untreated). Brefeldin A (2 μmol/L) blocked the membrane localization of active P-gp and α-Amanitin showed no significant difference (Fig. 2A). Together, these studies
suggested that membrane trafficking and not protein synthesis is required for the increase of active P-gp in temozolomide-treated GBM cells.

The studies shown in Fig. 2A assessed immune methods to detect conformational change for active P-gp. We next asked whether the active P-gp was functional using the Calcein-AM efflux assay in time-course studies. We compared vehicle- and temozolomide-treated GBM cells and showed a significant ($P < 0.05$) increase of Calcein-AM efflux (Fig. 2B). However, the efflux indicated a biphasic response to temozolomide treatment with a decrease between 8- and 12-hour treatment followed by restored efflux. As a control, we used the drug efflux inhibitor verapamil.

We next explored the underlying mechanisms in the biphasic response in Calcein-AM efflux (Fig. 2B). We pretreated the temozolomide-exposed with Brefeldin for 30 minutes and then performed a time-course study for functional P-gp using the Calcein-AM efflux assay. Brefeldin failed to block early phase of dye efflux (Fig. 2C) but significantly ($P < 0.05$) blocked dye efflux at the later phase (Fig. 2C). We asked whether transcription was responsible for the biphasic response shown in Fig. 2B. We there repeated the studies shown in Fig. 2B, in the presence or absence of 30 μmol/L of α-Amanitin (RNA Pol II inhibitor). The α-Amanitin significantly ($P < 0.05$) blunted the dye efflux at the later phase (Fig. 2D). Taken together, temozolomide induced a biphasic response with respect to the function of P-gp. The early phase entailed available membrane-bound active P-gp, whereas the latter phase appeared to be dependent on the transcription of MDRI and the trafficking of P-gp.

**Temozolomide activated the 5’ regulatory region of MDRI and increased MDR1 mRNA**

The results shown with α-Amanitin (Fig. 2D) suggested that temozolomide-induced efflux at the later phase might require gene transcription. We therefore studied the transcriptional regulation using a reporter gene system with different regions of the 5’ regulatory region of the MDRI gene, with respect to the initiation site ($–1202/+118$; Fig. 3A). These constructs were previously described in ref. (17).

GBM cells were transfected with the reporter gene constructs or with the empty vector. The transfectants

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Figure 3. Reporter gene studies with constructs from the 5’ regulatory region of MDRI. A, a diagram showing the relative positive of the MDRI constructs, relative to the initiation start site. B, pGL2 containing the different constructs of MDRI was transfected in U87 and T98G cells. After 72 hours, cell extracts were quantitated for luciferase activity and then normalized to total protein. The results are presented as RLU (±SD, n = 4). Each point represents the mean values of triplicates in four independent experiments. C, the reporter gene studies in B were repeated with the vector containing the $–136$ and then analyzed for luciferase activities at different times. The results are presented as in B. D, real time PCR was performed for MDR1 mRNA using total RNA from U87 and T98G cells, treated for different times with 200 μmol/L temozolomide (TMZ). The normalized results are presented as fold expression, relative untreated, mean ± SD, n = 4. Each point represents the mean values of triplicates in four independent experiments.
were treated with vehicle or 200 μmol/L temozolomide. After 72 hours, there was a significant ($P < 0.05$) increase in luciferase for the $-75/-136$ fragment (Fig. 3B). We therefore selected the $-136$ constructs in time-course studies. Temozolomide treatment resulted in rapid increases in luciferase at the 8- and 12-hour time point followed by reduced activation after 24 hours (Fig. 3C).

Real-time PCR for MDR1 mRNA with temozolomide-treated GBM cells showed an increase at the 2- to 4-hour time points (Fig. 3D). As luciferase was increased at 12 hours (Fig. 3C), the decrease in endogenous mRNA at this time period suggested that the transcript must be undergoing a rapid turnover. Taken together, this section describes studies that showed temozolomide-mediated activation of the 5′ regulatory region of MDR1 at $-75/-136$. This correlated with an increase in the respective mRNA.

**AP-1 activation in temozolomide-treated GBM cells binds to $-75/-136$ region of MDR1**

To understand how temozolomide regulated the expression of MDR1, we analyzed the $-75/-136$ region for transcription binding site and identified a potential AP-1 region (Fig. 4A). AP-1 is a heterodimeric transcription factor composed of c-Fos and c-Jun or homodimerized c-Jun (26). We first studied whether temozolomide can activate c-Jun and c-Fos by Western blots, using nuclear and cytoplasmic extracts from temozolomide-treated GBM cells. There was an increase in the total and phosphorylated c-Jun and c-Fos (Fig. 4B). The band densities are shown in the Supplementary Fig. S3.

**Figure 4.** AP-1 activation in temozolomide (TMZ)-treated GBM cells. A, a diagram showing the relative position of AP-1 in the MDR1 gene. B, Western blots were performed for total and phosphorylated c-Fos and c-Jun with nuclear and cytoplasmic extracts from vehicle and temozolomide (200 μmol/L)-treated U87 and T98G cells. The blots were stripped and reprobed for AH3, RPL26, and β-actin. Band density is shown in Supplementary Fig. S2. C, ChIP assay for the AP-1 binding site within the MDR1 gene in temozolomide- and vehicle-treated U87 and T98G.
of the cytoplasmic and nuclear extracts was determined with blots for ribosomal protein L26 and acetyl-histone 3, respectively. The significance of phosphorylated c-Jun and c-Fos was studied by examining the MDRI gene for AP-1 binding. ChIP assay with anti-c-Jun confirmed the binding of c-Jun to the region spanning −115/−121 of the MDRI gene (Fig. 4C).

**EGF-EGFR-AP-1 in MDRI induction by temozolomide**

The ChIP and Western blots assays supported a role for AP-1 in temozolomide-induced expression of the MDRI gene (Fig. 4). AP-1 can be activated by Janus N-terminal Kinase 1 (JNK1) and ERK1/2. The kinase activity of activated EGFR can activate ERK1/2 and JNK1 (27). As activation of EGFR would require autocrine production of its ligand (EGF), we asked whether EGF is produced by temozolomide-treated cells. EGF levels were studied by ELISA in the media and cell extracts. If the EGF is produced in the media, this would correlate with reduced EGF with cell extracts. Thus, the results are presented as EGF levels in the media/cell lysates. The results showed peaked EGF at 48 hours in the temozolomide-treated cells (Fig. 5A). We also asked whether this increase in EGF correlated with membrane receptor. We therefore asked this...
question by flow cytometry for membrane EGFR. The results indicated an increase in membrane EGFR by 2-fold in the temozolomide-treated GBM cells (Fig. 5B).

We asked whether EGF, via EGFR activation, induced MDR1 expression. To address this question we treated GBM cells with temozolomide in sera-free media containing exogenous rhEGF (1C50 = 50 ng/mL). Real-time PCR and Western blot analysis showed significant ($P < 0.05$) increases in MDR1 mRNA (Fig. 5C and Supplementary Fig. S4) and its corresponding P-gp protein (Fig. 5D), as compared with untreated GBM cells. Together, these studies supported a role for temozolomide in the induction of MDR1 to cell survival following temozolomide exposure.

We investigated whether the EGFR induced the expression of MDR1 through AP-1 activation. We used pharmacologic inhibitors to block kinases within the EGFR pathway: erlotinib (EGFR tyrosine kinase), PD98059 (ERK1), SP600125 (JNK), and U0126 (ERK1/2; Fig. 5C). Cells were first exposed to each drug for 24 hours and then treated with temozolomide and Western blot analysis showed significant ($P < 0.05$; Fig. 6A, left graph).

EGFR activation resulted in an increase in P-gp (Fig. 5C). Thus, EGFR activation is expected to protect GBM cells from temozolomide treatment. We therefore studied the effect of EGFR signaling on cell survival using two approaches. In the first, we added exogenous (50 ng/mL) 

Figure 6. EGF in the survival of temozolomide-treated GBM cells; in vivo decrease of GBM growth by erlotinib and temozolomide. A, cell viability was performed with MDR1 knockdown U87 and T98G that were treated with 200 µmol/L temozolomide. Controls were transfected with scrambled siRNA. The results are presented as mean ± SD, $n = 4$. B, cell viability was performed with U87 and T98G that were treated with 200 µmol/L temozolomide in the presence or absence of EGFR inhibitors. The results are presented as mean ± SD, $n = 4$. Each point represents the mean values of triplicates in four independent experiments. C, female nude BALB/c mice were injected with $5 \times 10^6$ T98G or U87 cells in the dorsal flank. After 5 days, the mice were treated with vehicle and either temozolomide or temozolomide plus erlotinib. The left part of the graph shows the mean volumes of the T98G ± SD ($n = 5$). The values for untreated mice are shown just before treatment (day 0). The values for the treated groups are shown at 5 days after the last treatment. The right section of the graph shows the mean volume of tumors at 2.5 and 3.0 months (mo). D, mice were injected with T98G in the dorsal flanks and then treated with erlotinib and/or temozolomide. After the second treatment with temozolomide, the tumors were excised and then studied for P-gp by Western blot analysis.
rhEGF in EGF-free media and showed a significant ($P < 0.05$) resistance to temozolomide as compared with untreated cells (Fig. 6A right graph). In the second approach, we blocked the effect of endogenously produced EGF by inhibiting the EGFR signaling molecules. This was done with the inhibitors shown in Fig. 5D, in the presence or absence of temozolomide. After 72 hours of temozolomide treatment, cell viability indicated significant ($P < 0.05$) cell death as compared to cultures with vehicle or temozolomide alone (Fig. 6B). Together, this section supported a role for EGFR signaling in the activation of AP-1 to increase the transcription of MDR1, thereby contributing to the resistance of GBM to temozolomide.

**In vivo treatment with erlotinib and temozolomide**

As activated EGFR seems to be important for the induction of *MDR1*, we asked whether blocking its signaling would cause a significant reduction in temozolomide resistance. We selected the EGFR tyrosine kinase inhibitor, erlotinib. On the basis of the *in vitro* studies, we proposed that erlotinib would sensitize the GBM to temozolomide. Nude BALB/c mice were injected with U87 and T98G in the dorsal flank. The mice were then divided into groups of five mice and then subjected to the following treatment: no treatment, temozolomide, erlotinib, temozolomide and erlotinib, vehicle. The mice received one injection of erlotinib. After 24 hours, the mice were treated with temozolomide up to 10 days at 2-day intervals. The mean volumes ± SD of the tumors are shown for the untreated group at the beginning (day 0) and end of the treatment time.

The results for both cell lines were similar. The left part of Fig. 6C shows the outcome with T98G at day of the last treatment. There was no difference between the untreated cells and erlotinib treatment. In contrast, there was a significant ($P < 0.05$) decrease in the tumors with temozolomide treatment. The tumor volumes were significantly ($P < 0.01$) decreased when the mice were treated with erlotinib and temozolomide as compared with temozolomide alone.

As the rate of tumor regression does not always translate into tumor control, we treated the mice with the full course of treatment and then monitored tumor growth. Because of humane endpoint, we could not keep the mice treated with vehicle. Mice treated with temozolomide alone had to be euthanized after one month as the tumor regrew. In contrast, at 2.5 to 3 months, there was no evidence of tumor regrowth in mice treated with erlotinib and temozolomide (Fig. 6C, right). Rather, the tumor volumes were decreased by 2.5 months and stayed the same up to 3 months. In summary, the *in vivo* studies recapitulated the *in vitro* findings, showing the effectiveness of erlotinib to temozolomide treatment for GBM.

EGFR signaling was important for the expression of P-gp (Fig. 5E). Thus, we asked whether blocking signaling with erlotinib would decrease P-gp. If so, this would make the tumor sensitive to temozolomide. We therefore repeated the *in vivo* studies, except, after two treatments with temozolomide the tumors were excised and then analyzed by Western blot analysis for P-gp. We compared the mice treated with temozolomide and/or erlotinib. The results showed a significant reduction in the band for P-gp when the mice were cotreated with erlotinib (Fig. 6D).

**Discussion**

The clinical outcome for patients diagnosed with GBM is often unfavorable. Patients resist the frontline chemotherapeutic agent, temozolomide. Resistance to temozolomide has shown to be multifactorial, including changes in the cell cycle, upregulation of mismatch repair genes, and MGMT (3). We report an additional resistance mechanism involving an increase in the active form of P-gp following temozolomide treatment (Fig. 1). The increase was biphasic with the initial enhancement caused by the presence of available intracellular P-gp, which move to the cell membrane (Fig. 1). The later phase was caused by an increase in the transcription of the *MDR1* gene, as indicated by reporter gene studies and real-time PCR (Fig. 3). The findings narrowed the temozolomide-sensitive site within the 5' untranslated region of *MDR1* and showed a functional AP-1 site (Fig. 4). Temozolomide activated the AP-1 site by increasing the release of EGF and its receptor (Fig. 5A and B). The ligand-receptor pair induced the expression of *MDR1* (Fig. 5C). Together, we showed the early enhancement of temozolomide depended on already synthesized P-gp and the later phase depended on the production of EGF and enhancement of its receptor to activate AP-1, which increased the transcription of *MDR1*.

We studied the active form of P-gp because of a need for conformational change to activate P-gp (28). The active P-gp was increased in GBM cells, acutely and chronically treated with temozolomide (Fig. 1A and D). The immunoreactive P-gp was confirmed for functionality using Calcein-AM dye exclusion (Fig. 2A). The functional studies indicated a bimodal response to temozolomide with an increase at the early phase, followed by significant decrease and then another increase (Fig. 1E). We referred to these two phases as early and late. Golgi inhibition by Brefeldin A and transcriptional inhibition with α-Amanitin were able to ameliorate the later phase of P-gp function (Fig. 2C and D), we concluded that the late phase required new gene transcription. As Brefeldin A and α-Amanitin did not affect the early response of Calcein-AM efflux (Fig. 2C and D), this indicated that the later phase of *MDR1* expression required new protein synthesis.

The functional studies with pharmacologic inhibitors correlated with time-dependent increases in *MDR1* RNA and its protein, P-gp (Fig. 3D). Reporter gene studies with the 5' regulatory region of *MDR1* identified the temozolomide-sensitive region containing AP-1 (Fig. 3B and C). Temozolomide phosphorylated AP-1, which could
interact with the MDRI gene within the temozolomide-sensitive region (Figs. 3C and 4C).

The tyrosine kinase receptor, EGFR, may act as an initiator of the MAPK pathway, which activates AP-1. Furthermore, activation of EGFR is dependent on dimerization and autophosphorylation induced by secreted EGF. The resistant GBM cells expressed EGFR and also produced EGF (Fig. 5A and B). The expression of this ligand–receptor complex was significant for the expression of MDRI (Fig. 5C). The effect of EGF-EGFR was an autocrine mechanism by which the EGF autostimulated the temozolomide-resistant GBM cells to activate AP-1 and induced MDRI expression (Fig. 5D and E). The significance of autocrine stimulation of EGF was studied in cell survival analyses using different approaches. The results indicated a protective role for EGFR through the expression of MDRI and, in the early phase, trafficking of active P-gp. The findings add to the literature on the involvement of activated EGFR in the resistance of GBM to chemotherapy, such as mechanisms mediated through PDGF receptor tyrosine kinase (29).

A summary of the findings is shown in Fig. 6D. At the initial phase, temozolomide induced the active P-gp in the cell membrane of GBM cells. Activation has been reported to require a conformational change of P-gp (30). Simultaneously, the GBM cells are beginning to prepare for sustained resistance by the autocrine stimulation of EGFR to activate intracellular kinases, which activated AP-1 for interaction on the MDRI gene. The newly transcribed transcripts are translated as active P-gp for membrane localization.

Taken together, our data showed an increase in P-gp synthesis, translocation, and function following treatment with temozolomide in human GBM cells. We were also able to show an essential role for EGFR signaling in the acquired resistance of GBM to temozolomide. EGFR inhibitors reduced the level of P-gp and sensitized the GBM cells to temozolomide. Combinatorial therapeutic approaches using EGFR inhibitors and alkylating agents such as temozolomide may provide added benefits to overcome chemoresistance. In fact, we combined erlotinib and temozolomide to treat GBM in nude mice and the results showed erlotinib as a sensitizing agent for temozolomide (Fig. 6C). It is unclear from the in vivo studies whether the tumor resurges after the combined treatment.
This observation is ongoing in the laboratory and brings up the question of whether all subsets of GBM are susceptible to the combined treatment.

The findings of this report are significant, considering that GBM is the most aggressive and common adult primary brain tumor. Resistance to therapy is a common feature of GBM, which ultimately leads to enhanced cellular necrosis and tissue damage. Temozolomide is the only frontline treatment approved by the FDA for GBM. However, GBM exhibits rapid and prolonged resistance to temozolomide, resulting in a short progression-free survival rate. Clinical data suggest that MDR1 polymorphisms have an important role in patient response efficiency to temozolomide. MDR1 codes for P-gp, which was the founding member of the ATP-binding cassette protein family of drug efflux pumps. Here, we show that temozolomide is able to activate cell-surface P-gp as well as allow for signal transduction cascades that enhanced P-gp function (Fig. 7). This study could lead to a combinational therapy with temozolomide to prevent the activation of P-gp.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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5. van Groenigen M, Valentijn LJ, Baas F. Identification of P-gp. Here, we show that temozolomide is able to activate cell-surface P-gp as well as allow for signal transduction cascades that enhanced P-gp function (Fig. 7). This study could lead to a combinational therapy with temozolomide to prevent the activation of P-gp.

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