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# Improved Brain Penetration and Antitumor Efficacy of Temozolomide by Inhibition of ABCB1 and ABCG2[1]

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## Abstract

The anticancer drug temozolomide is the only drug with proven activity against high-grade gliomas and has therefore become a part of the standard treatment of these tumors. P-glycoprotein (P-gp; ABCB1) and breast cancer resistance protein (BCRP; ABCG2) are transport proteins, which are present at the blood-brain barrier and limit the brain uptake of substrate drugs. We have studied the effect of P-gp and BCRP on the pharmacokinetics and pharmacodynamics of temozolomide, making use of a comprehensive set of *in vitro* transport experiments and *in vivo* pharmacokinetic and antitumor efficacy experiments using wild-type, Abcg2$^{-/-}$, Abcb1a/b$^{-/-}$, and Abcb1a/b;Abcg2$^{-/-}$ mice. We here show that the combined deletion of Abcb1a/b and Abcg2 increases the brain penetration of temozolomide by 1.5-fold compared to wild-type controls ($P < .001$) without changing the systemic drug exposure. Moreover, the same increase was achieved when temozolomide was given to wild-type mice in combination with the dual P-gp/BCRP inhibitor elacridar (GF120918). The antitumor efficacy of temozolomide against three different intracranial tumor models was significantly enhanced when Abcb1a/b and Abcg2 were genetically deficient or pharmacologically inhibited in recipient mice. These findings call for further clinical testing of temozolomide in combination with elacridar for the treatment of gliomas, as this offers the perspective of further improving the antitumor efficacy of this already active agent.

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Abbreviations: ABC, ATP-binding cassette; AUC, area under the curve; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; GBM, glioblastoma; MGMT, $O^6$-methylguanine-DNA methyltransferase; MRI, magnetic resonance imaging; P-gp, P-glycoprotein; WT, wild-type.

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## Introduction

High-grade gliomas, and in particular glioblastoma (GBM), are refractory to virtually all chemotherapy regimens. Whereas it is possible that the heterogeneity within these tumors favors the presence of innately resistant tumor cells, inadequate drug exposure of tumor cells because of the blood-brain barrier (BBB) is most likely also a major cause of the general lack of efficacy of chemotherapy [1]. The BBB restricts the entry of nearly all commonly used agents. Although this barrier is often disrupted in more central tumor areas, where leakiness can be visualized by contrast-enhanced magnetic resonance imaging (MRI), it is still functional in the more peripheral and surgically unresectable tumor regions that harbor many viable and proliferating tumor cells. Moreover, glioma tumor cells have the propensity to migrate deep into the surrounding normal brain tissue, where the BBB is also fully intact [2,3].

The principal components of the BBB are the endothelial cells that are linked together by tight junctions, limiting the paracellular movement of substances [4]. Moreover, transcellular routing is further restricted by the absence of fenestrae and the low endocytic activity of brain endothelial cells. Besides these more or less passive restraints, the BBB is also equipped with ATP-binding cassette (ABC) efflux transporters such as P-glycoprotein (P-gp, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) that together limit the brain penetration of almost all classical chemotherapeutics and novel targeted anticancer drugs [5,6].

For a long time, the standard treatment of GBM consisted of surgical resection followed by local radiotherapy, with or without nitrosourea-based chemotherapy. However, a large number of adjuvant nitrosourea-based chemotherapy trials have been conducted but did not demonstrate significant survival benefit [7,8]. At the last turn of the century, however, the orally bioavailable alkylating agent temozolomide was reported to have significant activity in the treatment of recurrent grade 3 and grade 4 gliomas, outperforming procarbazine in one study [9–11]. Then, about a decade ago, a large phase III trial showed a significant survival benefit for radiotherapy in combination with concomitant and adjuvant temozolomide compared to radiotherapy alone [12]. This landmark study set the basis for the new standard treatment of newly diagnosed GBM, where patients start temozolomide (75 mg/m$^2$/day for 42 days) concomitantly with radiotherapy, subsequently followed by six courses of temozolomide monotherapy (150 to 200 mg/m$^2$/day for 5 days with a 23-day rest period). Epigenetic silencing of the $O^6$-methylguanine-DNA methyltransferase (MGMT) promoter has been demonstrated to predict benefit to temozolomide chemotherapy [13]. Nevertheless, this drug is currently being prescribed to most GBM patients since it is generally well tolerated, a small survival benefit is seen in patients carrying nonsilenced MGMT promotors, and there is no real alternative [14].

Temozolomide is generally believed to penetrate the BBB relatively well. Indeed, the brain penetration is apparently high enough to improve the median survival of GBM patients from 12.1 to 14.6 months [12]. Nevertheless, we here show that P-gp and BCRP collectively limit the brain penetration of this drug. Using drug transporter knockout mouse models, we show that the temozolomide brain penetration is increased when these two drug transporters are absent or inhibited, while the plasma clearance of the drug remains unaffected. Importantly, we show that the efficacy of temozolomide against three independent experimental intracranial tumor models is significantly improved when P-gp and BCRP are genetically absent or pharmacologically inhibited.

## Materials and Methods

### *Reagents*

Temozolomide (Temodal 20 mg hard capsules) originated from Schering Plough BV (Utrecht, The Netherlands). Elacridar (GF120918) was a generous gift from GlaxoSmithKline Inc. (Research Triangle Park, NC). Erlotinib was kindly provided by OSI Pharmaceuticals, Inc., (Melville, NY). Zosuquidar was a generous gift of Eli Lilly (Indianapolis, IN). Gefitinib was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Bovine Serum Albumin (BSA) fraction V was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany) and were used as supplied.

### *Preparation of Drug Solutions*

The content of a temozolomide capsule containing 20 mg of active substance was dissolved in 0.4 ml ethanol and 3.6 ml saline to yield a solution of 5.0 mg/ml and was used within 60 minutes after preparation. Elacridar was prepared freshly the day before each experiment and suspended at 5 mg/ml in a mixture of hydroxypropyl methylcellulose (0.5 g/l)/1% polysorbate 80 (v/v). The suspension was mixed for 2 minutes using a Polytron PT1200 homogenizer (Kinematica AG, Littau, Switzerland). Additionally, the suspension was kept protected from light and stirred continuously before and during administration. Gefitinib was suspended in 0.5% (v/v) Tween 20 and 0.25% (w/v) carboxymethylcellulose in water at a concentration of 10 mg/ml.

### *Analytical Methods*

Based on previous work by Kim et al. [15], we have developed a high-performance liquid chromatographic (HPLC) assay for the determination of temozolomide in medium used for *in vitro* transport experiments and in mouse plasma and brain tissue homogenates for *in vivo* pharmacokinetic studies. Separation and quantification were achieved using a Symmetry $C_{18}$ column (150 × 2.0 mm; ID) together with a mobile phase of 7.5% of methanol in 0.5% acetic acid in water, delivered at a flow rate of 0.2 ml/min and UV detection at 330 nm (PDA996 photodiode array detector; Waters, Milford, MA, or SF 757 detector; Kratos, Ramsey, NJ). Medium from Transwell experiments was diluted 10-fold with 0.2% acetic acid in water, and 50 µl was injected directly into the HPLC system. Temozolomide was extracted from the acidified plasma and brain tissue homogenate samples (200 µl) with 1.0 ml ethyl acetate. The dried extracts were subsequently dissolved in 100 µl of 5% methanol in 0.2% acetic acid in water, and 50 µl was injected into the HPLC system. External calibration was used. The lower and upper limits of quantitation were 0.020 µg/ml and 10.0 µg/ml, respectively. Samples above the upper limit of quantification were first diluted with acidified blank human plasma. All samples from *in vivo* studies were analyzed twice in two independent analytical series and repeated once more when the duplicates differed by more than 10%.

### *In Vitro Transport Experiments*

The parental LLC porcine kidney (PK1) cell line and its Mdr1a transduced subline [16] and the parental Madine Darby Canine Kidney (MDCKII) cell line and its Bcrp1 transduced subline [17] were used to establish whether temozolomide is a substrate of Mdr1a (P-gp) and Bcrp1, respectively. Cells were seeded on Transwell microporous polycarbonate membrane filters (3.0 µm pore size, 24 mm diameter; Costar Corning, NY) at a density of 1 × 10$^6$ cells