Brain Pharmacokinetics of Ganciclovir in Rats with Orthotopic BT4C Glioma

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ABSTRACT

Ganciclovir (GCV) is an essential part of the Herpes simplex virus thymidine kinase (HSV-tk) gene therapy of malignant gliomas. The purpose of this study was to investigate the brain pharmacokinetics and tumor uptake of GCV in the BT4C rat glioma model. GCV’s brain and tumor uptakes were investigated by in vivo microdialysis in rats with orthotopic BT4C glioma. In addition, the ability of GCV to cross the blood-brain barrier and tumor vasculature was assessed with in situ rat brain perfusion. Finally, the extent to which GCV could permeate across the BT4C glioma cell membrane was assessed in vitro. The areas under the concentration curve of unbound GCV in blood, brain extracellular fluid (ECF), and tumor ECF were 6157, 1658, and 4834 μM min, respectively. The apparent maximum unbound concentrations achieved within 60 minutes were 46.9, 11.8, and 25.8 μM in blood, brain, and tumor, respectively. The unbound GCV concentrations in brain and tumor after in situ rat brain perfusion were 0.41 and 1.39 nmol/g, respectively. The highly polar GCV likely crosses the fenestrated tumor vasculature by paracellular diffusion. Thus, GCV is able to reach the extracellular space around the tumor at higher concentrations than that in healthy brain. However, GCV uptake into BT4C cells at 100 μM was only 2.1 pmol/mg of protein, and no active transporter-mediated disposition of GCV could be detected in vitro. In conclusion, the limited efficacy of HSV-tk/GCV gene therapy may be due to the poor cellular uptake and rapid elimination of GCV.

INTRODUCTION

Gliomas are the most common primary brain tumors, and the majority of them are malignant. Glioblastoma multiforme is considered the most common and aggressive form of all brain tumors (Louis et al., 2007). In patients with glioblastoma multiforme, the life expectancy is only 1 year after diagnosis (Kanu et al., 2009). Currently, the treatment of malignant gliomas consists of surgery followed by radiotherapy and chemotherapy, but in spite of therapy, malignant gliomas almost invariably recur, and after recurrence, the median survival is a mere 2–3 months. Thus, there is an enormous need to devise more-efficient therapies.

Herpes simplex thymidine kinase (HSV-tk) gene therapy with ganciclovir (GCV) represents a promising new therapeutic strategy for the treatment of recurrent malignant gliomas (Pulkkanen and Ylä-Herttuala, 2005). After surgical removal of the tumor, the adenovirus-mediated HSV-tk gene is targeted to the remaining dividing cells on the walls of the tumor cavity (Westphal et al., 2013). Tumor cells transduced with the HSV-tk gene convert GCV to a GCV monophosphate (GCV-MP), which is further phosphorylated into the cytotoxic GCV triphosphate (GCV-TP) by cellular kinases (Fig. 1) (Reardon, 1989). The cytotoxic GCV-TP inhibits the activity of the DNA polymerase and thus prevents DNA replication, ultimately resulting in cell death mainly through apoptosis. Despite promising results in preclinical trials, at best only a modest increase in median survival has been achieved with HSV-tk/GCV gene therapy in the clinical trials conducted in patients with recurrent gliomas (Tobias et al., 2013; Westphal et al., 2013). It has been speculated that the limited transduction efficiency of tumor cells with the HSV-tk gene and tumor heterogeneity, which challenge vector targeting and delivery, may impair the efficacy of the gene therapy concept (Candolfi et al., 2006; Tobias et al., 2013). However, adequate and stable GCV levels are needed to achieve cytotoxic GCV-TP levels inside the tumor cells. Thus, another less well recognized reason for the limited efficacy of HSV-tk/GCV gene therapy may be the poor delivery of GCV into the tumor cells. However, brain pharmacokinetics and access to tumor cells of GCV have not been studied; i.e., the role of pharmacokinetic factors in the efficacy of HSV-tk/GCV gene therapy remains unresolved.

Factors such as hypoxia, intratumoral pressure gradients, and the abnormal vasculature can limit drug uptake from the systemic circulation into the extracellular spaces surrounding tumor cells (Jain et al., 2007). It is important to be aware that these factors limit the

ABBRREVATIONS: ABC, ATP-binding cassette; ANOVA, analysis of variance; AUC, area under the concentration curve; BBB, blood-brain barrier; [14C]CPT, [14C]camptothecin; ECF, extracellular fluid; GCV, ganciclovir; GCV-MP, ganciclovir monophosphate; GCV-TP, ganciclovir triphosphate; GF120918, N-[4-{[1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinolinyl]ethyl}-1-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; HSV-tk, Herpes simplex virus thymidine kinase; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MK-571, 3-[3-[2-[(7-chloroquinolin-2-yl)vinyl]phenyl]-2-dimethylcarbamoyl-benzenesulfanyl][methylsulfanyl] propionic acid; MRP, multidrug resistance–associated protein; PS, permeability–surface area product; [3H]TAX, [3H]paclitaxel.
tumor uptake of lipophilic cancer drugs, for which tumor uptake is governed by tumor perfusion rather than their permeability across cell membranes. Therefore, lipophilic cancer drugs may reach higher concentrations in healthy brain than in the tumor. However, in the case of polar molecules, it is likely that tumor or brain uptakes are predominantly limited by poor permeability across the blood-tumor barrier or blood-brain barrier (BBB). Thus, the angiogenic and fenestrated tumor vasculature present in high-grade gliomas may result in higher drug concentrations in the tumor than those found in healthy brain tissue. This phenomenon has been demonstrated for temozolomide and methotrexate not only in rodents but also in humans (de Lange et al., 1995; Blakeley et al., 2009; Rosso et al., 2009). Because it is a highly polar molecule, GCV (Table 1) would be placed in the category of a drug with poor permeability across biologic membranes, and the GCV concentration in tumor likely exceeds the concentration in healthy brain. Even if GCV was able to reach the tumor extracellular fluid (ECF) across the compromised BBB, its high polarity may limit drug uptake into the tumor cells and its subsequent transformation into GCV-TP. There may be yet one more pharmacokinetic hurdle to be overcome: In patients, the tumor mass is removed from the brain and the HSV-tk gene is targeted to the remaining dividing cells on the walls of the tumor cavity. As a result, the hydrophilic GCV may prefer to remain in the tumor cavity, which is filled with aqueous ECF, rather than crossing the cell membranes of the remaining glioma cells.

The objective of the present study was to assess the brain pharmacokinetics of GCV in rats with malignant BT4C glioma by utilizing the pharmacokinetic application of in vivo microdialysis. Furthermore, GCV permeation across the BBB, the tumor vasculature, as well as the glioma pharmacokinetic application of in vivo microdialysis. Furthermore, GCV uptake studies in BT4C glioma cells in vitro. In addition, the role of active transport mechanisms in GCV uptake was assessed in vitro.

Materials and Methods

Drugs and Reagents. GCV and acyclovir were obtained from MB Biomedicals (Santa Ana, CA). [3H]Ganciclovir ([3H]GCV) (4.2 Ci/mmol) and [3H]Paclitaxel ([3H]TAX) (5.4 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [3H]Camptothecin ([3H]CPT) (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). OptiPhase SuperMix scintillation cocktail was obtained from PerkinElmer (Waltham, MA). N-(4-[2-(1,2,3,4-Tetrahydro-6,7-dimethoxy-2-isquinolinyl)ethyl]-phenyl)-9,10-di{[3-[2-(7-Chloroquinolin-2-yl)vinyl][phenyl]-[2-dimethylcar-bamoylethylsulfanyl]methylsulfanyl]} propionic acid (MK-571) was obtained from Cayman Chemical Company (Ann Arbor, MI). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless mentioned otherwise. All solvents and chemicals were of high-performance liquid chromatography grade or higher. Laboratory Ultrapure water was produced using an Elix system (Millipore, Billerica, MA).

BT4C Rat Glioma Model. BT4C rat glioma cells (Laerum et al., 1977; Sandmair et al., 2000) were obtained from Ark Therapeutics (London, UK). BT4C glioma cells were inoculated intracranially in inbred male BDII rats aged 3–4 months (Charles River Laboratories, Lille, France) as described previously (Tyynen et al., 2002). Briefly, 10,000 BT4C cells in 5 μl of Optitop medium were inoculated over right the corpus callosum into brain parenchyma under stereotactic guidance. Coordinates were 1 mm caudal to bregma, 2 mm right of sagittal suture, and a depth of 2.5–3.0 mm. Inoculations were done slowly over 5 minutes to avoid backflow, and the needle was left in place for another 5 minutes. Sham inoculations on the opposite brain hemisphere (termed here “healthy brain”) were performed by injecting 5 μl of Optitop medium without BT4C cells. All procedures with the animals were performed according to appropriate European Community guidelines and Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised in 1985). The procedures were reviewed and approved by the Finnish National Animal Experiment Board.

In Situ Rat Brain Perfusion Technique. Between day 15 and day 24 after inoculation, the rats with brain BT4C glioma were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and xylazine (5 mg/ml), and their right carotid artery system was exposed. The right external carotid artery was ligated, and the right common carotid artery was cannulated with PE-50 catheters filled with 100 IU/ml heparin. A GCV concentration of 100 μM was perfused for 60 seconds (10 ml/min), and the brain vasculature was washed.

### TABLE 1

<table>
<thead>
<tr>
<th>Molecular Mass</th>
<th>Polar Surface Area</th>
<th>Calculated Partition Coefficient</th>
<th>H-Bond Donors</th>
<th>H-Bond Acceptors</th>
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<td>139</td>
<td>-2.1</td>
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<td>9</td>
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Fig. 1. The structure and activation pathway of GCV.
with 2 ml of drug-free perfusion buffer. The tumor and a part of the healthy brain tissue from the right brain hemisphere were removed for GCV concentration analysis. The in situ rat perfusion technique has been described in detail previously (Gynter et al., 2008). 

Non-specific Tissue Binding. Non-specific tissue binding of GCV was determined in rat brain and BT4C tumor tissue homogenates as well as in rat plasma in vitro using equilibrium dialysis. Brain and tumor tissues were homogenized in three volumes of phosphate-buffered saline at pH 7.4 on ice by using an ultrasonic homogenizer, after which GCV was added into the homogenate. Equilibrium dialysis was performed for 400 μl of tissue homogenate or plasma and 600 μl of buffer for 4 hours at 37°C in a single-use RED Plate with an 8-kDa cutoff dialysis membrane (Thermo Scientific, Rockford, IL). The dialysis GCV concentrations were determined, and then the buffer-to-homogenate/ plasma concentration ratios of GCV were calculated. The concentration ratios were used to calculate the unbound fraction of GCV in brain and tumor (f<sub>u</sub>brain). A previously described approach to account for the effect of tissue dilution on unbound fraction was used to calculate the brain unbound fraction (Kalvass and Maurer, 2002):

\[
\text{f}_{u,\text{tissue}} = \frac{1}{D} \left( \frac{1}{(1/f_{u,\text{homogenate}})} - 1 \right) + \frac{1}{D}
\]

where D represents the dilution factor of brain or tumor tissue and f<sub>u, homogenate</sub> is the ratio of concentrations determined from the buffer and tissue homogenates. As plasma was not diluted prior to equilibrium dialysis, the f<sub>u, plasma</sub> value could be obtained directly from the buffer-to-plasma ratio.

In Vivo Microdialysis. Extracellular unbound brain, tumor, and blood levels of GCV were determined using a triple-probe in vivo microdialysis approach after a 25 mg/kg i.p. bolus injection of GCV. The 25 mg/kg dose of GCV administered twice daily has been found to be effective in rats with BT4C gliomas expressing HSV-tk (Tyynelä et al., 2002). First, the rats were anesthetized with the combination of ketamine/medetomidine (60/0.4 mg per kg i.p.), and intracerebral guide cannulas (MAB 9.9.IC; AgnTho, Lidingö, Sweden) were implanted stereotactically into the brain 19–24 days after inoculation. One cannula was implanted just above the tumor (coordinates measured from bregma and the top of the skull: anteroposterior: −1.0; lateral: −2.0; dorsoventral: −1.0) and another one on the opposite side of the brain into healthy tissue (coordinates: anteroposterior: −1.0; lateral: −2.0; dorsoventral: −1.0). The cannulas were fixed into the skull by anchor screws and dental cement. Once the cannulas were tightly secured, the intracerebral microdialysis probes (MAB 9.9.4; 6-kDa cutoff, 4-mm active membrane length; AgnTho’s) were carefully inserted into the brain through the guide cannulas. A single dose of buprenorphine (0.02 mg/kg s.c.) was given to relieve any postoperative pain, and the animals were woken by an injection of pentobarbital to prevent blood clotting.

The in vivo recovery values of the probes were obtained directly from the buffer-to-plasma ratio. The relative recovery (R<sub>n</sub>) was inserted into the vein using a plastic introducer. The Ringer’s solution used in the peripheral probe contained an additional 20 IU/ml of dalfopristin to prevent blood clotting. Each probe was calibrated in vivo by a retrodialysis by drug method (Bouw and Hammarlund-Udenaes, 1998). Briefly, 0.5 μM GCV (C<sub>pu</sub>) was added to the perfusion solution, and five samples in fractions of 20 minutes were collected at a flow rate of 1 μl/min. The relative recovery (R) of GCV was estimated for each probe and rat, and the mean C<sub>pu</sub> value of the last three samples during the retrodialysis was used to calculate the in vivo recovery of GCV with the equation:

\[
R = \frac{C_{\text{pu}} - C_{\text{null}}}{C_{\text{pu}}}
\]

The in vivo recovery values of the probes were 45.4, 17.4, and 12.2% for probes in blood, brain, and tumor, respectively. After the retrodialysis, the perfusion solution was changed back to blank Ringer’s solution without GCV. A washout period of 80 minutes (the first 20 minutes at a flow rate of 5 μl/min, and 60 minutes at 1 μl/min) was allowed before systemic GCV administration. Finally, GCV was administered intraperitoneally, and dialysate was collected in 20-minute fractions for 300 minutes into polypropylene vials (AgnTho’s). The dialysate samples were stored at −80°C until analyzed. After the termination of each experiment, the rats were decapitated and the brains were rapidly removed and frozen in isopentane cooled with dry ice. To verify the correct probe location in the tumor and in the healthy tissue, the brains were cut into 30-μm slices with a cryostat (Bright Instrument, Huntingdon, UK). Only animals with successful probe placement were included in the pharmacokinetic analysis.

Brain Tissue and BT4C Tumor Sample Preparation. Brain tissue and BT4C tumor samples were weighed and homogenized with ultrapure water (1:3). An aliquot of 50 μl was taken, and GCV was isolated from the samples by protein precipitation with 150 μl of acetonitrile. Samples were vortexed and centrifuged for 10 minutes at 10,000 g at 4°C. Then 100 μl of supernatant was mixed with 100 μl of 300 mM internal standard (acyclovir) in water prior to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. In vivo microdialysis samples were diluted with ultrapure water, and a 20-μl aliquot was mixed with 180 μl of 300 mM internal standard prior to LC-MS/MS analysis.

Cell Culture. BT4C glioma cells were maintained in 75-cm² cell culture flasks in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (American Type Culture Collection, Manassas, VA) and 1% penicillin-streptomycin (American Type Culture Collection) at 37°C in a humidified atmosphere containing 5% CO₂ and subcultured twice per week. The cells were seeded onto 24-well plates (Nunc, Rochester, NY) at 0.3 × 10⁵ or 1 × 10⁶ cells/well and cultured for 3 or 2 days, respectively.

Expression of ATP-Binding Cassette Transporter Genes by Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction. The total RNA extraction and quantification of the ATP-binding cassette (ABC) transporter genes (Mrkd1, Abcb1; R. erythraea, 2009; Abcc1, Abcc2, Abcc5–6) from cultured BT4C cells, rat BT4C tumor, healthy rat brain tissue from the noninoculated cerebral hemisphere, and rat liver tissue were performed as described previously (Soininnen et al., 2012). The TaqMan gene expression assays were Rat0561753_m1 (Abcb1), Rn00710558_m1 (Abcc2), Rn00574093_m1 (Abcc1), Rn00563231_m1 (Abcc2), Rn01452854_m1 (Abcc3), Rn01465702_m1 (Abcc4), Rn00588341_m1 (Abcc5), Rn00578778_m1 (Abcc6), and Rn00678697_m1 (Actb) (Applied Biosystems, Foster City, CA).

Cellular Uptake In Vitro. The uptake studies were carried out as described previously (El Hafny et al., 1997) with minor modifications. Briefly, BT4C cells grown onto 24-well plates were washed twice with uptake buffer (129 mM NaCl, 0.63 mM CaCl₂, 2.5 mM KCl, 0.74 mM MgSO₄, 7.4 mM Na₂HPO₄, 1.3 mM KH₂PO₄, 5.5 mM t-glucose, and 10 mM Hepes [pH 7.4]) and then preincubated with or without the P-glycoprotein inhibitor GF120918 (Wallstab et al., 1999) (3 μM), the inhibitor of multidrug resistance-associated protein (MRP) family transporters MK-571 (Weiss et al., 2007) (50 μM), or GCV (100 μM) at 37°C for 30 minutes. [³H]TAX and [³H]CPT were used as positive reference compounds to assess the functionality of P-glycoprotein and MRP family transporters, as these compounds have been shown to be ABC transporter substrates (Kemper et al., 2003; Tian et al., 2005). After the preincubation, [³H]GCV (0.5 μCi/ml, 0.12 μM) and the reference compounds [³H]TAX (0.5 μCi/ml, 9.2 nm) and [³H]CPT (0.5 μCi/ml, 33 nM) prepared either in the fresh uptake buffer or in the uptake buffer including GF120918 (3 μM), MK-571 (50 μM), or GCV (100 μM) were added to the cells and incubated at 37°C on a stirring platform for 20 minutes. The cellular uptake was stopped by rapidly aspirating the drug solutions, and the cells were washed three times with ice-cold uptake buffer. Cells were lysed with 0.1 M NaOH. Cellular protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. Radioactivity of the samples was analyzed with liquid scintillation counting (1450 MicroBeta TriLux Liquid Scintillation Counter; Wallac Ltd., Turku, Finland) after the addition of 500 μl of OptiPhase SuperMix scintillation cocktail. The amount of [³H]GCV, [³H]TAX, or [³H]CPT retained in the BT4C cells was normalized to the protein concentration and expressed as picomoles per milligram of protein.

Liquid Chromatographic and Mass Spectrometric Conditions. GCV concentration in the in vivo microdialysis and in situ rat brain perfusion samples were analyzed with LC-MS/MS. An Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Palo Alto, CA) was used together with a Hypercarb (50 mm × 2.1 mm) porous graphite carbon column. A Rapid Resolution LC in-line filter (2 mm, max 600 bar, 0.2 μm; Agilent Technologies) was used for protecting the analytical column from possible
contaminants. The high-performance liquid chromatography eluents were water (A) and acetonitrile (B), both containing 0.1% formic acid. A gradient elution with 5–90% B was applied in 0.5–7 minutes, followed by 1 minute of isocratic elution with 90% B and column equilibration, giving a total time of 12 minutes/injection. The eluent flow rate was 0.2 ml/min, whereas the column temperature was 60°C and injection volume 10 µl. The data were acquired with an Agilent 6410 Triple Quadrupole Mass Spectrometer equipped with an electrospray ionization source (Agilent Technologies). The following mass spectrometry conditions were used: electrospray ionization, positive ion mode; drying gas (nitrogen) temperature, 300°C; drying gas flow rate, 10 l/min; nebulizer pressure, 40 psi; and capillary voltage, 4000 V. Analyte detection was performed using multiple reaction monitoring, the transitions being 256.2 → 152.1 and 226.1 → 152.1 for GCV and acyclovir (internal standard), respectively. Fragmentor voltages and collision energies used for GCV were 100 V and 8 V, whereas for acyclovir the parameters were 75 V and 8 V, respectively. The pressure of the collision cell nitrogen was adjusted to 2.9 × 10⁻⁵ Torr. The divert valve was programmed to allow eluent flow into the mass spectrometer from 2–11 minutes for each run. Agilent MassHunter Workstation Acquisition software (Data Acquisition for Triple Quadrupole Mass Spectrometer, version B.03.01; Agilent Technologies) was used for data acquisition, whereas Quantitative Analysis (B.04.00) software was used for the data processing and analysis.

Data Analyses. An unpaired two-tailed t test was used to test the statistical significance of differences between brain and tumor GCV concentration after in situ rat brain perfusion. In the microdialysis experiments, sample concentrations of GCV were corrected with the in vivo recovery values determined for each probe. The unbound area under the concentration curve (0–300 minutes) (AUCu,0–300 minute) values were calculated from the individual data with the trapezoid rule using GraphPad Prism 5.03 software (GraphPad Software, Inc., La Jolla, CA). The apparent pharmacokinetic parameters—maximum unbound concentration (Cmax), time to reach Cmax (tmax), and elimination half-lives (t1/2) — in the brain, tumor, and blood—were calculated from microdialysis data by using a one-compartment model and WinNonlin Professional v5.0.1 software (Pharsight Corporation, Mountain View, CA). The statistical significance of differences between GCV AUCu,0–300 minute in blood, brain, and tumor was tested by one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparisons test with GraphPad Prism 5.03 Software.

To evaluate the effects of GF120918, MK-571, and unlabeled GCV on the cellular uptake of [³H]GCV, [³H]TAX, or [³H]CPT in the BT4C cells in vitro, the statistical significances of differences between different treatments were tested by one-way ANOVA followed by Dunnett’s test by using GraphPad Prism 5.03 software. Statistical significance was set at P < 0.05. Statistical significance of differences between the expressions of the ABC transporter genes in the tumors was confirmed from brain slices after the in vivo microdialysis experiments (data not shown). One probe out of seven was not correctly positioned in the tumor, and the corresponding GCV concentration data were excluded from the pharmacokinetic analysis.

Expression and Functionality of ABC Transporter Genes. Expression of ABC transporter genes (Mdr1, Abcb1; Bcrp1, Abcg2; Mrpl–6, Abcc1–6) in BT4C cells, rat brain BT4C tumor, healthy rat brain tissue, and rat liver was assessed by using real-time quantitative reverse-transcription polymerase chain reaction analysis. Expressions of Abcb1, Abcg2, Abcc1, and Abcc3–5 genes are shown in Fig. 4. Expression of Abcc2 was not detected in BT4C cells, rat brain BT4C tumor, or healthy rat brain tissue. Expression of Abcc2 in rat liver tissue was 3.1 × 10⁻² ± 8.9 × 10⁻⁴ (mean ± S.E.M., n = 6). Similarly, expression of Abcc6 was not detected in BT4C cells or rat brain BT4C tumor. Expression of Abcc6 in healthy rat brain tissue (8.7 × 10⁻⁶ ± 2.7 × 10⁻⁶; mean ± S.E.M., n = 6) was significantly lower (t test, P < 0.001) than in rat liver tissue (4.0 × 10⁻² ± 1.4 × 10⁻⁴; mean ± S.E.M., n = 6).

The functionality of ABC transporters, such as P-glycoprotein and MRP, in the BT4C cells was confirmed by using two positive controls: [³H]TAX for P-glycoprotein–mediated transport and [³H]CPT for MRP family–mediated transport. The cellular uptake of [³H]TAX was significantly increased in the presence of a potent P-glycoprotein inhibitor (GF120918) (Fig. 5); i.e., 31% of the [³H]TAX amount was inhibited with GF120918, evidence for the functionality of P-glycoprotein in the BT4C cells. Similarly, the cellular uptake of [³H]CPT was significantly increased in the presence of a potent inhibitor

| TABLE 2
| Apparent pharmacokinetic parameters of unbound GCV calculated from microdialysis data |
|-------------------|-------------------|-------------------|-------------------|-------------------|
|                  | AUCu,0–300 minute | Cmax              | tmax              | t1/2              |
| Blood             | 6157 ± 1207       | 46.9 ± 10.8       | 60                | 77                |
|                   | (µM·min)          | (µM)              | (min)             | (%)               |
| Brain ECF         | 1658 ± 239        | 11.8 ± 1.7        | 60                | 116               |
| Tumor ECF         | 4834 ± 1182**     | 25.8 ± 6.2        | 60                | 143               |

*Apparent value (fraction 40–60 minutes).
**P < 0.01 vs. brain ECF value.

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The statistical significance of the difference between concentrations of the MRPs (MK-571) (Fig. 5). The uptake of [3H]CPT was increased by 2.6-fold (from 0.4 to 1.04%), indicating that MRPs family transporters were inhibited in the BT4C cells. In addition, 100 μM unlabeled GCV was not able to alter the cellular uptake of [3H]TAX or [3H]CPT (Fig. 5), suggesting that GCV did not inhibit P-glycoprotein or MRPs family–mediated transport.

**Cellular Uptake of GCV in BT4C Glioma Cells In Vitro.** The uptake of [3H]GCV into the BT4C glioma cells and the role of active transporter–mediated disposition were assessed in vitro (Fig. 5). The cellular uptake of [3H]GCV was shown to be extremely poor (2.1 pmol/mg of protein), corresponding to only 0.12% of the [3H]GCV amount to which BT4C glioma cells had been initially exposed. Because GCV has been proposed to undergo ABC transporter–mediated transport in vitro (Adachi et al., 2002; Hu and Liu, 2010), we evaluated the effect of potent ABC transporter inhibitors (GF120918 and MK-571) on uptake of [3H]GCV in the BT4C glioma cells. The inhibitors were not able to increase the cellular uptake of [3H]GCV into the BT4C glioma cells (Fig. 5). In addition, the effect of a higher GCV concentration on the cellular uptake of [3H]GCV was tested to reveal other active transport mechanisms, but the [3H]GCV uptake into the BT4C glioma cells was not changed in the presence of 100 μM unlabeled GCV. These results indicate that [3H]GCV was not undergoing active transporter–mediated disposition in the BT4C cells.

**Discussion**

The results of in situ rat brain perfusion and in vivo microdialysis experiments revealed that GCV is able to cross both the BBB and tumor vasculature despite its high polarity. This rather unexpected result could be explained by the high driving force created by the high unbound blood concentrations. In addition, no evidence for the involvement of an active ABC transporter mechanism in the GCV disposition was found in our in vitro studies, although several active ABC transporters were demonstrated to be expressed and functional in the BT4C glioma cells. Polar GCV has a low PS product across the functional BBB, and the GCV concentrations in brain tumor were significantly higher than those in healthy brain tissue in both the in vivo microdialysis experiment and the in situ brain perfusion studies. This is most likely due to a compromised BBB in the tumor, as a result of angiogenesis, which allows hydrophilic small molecules such as GCV to pass the BBB and enter the tumor ECF by passive paracellular diffusion.

Although our present in vivo data suggest that GCV reaches the BT4C glioma ECF at high micromolar concentrations, high ECF concentrations do not necessarily reflect pharmacologically effective concentrations in the target site, i.e., in the intracellular compartment of tumor cells. Indeed, the present data indicate that the cellular uptake of GCV in BT4C glioma cells in vitro is extremely poor: only 0.12% of the extracellular concentration is actually taken up into the cells. The poor uptake into the BT4C glioma cells is probably due to the high polarity of GCV, which hinders its permeability across cell membranes. Thus, although the GCV C\textsubscript{u,max} in tumor ECF was as high as 30 μM at a dose of 25 mg/kg i.p., one could estimate that the intracellular concentrations of GCV would only be in the nanomolar range. However, 25 mg/kg of GCV twice daily has been shown to be adequate in nonclinical efficacy studies with HSV-tk/GCV gene therapy in the BT4C rat glioma model. Furthermore, the present in vivo data show that GCV is rapidly eliminated from blood and tumor ECF, reaching a level of <10 μM within 5 hours after administration, which raises concerns about the sufficient duration of a minimal effective concentration at the target site. In addition, in humans GCV is rapidly eliminated from plasma (elimination half-life of 2.5 hours) (Fletcher et al., 1986), but there are no brain concentration data available. When given twice daily, which has been the protocol in both nonclinical (Sandmair et al., 2000) and clinical HSV-tk/GCV gene therapy efficacy studies (Sandmair et al., 2000; Westphal et al., 2013), the GCV concentration in the tumor is likely to decline below the effective level between doses. On the basis of the nonclinical brain pharmacokinetic data obtained in the present study, one could speculate that the efficacy of HSV-tk/GCV gene therapy could be enhanced by achieving an elevated and prolonged GCV concentration at the target site. However, high-dose GCV treatment is commonly associated with a range of serious hematologic adverse effects. In addition, intravenous injection of GCV causes pain and phlebitis at the injection site due to the high pH of the drug solution. These adverse effects limit the use of higher GCV doses in patients. Therefore, instead of using higher GCV doses, shorter dosing intervals, constant-rate infusion, controlled-release formulation, or innovative drug delivery technology should be considered as ways to achieve enhanced glioma cell uptake of GCV and to prolong the duration of pharmacologically effective drug levels at the target site. However, it should be noted

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**Fig. 2.** Brain and tumor uptake of GCV after in situ rat brain perfusion (100 μM GCV was perfused for 60 seconds at 10 ml/min) in rats with BT4C glioma. GCV total and unbound concentrations were 0.55 ± 0.16 and 0.41 ± 0.12 nmol/g in brain and 1.78 ± 0.28 and 1.39 ± 0.22 nmol/g in tumor (mean ± S.E.M., n = 4), respectively. The statistical significance of the difference between concentrations was determined with unpaired two-tailed t test. **P < 0.01.

**Fig. 3.** Unbound GCV concentration in blood, brain, and tumor ECF after administration of a single 25-mg/kg i.p. dose in rats with BT4C glioma. Data are mean ± S.E.M. (n = 6–7).
that not only are there species differences in tumor structure and microenvironment, but also the different HSV-tk/GCV gene therapy protocols between the BT4C rat glioma model and human patients with malignant glioma limit the translation of the present nonclinical results to humans. In patients, the tumor mass is removed from the brain, whereas in the BT4C rat glioma model, the tumor core is not removed before gene therapy. After removal of the tumor, a cavity filled with ECF is formed. Therefore, the volume of aqueous ECF may increase, leading to a reduced GCV concentration in the vicinity of the remaining glioma cells. Furthermore, this would lead to therapeutically inadequate GCV concentrations inside the glioma cells.

This nonclinical pharmacokinetic study revealed that the currently used GCV treatment protocol associated with HSV-tk/GCV gene therapy

![Fig. 4. Expression of Mdr1 (Abcb1), Bcrp1 (Abcg2), Mrp1 (Abcc1), and Mrp3–5 (Abcc3–5) genes in BT4C glioma cells, rat brain BT4C tumor, healthy rat brain tissue, and rat liver at mRNA level measured by real-time quantitative reverse-transcription polymerase chain reaction. Values are normalized to the expression of the β-actin (Actb) gene. Data are mean ± S.E.M., n = 6, except n = 9 in Mdr1 in liver tissue. ND, not detected. Statistical significances of differences were tested by one-way ANOVA followed by Tukey's test. *P < 0.05; **P < 0.01; ***P < 0.001.]

![Fig. 5. Cellular uptake (picomoles per milligram of protein) of [3H]GCV, [3H]TAX, and [3H]CPT in the BT4C glioma cells and the effect of different treatments (3 μM GF120918, 30 μM MK-571, and 100 μM unlabeled GCV) on the cellular uptake of [3H]GCV, [3H]TAX, and [3H]CPT. Data are mean ± S.E.M. from three independent experiments conducted in duplicate. Statistical significances of differences between different treatments were tested by one-way ANOVA followed by Dunnett's test. **P < 0.01; ***P < 0.001.]

produces high extracellular GCV concentrations in brain and tumor ECF in the BT4C rat glioma model. However, the results suggest that the target site (intracellular) concentrations are significantly lower and that the unbound blood and tumor extracellular GCV levels decrease rapidly, reaching zero level between doses. In conclusion, poor delivery of GCV into the tumor cells and especially the rapid elimination may be one reason for the limited efficacy of HSV-tk/GCV gene therapy. Thus, GCV brain pharmacokinetics should be studied in patients to optimize GCV dose and dose regimen. To achieve higher and more sustained GCV levels at the target site, GCV could be administered as a constant-rate infusion or as a controlled-release formulation.

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