Brain Pharmacokinetics of Ganciclovir in Rats with Orthotopic BT4C Glioma

Mikko Gynther, Tiina M. Kääriäinen, Jenni J. Hakkarainen, Aaro J. Jalkanen, Aleksanteri Petsalo, Marko Lehtonen, Lauri Peura, Jere Kurkipuro, Haritha Samaranayake,1 Seppo Ylä-Herttuala, Jarkko Rautio, and Markus M. Forsberg

Faculty of Health Sciences, School of Pharmacy (M.G., T.M.K., J.J.H., A.J.J., A.P., M.L., L.P., J.R., M.M.F.) and A. I. Virtanen Institute for Molecular Sciences (J.K., H.S., S.Y.-H.), University of Eastern Finland, Kuopio, Finland

Received July 4, 2014; accepted October 27, 2014

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; [3H]CPT, [3H]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-isouquinolin-1(7H)-yl]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-dimethylcar-bamoylethylsulfanyl]methylsulfanyl] propionic acid; MRP, multidrug resistance–associated protein; PS, permeability–surface area product; [3H]TAX, [3H]paclitaxel.

This work was financially supported by the Finnish Funding Agency for Innovation (TEKES)/European Regional Development Fund (ERDF) [Grant 70043/10].

1Current affiliation: Aurealis Pharma Ltd., Kuopio, Finland.

This work was financially supported by the Finnish Funding Agency for Innovation (TEKES)/European Regional Development Fund (ERDF) [Grant 70043/10].

1Current affiliation: Aurealis Pharma Ltd., Kuopio, Finland.
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 another pharmacokinetic hurdle to be overcome: In patients, the 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 cell membrane was assessed with in situ rat brain perfusion technique and with 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-(1,3,4-Tetrahydro-6,7-dimethoxy-2-isquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918) was a gift from GlaxoSmithKline (Research Triangle Park, NC). 3-[[3-[2-(7-Chloroquinolin-2-yl)vinyl]phenyl]-[2-dimethylcarbamoylthiophenyl]-methoxy]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 BDIX rats aged 3–4 months (Charles River Laboratories, Lille, France) as described previously (Tyynelä et al., 2002). Briefly, 10,000 BT4C cells in 5 μl of Optitrem 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 right to fs a g i t t a l s u t u r e , a n d d e p t ho f 2 . 5 – 3 . 0 m m. 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 Optitrem 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

Selected physicochemical properties of GCV

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Mass</td>
<td>255.23 Da</td>
</tr>
<tr>
<td>Polar Surface Area</td>
<td>139 Å²</td>
</tr>
<tr>
<td>Calculated Partition Coefficient</td>
<td>2.1</td>
</tr>
<tr>
<td>H-Bond Donors</td>
<td>5</td>
</tr>
<tr>
<td>H-Bond Acceptors</td>
<td>9</td>
</tr>
</tbody>
</table>

**Fig. 1.** The structure and activation pathway of GCV.

![Structure and Activation Pathway of GCV](image-url)
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 (Gynther 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><sub>tissue</sub>). 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):

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

where D represents the dilution factor of brain or tumor tissue and f<sub>u</sub><sub>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 (Tynnell et al., 2002). First, the rats were anesthetized with the combination of ketamine/medetomidine (600.4 mg per kg i.p.) and intracerebral guide cannulas (MAB 9.9IC; AgnTho’s, 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; 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 atipamezole (1 mg/kg s.c.). The rats were allowed to recover from the surgery for 40 hours.

After the 40-hour recovery period, the animals were anesthetized (ketamine/medetomidine 600.4 mg per kg i.p.) and placed on a heating pad to maintain normal body temperature during the experiment. The intracerebral probes were attached to the microdialysis tubings, and Ringer’s solution containing 138 mM NaCl, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂, 11 mM NaHCO₃, 1 mM NaH₂PO₄, and 11 mM H-glucose (pH 7.4) was allowed to flow through the system with a flow rate of 1 μl/min. The left femoral vein was exposed, and a concentric perfusion probe (MAB11.20.10; 6-kDa cutoff, 10-mm active membrane length; AgnTho’s) 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 dalteparin to prevent blood clotting.

Each probe was calibrated in vivo by a retrodialysis by drug method (Bouw et al., 2012) and, during a period of 24–48 hours, 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 10,000g at 4°C. Then 100 μl of supernatant was mixed with 100 μl of 300 nM 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 nM 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 × 10⁴ or 1 × 10⁵ cells/well and cultured for 5 or 3 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 (Mdr1, Abcb1; Bcrp1, Abcg2; Mpi1–6, Abcc1–6) 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 (Soininen et al., 2012). The TaqMan gene expression assays were Rn0561753_m1 (Abcb1), Rn00710585_m1 (Abcg2), Rn0574093_m1 (Abcc1), Rn00563231_m1 (Abcc2), Rn01452854_m1 (Abcc3), Rn01467572_m1 (Abcc4), Rn00588341_m1 (Abcc5), Rn00557877_m1 (Abcc6), and Rn01067869_m1 (Abct) (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 d-glucose, and 10 mM Hepes (pH 7.4)) and then preincubated with or without the P-glycoprotein inhibitor GI2910981 (Wallstall 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 references 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) were added to both the fresh uptake buffer in or the uptake buffer including GI2910981 (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 concentrations in the in vitro microdialysis and in situ rat brain perfusion experiments were analyzed with LC-MS/MS. An Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Palo Alto, CA) was used together with a Hypersarc (50 mm × 2.1 mm) porous graphitic 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
in the brain, tumor, and blood (Pharsight Corporation, Mountain View, CA). The statistical significance of using a one-compartment model and WinNonlin Professional v5.0.1 software was tested by one-way ANOVA followed by Dunnett’s test (Table 2). In the study of Liu et al. (2004), the PS product of 28 drug-like molecules was determined and only two of these molecules had lower PS products than GCV.

**In Vivo Pharmacokinetics and Tumor Uptake of GCV.** GCV’s ability to reach the extracellular compartment of healthy brain tissue and BT4C glioma after a single dose of 25 mg/kg i.p. was determined with in vivo microdialysis. The time-concentration profiles of unbound GCV in blood, healthy brain tissue, and tumor are presented in Fig. 3, and the apparent in vivo pharmacokinetic parameters $AUC_{u,0-300\text{min}}$, $C_{\text{max}}$, $t_{\text{max}}$, and $t_{1/2\text{g}}$ calculated from the microdialysis data are presented in Table 2. The $C_{\text{max}}$ of GCV was 2.2-fold higher in tumor ECF than in healthy brain ECF, and there was a 2.9-fold difference between brain and tumor $AUC_{u,0-300\text{min}}$ values ($P < 0.01$) (Table 2). The brain/blood and tumor/blood $AUC_{u,0-300\text{min}}$ ratios ($K_{\text{p,ibu}}$) of GCV were 0.27 and 0.79, respectively. The location of the microdialysis probes in the tumors was confirmed from brain slices after the in vivo experiments (data not shown). The probe outside 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; Berp1, Abcg2; Mrpl6–6, 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 Abcc5–5 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 $3.1 \times 10^{-7} \pm 8.9 \times 10^{-4}$ (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 was $8.7 \times 10^{-6} \pm 2.7 \times 10^{-6}$ (mean ± S.E.M., $n = 6$) was significantly lower ($t$ test, $P < 0.001$) than in rat liver tissue ($4.0 \times 10^{-5} \pm 1.4 \times 10^{-4}$ (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: $[{}^{3}\text{H}]\text{TAX}$ for P-glycoprotein and $[{}^{3}\text{H}]\text{CPT}$ for MRP. The $[{}^{3}\text{H}]\text{CPT}$ was significantly increased in the presence of a potent P-glycoprotein inhibitor (GF120918) (Fig. 5); i.e., 31% of the $[{}^{3}\text{H}]\text{TAX}$ amount was 3.1% of the $[{}^{3}\text{H}]\text{CPT}$ amount in BT4C cells, rat brain BT4C tumor, healthy rat brain tissue, and rat liver. Similarly, the cellular uptake of $[{}^{3}\text{H}]\text{TAX}$ was significantly increased in the presence of a potent inhibitor (GF120918) (Fig. 5). Two positive controls were tested by one-way ANOVA followed by Tukey’s test or unpaired two-tailed $t$ test (Mrp6, Abcc6) by using GraphPad Prism 5.03 software. Statistical significance was set at $P<0.05$ in all analyses.

**Results**

**GCV Permeability across BBB and Tumor Vasculature in Rats with BT4C Glioma.** GCV’s ability to cross the BBB and the tumor vasculature was determined with in situ rat brain perfusion technique in rats with BT4C glioma. GCV was able to reach both healthy brain tissue and tumor after 60 seconds of perfusion with a 10-ml/min flow rate, but the uptake in the tumor was significantly higher than that in healthy brain tissue. The unbound fraction of GCV was determined with equilibrium dialysis, and the $f_u$ values were used to calculate the unbound tissue concentrations in brain and tumor. The unbound fraction of GCV was high in both tumor and healthy brain, and there was no significant differences between tumor and healthy brain $f_u$ values (Table 2). In addition, the unbound fraction was determined in rat plasma and found to be near 100%, which is in accordance with the literature (Fletcher et al., 1986). Figure 2 shows the total and unbound GCV concentrations in healthy brain tissue and tumor after in situ brain perfusion. GCV’s permeability—surface area (PS) product across the functional BBB was also determined with the in situ rat brain perfusion technique. GCV’s PS product was $0.91 \times 10^{-4} \pm 0.27 \times 10^{-4} \text{ml/s per g}$ (mean ± S.E.M.; $n = 4$), which is lower than the PS product of urea, a small polar molecule used as a reference molecule for poor BBB permeation (Killian and Chikhale, 2001; Gynther et al., 2008). Furthermore, in the study of Liu et al. (2004), the PS product of 28 drug-like molecules was determined and only two of these molecules had lower PS products than GCV.

**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) ($AUC_{u,0-300\text{min}}$) 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 ($C_{\text{max}}$), time to reach $C_{\text{max}}$ ($t_{\text{max}}$), and elimination half-lives ($t_{1/2\text{g}}$) 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 $AUC_{u,0-300\text{min}}$ 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 $[{}^{3}\text{H}]\text{GCV}$, $[{}^{3}\text{H}]\text{TAX}$, or $[{}^{3}\text{H}]\text{CPT}$ 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 were tested by one-way ANOVA followed by Tukey’s test or unpaired two-tailed $t$ test (Mrp6, Abcc6) by using GraphPad Prism 5.03 software. Statistical significance was set at $P < 0.05$ in all analyses.

**Table 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Blood</th>
<th>Brain ECF</th>
<th>Tumor ECF</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{u,0-300\text{min}}$</td>
<td>6157 ± 1207</td>
<td>1658 ± 239</td>
<td>4834 ± 1182**</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>46.9 ± 10.8</td>
<td>11.8 ± 1.7</td>
<td>25.8 ± 6.2</td>
</tr>
<tr>
<td>$t_{\text{max}}$</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>$t_{1/2\text{g}}$</td>
<td>77</td>
<td>116</td>
<td>143</td>
</tr>
<tr>
<td>$f_u$</td>
<td>97.0 ± 2.4</td>
<td>74.3 ± 1.2</td>
<td>78.0 ± 8.6</td>
</tr>
</tbody>
</table>

*Apparent value (fraction 40-60 minutes).

**P < 0.01 vs. brain ECF value.**
was determined with unpaired two-tailed test. **P < 0.01.

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 mechanisms 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 concentration 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

![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.](image)

![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).](image)
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
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.

Acknowledgments

The authors thank Jaana Leksäinen for excellent technical assistance and Dr. Ewen MacDonald for revision of the language.

Authorship Contributions

Participated in research design: Forsberg, Gynther, Hakkarainen, Jalkanen, Kaariainen, Peura, Rautio, Ylä-Herttuala.

Conducted experiments: Gynther, Hakkarainen, Jalkanen, Kurkipuro, Kaariainen, Peura, Samaranayake.

Contributed new reagents or analytic tools: Lehtonen, Petsalo.

Performed data analysis: Forsberg, Gynther.

Wrote or contributed to the writing of the manuscript: Forsberg, Gynther, Hakkarainen, Jalkanen, Kaariainen.

References


Address correspondence to: Mikko Gynther, School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Yliopistonranta 1 C, 70211 Kuopio, Finland. E-mail: mikko.gynther@uef.fi