The Role of the Breast Cancer Resistance Protein (ABCG2) in the Distribution of Sorafenib to the Brain

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ABSTRACT

ATP-binding cassette transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) have been shown to work in concert to restrict brain penetration of several tyrosine kinase inhibitors. It has been reported that P-gp is dominant in limiting transport of many dual P-gp/BCRP substrates across the blood-brain barrier (BBB). This study investigated the influence of P-gp and BCRP on the central nervous system (CNS) penetration of sorafenib, a multitargeted tyrosine kinase inhibitor currently being evaluated in clinical trials for glioma. In vitro studies showed that BCRP has a high affinity for sorafenib. Sorafenib inhibited P-gp, but did not seem to be a P-gp substrate in vitro. CNS distribution studies showed that transport of sorafenib to the brain was restricted because of active efflux at the BBB. The brain-to-plasma equilibrium-distribution coefficient (area under the concentration-time profiles for plasma/area under the concentration-time profiles for brain) was 0.06 in

wild-type mice. Steady-state brain-to-plasma concentration ratio of sorafenib was approximately 0.36 ± 0.056 in the *Bcrp1*($-/-$) mice, 0.11 ± 0.021 in the *Mdr1a/b*($-/-$) mice, and 0.91 ± 0.29 in the *Mdr1a/b*($-/-$)/*Bcrp1*($-/-$) mice compared with 0.094 ± 0.007 in the wild-type mice. Sorafenib brain-to-plasma ratios increased on coadministration of the dual P-gp/BCRP inhibitor elacridar such that the ratio in wild-type mice (0.76 ± 0.24), *Bcrp1*($-/-$) mice (1.03 ± 0.33), *Mdr1a/b*($-/-$) mice (1.3 ± 0.29), and *Mdr1a/b*($-/-$)/*Bcrp1*($-/-$) mice (0.73 ± 0.35) were not significantly different. This study shows that BCRP and P-gp together restrict the brain distribution of sorafenib with BCRP playing a dominant role in the efflux of sorafenib at the BBB. These findings are clinically relevant to chemotherapy in glioma if restricted drug delivery to the invasive tumor cells results in decreased efficacy.

Introduction

ATP-binding cassette (ABC) transporters limit drug distribution to sites of action. P-glycoprotein (P-gp; *ABCB1*) and breast cancer resistance protein (BCRP; *ABCG2*) are two important members of the ABC transporters that transport a

variety of structurally diverse drugs that are currently used in the clinic (Sun et al., 2003; Begley, 2004; Löscher and Potschka, 2005). These two transporters constitute a vital part of the defense mechanism at the blood-brain barrier (BBB), where they prevent the passage of molecules into the brain by effluxing them back into the blood. While they help minimize the neurotoxic side effects of drugs that would otherwise penetrate the brain, they also limit brain distribution of drugs intended to target diseases of the central nervous system (CNS), such as brain tumors. There is considerable overlap in the substrate specificity between P-gp and BCRP (Litman et al., 2000) and a number of drugs have been reported to be dual substrates for P-gp and BCRP (de Vries et al., 2007; Polli et al., 2008; Chen et al., 2009; Agarwal et al.,

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ABBREVIATIONS: ABC, ATP-binding cassette; CNS, central nervous system; BBB, blood-brain barrier; MDR1, multidrug resistance protein 1; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; TKI, tyrosine kinase inhibitor; A-to-B, apical-to-basolateral; B-to-A, basolateral-to-apical; B/P, brain-to-plasma; *Mdr1*, gene encoding the murine P-glycoprotein; *MDR1*, gene encoding the human P-glycoprotein; *Bcrp1*, gene encoding the murine breast cancer resistance protein; MS, mass spectrometry; LC, liquid chromatography; AG1478, tyrphostin; MDCKII, Madin-Darby canine kidney II; GF120918 (elacridar), *N*-[4-[2-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl)ethyl]-5-methoxy-9-oxo-10*H*-acridine-4-carboxamide]; LY335979 (zosuquidar), (*R*)-4-((1*aR*,6*R*,10*bS*)-1,2-difluoro-1,1*a*,6,10*b*-tetrahydrodibenzo-(*a,e*)cyclopropa(c)cycloheptan-6-yl)- α -((5-quinoloyloxy)methyl)-1-piperazine ethanol, trihydrochloride; Ko143, (3*S*,6*S*,12*aS*)-1,2,3,4,6,7,12,12*a*-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino(1',2':1,6)pyrido(3,4-*b*)indole-3-propanoic acid 1,1-dimethylethyl ester; FVB, Friend leukemia virus strain B; DMSO, dimethyl sulfoxide; AUC, area under the curve.

2010). Furthermore, it has been postulated that P-gp is the dominant transporter and BCRP plays a minor role in the efflux of many dual substrates at the BBB (Lee et al., 2005; Zhao et al., 2009).

Tyrosine kinase inhibitors (TKIs) inhibit tyrosine kinases that are a driving force for several aggressive tumors (Zwick et al., 2001). Studies evaluating the interaction of tyrosine kinase inhibitors with ABC transporters have shown that several TKIs are substrates for and/or inhibit the function of both P-gp and BCRP (Dai et al., 2003; Shi et al., 2007; Chen et al., 2009; Hu et al., 2009; Polli et al., 2009; Shukla et al., 2009; Agarwal et al., 2010). These interactions may alter selected pharmacokinetic processes and hence the tissue distribution of many drugs. This is important when the compounds are used for diseases in tissues such as brain, because active efflux at the blood-brain barrier can limit CNS distribution, thereby limiting their efficacy.

Sorafenib (Nexavar) is an oral, biaryl-urea RAF kinase inhibitor that acts against both vascular endothelial growth factor and platelet-derived growth factor receptors, simultaneously targeting both tumor cell proliferation and angiogenesis (Wilhelm et al., 2006). It was the first multikinase inhibitor approved by the U.S. Food and Drug Administration for the treatment of patients with renal cell carcinoma (Bellmunt et al., 2010) and was later granted approval for treatment of unresectable hepatocellular carcinoma. It also inhibits c-KIT, FLT-3, and several other tyrosine kinase receptors, making it a therapeutic option in several solid tumors such as breast, colon, and non-small cell lung cancer (Wilhelm et al., 2008). Moreover, given its inhibitory action on signal transducer and activator of transcription 3 (Siegelin et al., 2010; Yang et al., 2010) and its potent antiangiogenic effect, it is being evaluated in clinical trials for brain tumors such as glioma (Nabors et al., 2007; Wen et al., 2009; Scott et al., 2010). In contrast to other solid tumors, brain tumors reside in what is considered to be a pharmacological sanctuary site, separated from the systemic circulation by the blood-brain barrier. Glioma is highly invasive with tumor cells infiltrating normal brain areas several centimeters away from the tumor core (Kuratsu et al., 1989; Silbergeld and Chicoine, 1997). This infiltrative growth pattern makes it difficult to target the invasive tumor cells that may reside behind an intact BBB and might receive low concentrations of drug because of efflux by P-gp and BCRP (Blakeley et al., 2009). Clearly, sorafenib efficacy against brain tumors such as glioma depends in part on its ability to cross the BBB and achieve therapeutic concentrations in the brain, especially in the invasive tumor cells. This distribution process also depends on the interaction of sorafenib with drug efflux transporters present at the brain endothelial cells.

Results from studies investigating the interaction of sorafenib with efflux transporters have suggested that sorafenib is minimally transported by P-gp or BCRP and transporter-related interaction might not alter the systemic pharmacokinetics of sorafenib (Hu et al., 2009). In contrast, Lagas et al. (2010) reported that sorafenib is a substrate for both P-gp and BCRP that limit its brain accumulation. Furthermore, another study (Gnoth et al., 2010) reported that P-gp does not affect plasma sorafenib exposure and might not limit transport of sorafenib across the BBB. Therefore, the interaction of sorafenib with transporters in the BBB is not fully elucidated. In the current study, we investigated whether sor-

afenib, similar to many other TKIs, is a substrate for P-gp and BCRP and whether these two transporters prevent sorafenib from traversing the BBB, thereby limiting its distribution to the CNS.

The aim of this study was to identify and better understand the interaction of the multikinase inhibitor sorafenib with P-gp and BCRP and examine the effect of this interaction on the transport of sorafenib to the brain. This is of particular importance for the treatment of invasive brain tumors such as glioma, because subtherapeutic concentrations in the brain would limit the effectiveness of therapy at the invasive sites.

Materials and Methods

Chemicals and Reagents. [^3H]sorafenib (3.5 Ci/mmol, purity 98.4%), [^3H]mitoxantrone (3 Ci/mmol, purity 95.1%), and [^3H]vinblastine (18.2 Ci/mmol, purity 97.4%) were purchased from Moravsek Biochemicals (La Brea, CA). [^3H]prazosin (70 Ci/mmol, purity 97%) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Unlabeled sorafenib tosylate and tyrphostin (AG1478) was purchased from LC Laboratories (Woburn, MA). Elacridar [GF120918; *N*-[4-[2-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl)ethyl]-5-methoxy-9-oxo-10*H*-acridine-4-carboxamide]] was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Ko143 [(3*S*,6*S*,12*aS*)-1,2,3,4,6,7,12,12*a*-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxypyrazino[1',2':1,6]pyrido[3,4-*b*]indole-3-propanoic acid 1,1-dimethylethyl ester] was generously provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands), and zosuquidar [LY335979; (*R*)-4-((1*aR*,6*R*,10*bS*)-1,2-difluoro-1,1*a*,6,10*b*-tetrahydrodibenzo-*(a,e)*cyclopropa(c)cycloheptan-6-yl)- α -((5-quinoloyloxy)methyl)-1-piperazine ethanol, trihydrochloride] was a gift from Eli Lilly & Co. (Indianapolis, IN). All other chemicals used were HPLC or reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO).

In Vitro Studies. In vitro studies were conducted in epithelial Madin-Darby canine kidney II (MDCKII) cells expressing either human P-gp (MDCKII-*MDR1* cell line) or murine BCRP (MDCKII-*Bcrp1* cell line) and were generously provided by Dr. Piet Borst and Dr. Alfred H. Schinkel (The Netherlands Cancer Institute). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and amphotericin B (250 ng/ml) (all Sigma-Aldrich) and maintained at 37°C with 5% CO_2 under humidifying conditions.

Intracellular Accumulation. Intracellular accumulation of sorafenib was examined in these cells using 24-well polystyrene plates (Thermo Fisher Scientific, Waltham, MA). Cells were seeded at a density of 2×10^5 cells/well, and confluent monolayers were obtained in 3 days. On the day of the experiment, growth media were aspirated, and the cells were washed twice with prewarmed (37°C) cell assay buffer. The experiment was started after a 30-min preincubation during which the cells were equilibrated with 1 ml of cell assay buffer. One milliliter of a tracer solution (2 ng/ml) of [^3H]sorafenib was then added to the cells, and the plates were incubated for 1 h in an orbital shaker maintained at 37°C. At the end of the 1-h accumulation, the experiment was ended by aspirating the radiolabeled drug solution from the wells and washing the cells with ice-cold phosphate-buffered saline. A 1% Triton X-100 solution (0.5 ml) was added to each well to solubilize the cells, and the protein concentration in the solubilized cell fractions was determined by the BCA protein assay (Thermo Fisher Scientific). Radioactivity associated with a 150- μl sample was determined by liquid scintillation counting (LS-6500; Beckman Coulter, Fullerton, CA). The radioactivity in the cell fractions was normalized by the respective protein concentrations, and drug accumulation in the cells was expressed as amount of accumulated radioactivity (dpm) per microgram of protein. [^3H]vin-

blastine and [^3H]prazosin were included in the accumulation studies as positive control for P-gp and BCRP, respectively.

Directional Flux Studies in MDCKII Cells. Transport assays were carried out by using six-well Transwells (Corning Glassworks, Corning, NY). The cells were seeded at a density of 2×10^5 cells/well until they formed confluent polarized monolayers. On the day of the experiment, the monolayers were washed with prewarmed (37°C) cell assay buffer, and after a 30-min preincubation, the experiment was initiated by addition of a tracer solution (2 ng/ml) of radiolabeled sorafenib in cell assay buffer to the donor side. The receiver compartment was sampled at predetermined times (0, 10, 20, 30, 45, 60, and 90 min) after addition of drug to the donor side. The volume sampled (200 μl) was immediately replaced with fresh assay buffer. The transport of sorafenib was measured in two directions: apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A). When an inhibitor was used in the flux study, the inhibitor [1 μM LY335979 (Dantzig et al., 1999) or 200 nM Ko143 (Allen et al., 2002)] was added during the preincubation step, followed by determination of A-to-B and B-to-A flux with the inhibitor present in both compartments throughout the course of the experiment. [^3H]sorafenib was quantified by liquid scintillation counting. The apparent permeability of sorafenib was calculated as described below.

Permeability Calculations. The apparent permeability (P_{app}) was calculated by the following equation:

$$P_{app} = \frac{\left(\frac{dQ}{dt}\right)}{A \times C_0} \quad (1)$$

where, dQ/dt is the mass transport rate (determined from the slope of the amount transported versus time plot), A is the apparent surface area of the cell monolayer (4.67 cm^2), and C_0 is the initial donor concentration. The efflux ratio, defined as the ratio of P_{app} in the B-to-A direction to the P_{app} in the A-to-B direction, was used to estimate the magnitude of P-gp- or BCRP-mediated efflux.

Calculation of K_m value. The affinity of BCRP for sorafenib ($K_{m,app}$) was determined by calculating the basolateral-to-apical permeability in MDCKII-*Bcrp1* cells, with a range of concentrations (2 ng/ml to 30 $\mu\text{g/ml}$) applied to the donor side. The B-to-A permeability was plotted as a function of sorafenib concentration. Phoenix WinNonlin 6.1 (Pharsight, Mountain View, CA) was used to fit an inhibitory E_{max} model to the data,

$$E = E_{max} \left(1 - \frac{C}{K_{m,app} + C} \right) \quad (2)$$

where, E is the B-to-A permeability of sorafenib, E_{max} is the maximum permeability seen at the lowest sorafenib concentration, $K_{m,app}$ is the affinity constant determined by the concentration of sorafenib at which half-maximal inhibition is seen, and C is the concentration of sorafenib in the donor compartment.

P-gp and BCRP Inhibition Assays. Inhibition studies were conducted by using prototypical probe substrates, [^3H]vinblastine for P-gp and [^3H]prazosin or [^3H]mitoxantrone for BCRP. Intracellular accumulation of these substrates at 60 min was determined in MDCKII-*MDR1* or *Bcrp1* cells in the presence of different concentrations of sorafenib spanning 20 ng/ml to 60 $\mu\text{g/ml}$. The increase in cellular accumulation relative to control (no sorafenib treatment) was measured as a function of sorafenib concentration. A sigmoid E_{max} model given by the following equation was fit to the data by using Phoenix WinNonlin 6.1:

$$E = E_0 + (E_{max} - E_0) \frac{C^\gamma}{IC_{50}^\gamma + C^\gamma} \quad (3)$$

where E is the fold increase in the accumulation of substrate seen in the presence of sorafenib relative to control (no sorafenib), E_0 is the accumulation of probe in the absence of sorafenib normalized to unity, E_{max} is the maximum increase in accumulation of probe, IC_{50}

is the concentration of sorafenib at which half-maximal effect is seen, C is the concentration of sorafenib, and γ is the shape factor that determines the slope of the curve.

In Vivo Studies. In vivo studies were conducted in male FVB (wild type), *Mdr1a/b*($-/-$) (P-gp knockout), *Bcrp1*($-/-$) (BCRP knockout), and *Mdr1a/b*($-/-$)/*Bcrp1*($-/-$) (triple knockout) mice of a FVB genetic background (Taconic Farms, Germantown, NY). All animals were 8 to 10 weeks old at the time of experiment. Animals were maintained under temperature-controlled conditions with a 12-h light/dark cycle and unlimited access to food and water. All studies were carried out in accordance with the guidelines set by the *Principles of Laboratory Animal Care* (National Institutes of Health, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Plasma and Brain Pharmacokinetics of Sorafenib. Sorafenib dosing solution was prepared on the day of the experiment in a vehicle containing DMSO, propylene glycol, and saline (3:5:2 v/v/v) at a concentration of 5 mg/ml. FVB (wild type) mice received an intravenous dose of 10 mg/kg sorafenib by injection into the tail vein. Blood and brain were sampled at 15 min and 0.5, 1, 2, 4, and 6 h after dose ($n = 4$ at each time point). At the desired time point, animals were euthanized by using a CO_2 chamber. Blood was collected by cardiac puncture and transferred to heparinized tubes. Plasma was isolated from blood by centrifugation at 3000 rpm for 10 min at 4°C . Whole brain was immediately removed from the skull, rinsed with cold saline, and flash-frozen in liquid nitrogen. Plasma and brain specimens were stored at -80°C until analysis by LC-MS/MS.

Steady-State Brain Distribution of Sorafenib in FVB Mice. Steady-state brain and plasma concentrations of sorafenib were determined in FVB wild-type, *Mdr1a/b*($-/-$), *Bcrp1*($-/-$), and *Mdr1a/b*($-/-$)/*Bcrp1*($-/-$) mice. Alzet osmotic minipumps (Durect Corporation, Cupertino, CA) were used to maintain a constant rate infusion in the peritoneal cavity. The procedure was the same as that used previously for gefitinib (Agarwal et al., 2010). In brief, a 50 mg/ml solution of sorafenib in DMSO was filled in the minipumps (model 1003D), and the pumps were equilibrated by soaking them overnight in sterile saline solution at 37°C . Mice were anesthetized by intraperitoneal administration of 100 mg/kg ketamine and 10 mg/kg xylazine (Boynnton Health Service Pharmacy, Minneapolis, MN). The primed pumps were surgically inserted into the peritoneal cavity, and the animals were allowed to recover for 1 h on a heated pad. The animals were euthanized 48 h after surgery followed by collection of brain and blood as described earlier. Plasma and brain specimens were stored at -80°C until analysis by LC-MS/MS. The pumps operated at a constant flow rate of 1 $\mu\text{l/h}$, resulting in an intraperitoneal infusion rate of 50 $\mu\text{g/h}$ (2 mg/h/kg). Sorafenib half-life in plasma and brain after an intravenous dose was determined to be 1.6 and 0.9 h, respectively. Therefore an infusion lasting 48 h was considered to be sufficiently long to attain steady state in both plasma and brain.

Influence of Elacridar on Brain Distribution of Sorafenib. In a separate study, FVB wild-type, *Mdr1a/b*($-/-$), *Bcrp1*($-/-$), and *Mdr1a/b*($-/-$)/*Bcrp1*($-/-$) mice ($n = 12$ per group) were divided into three cohorts ($n = 4$) and administered 10 mg/kg sorafenib intravenously. One cohort (elacridar pretreatment) received 10 mg/kg elacridar (10:7:3 v/v/v DMSO, propylene glycol, saline), and a second cohort (vehicle control) was administered vehicle (10:7:3 v/v/v DMSO, propylene glycol, saline), via intravenous injection 30 min before sorafenib was administered. The third cohort did not receive any pretreatment and served as control. Blood and brain were collected at 60 min after the sorafenib dose and processed as described earlier. Sorafenib brain concentrations in the presence and absence of elacridar in the wild-type mice were compared with the three transgenic mouse groups.

Analysis of Sorafenib by Liquid Chromatography/Tandem Mass Spectrometry. The concentration of sorafenib in mouse plasma and brain homogenate was determined by high-pressure liquid chromatography coupled with mass spectrometry. Before analysis, frozen samples were thawed at room temperature. Brain samples were homogenized by using three volumes of ice-cold 5%

bovine serum albumin in phosphate-buffered saline using a tissue homogenizer (Thermo Fisher Scientific). A 50- μ l aliquot of plasma and a 100- μ l aliquot of brain homogenate were spiked with 20 ng of internal standard, tyrphostin (AG1478), and extracted by vigorous vortexing with 1 ml of ice-cold ethyl acetate. Samples were centrifuged at 7500 rpm for 15 min at 4°C, and a volume of 750 μ l of the organic layer was transferred to fresh polypropylene tubes and dried under nitrogen. Samples were reconstituted in 100 μ l of mobile phase and transferred to glass auto sampler vials. A volume of 10 μ l was injected in the high-pressure liquid chromatography system using a temperature-controlled autosampling device maintained at 10°C. Chromatographic analysis was performed using an Agilent Technologies (Santa Clara, CA) model 1200 separation system. Separation of analytes was achieved by using an Agilent Technologies Eclipse XDB-C18 RRHT threaded column (4.6 mm i.d. $\times$ 50 mm, 1.8 μ) fitted with an Agilent Technologies C18 guard column (4.6 mm i.d. $\times$ 12.5 mm, 5 μ). The mobile phase was composed of acetonitrile/20 mM ammonium formate (containing 0.1% formic acid) (68:32 v/v) and delivered at a flow rate of 0.25 ml/min. The column effluent was monitored by using a Thermo Finnigan TSQ Quantum 1.5 detector (Thermo Fisher Scientific). The instrument was equipped with an electrospray interface and controlled by the Xcalibur version 2.0.7 data system (Thermo Fisher Scientific). The samples were analyzed by using an electrospray probe in the positive ionization mode operating at a spray voltage of 4500V for both sorafenib and the internal standard. The spectrometer was programmed to allow the $[MH]^+$ ion of sorafenib at m/z 465.06 and that of internal standard at m/z 316.67 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 30 V for sorafenib and 9 V for tyrphostin. The product ions for sorafenib (m/z 251.9) and the internal standard (m/z 300.9) were monitored through the third quadrupole (Q3). The scan width and scan time for monitoring the two product ions were 1.5 m/z and 0.5 s, respectively. The assay was sensitive over a range of 2.5 ng/ml to 1 μ g/ml with the coefficient of variation being less than 15% over the entire range.

Pharmacokinetic Calculations. Pharmacokinetic parameters from the concentration-time data in plasma and brain were obtained by noncompartmental analysis performed with Phoenix WinNonlin 6.1. We used a built-in capability of noncompartmental analysis, which uses an extension of the Nedelman and Jia approach (Bailer, 1988; Nedelman and Jia, 1998) to analyze data from sparse sampling and estimate the variance in the area under the curve (AUC). The terminal rate constants (λ_z) for plasma and brain were determined from the last three data points of the respective concentration-time profiles. The areas under the concentration-time profiles for plasma (AUC_{plasma}) and brain (AUC_{brain}) from time 0 to infinity were calculated by using the linear trapezoidal method. The AUC from the last measured time point to infinity was estimated by dividing the last measured concentration by the respective terminal rate constant. The percentage of AUC extrapolated was less than 10% in both brain and plasma. In the case of plasma, the AUC from time 0 to the first measured time point was obtained by back-extrapolation of the first two data points to time 0.

In the intraperitoneal infusion studies, the apparent plasma clearance (CL_{app}) was calculated by using the equation,

$$CL_{\text{app}} = \frac{k(0)}{C_{ss}} \quad (4)$$

where, $k(0)$ is the rate of infusion into the peritoneal cavity normalized to body weight (ng/h/kg), and C_{ss} is the plasma concentration at steady state (ng/ml).

Statistical Analysis. Comparison between groups were made using SigmaStat, version 3.1 (Systat Software, Inc., San Jose, CA). Statistical difference between two groups was tested by using the two-sample t test, and significance was declared at $p < 0.05$. Multiple groups were compared by one-way analysis of variance with the

Holm-Sidak post hoc test for multiple comparisons at a significance level of $p < 0.05$.

Results

Intracellular Accumulation of Sorafenib in MDCKII Cells. Intracellular accumulation of sorafenib was examined in polarized epithelial MDCKII cells that overexpressed the transporter proteins, P-gp or BCRP. Prazosin, a prototypical BCRP substrate, was included as a positive control and showed significantly lower accumulation in the MDCKII-*Bcrp1* cells compared with wild type ($\sim 10\%$ of wild type). Likewise, sorafenib accumulation in the *Bcrp1*-transfected cells was 5-fold lower compared with the wild-type cells ($p < 0.05$; Fig. 1A). This suggested that BCRP limits intracellular accumulation of sorafenib. In the *MDR1*-transfected cells, accumulation of the P-gp substrate vinblastine was significantly reduced compared with the wild type ($\sim 10\%$ of wild type). However, accumulation of sorafenib in the *MDR1*-transfected cells was not statistically different from that in wild-type cells, indicating that P-gp might not be involved in efflux of sorafenib from the cells (Fig. 1B).

Fig. 1. Intracellular accumulation of [^{3} H]sorafenib in MDCKII cells. A, accumulation in wild-type and *Bcrp1*-transfected cells. Accumulation of the sorafenib in the *Bcrp1*-transfected cells (gray bars) was approximately 5-fold lower than that in the wild-type cells (black bars). B, accumulation in wild-type and *MDR1*-transfected cells. Accumulation of the prototypical substrate vinblastine in the *MDR1*-transfected cells (gray bars) was approximately 10-fold lower than that in the wild-type cells (black bars). In comparison, no such difference was seen in the accumulation of sorafenib between the two cell types. Mean $\pm$ S.D. ($n = 6$). *, $p < 0.05$.

Directional Permeability of Sorafenib across MDCKII Cells. Transcellular transport of [^3H]sorafenib was studied by using MDCKII cells. In the MDCKII-*Bcrp1* cells, the rate of drug transported was significantly increased in the B-to-A direction compared with wild-type cells (Fig. 2A; $p < 0.05$). The BCRP inhibitor Ko143 completely inhibited BCRP-mediated active transport in the *Bcrp1* cells such that there was no difference in the amount of sorafenib transported in the two directions (Fig. 2B). The apparent permeability of sorafenib in the B-to-A direction was 15-fold greater than the A-to-B permeability in the *Bcrp1* transfects ($p < 0.05$). This difference in permeability was abolished upon treatment with Ko143 (Fig. 3). Directional flux experiments in the *MDR1*-transfected cells showed no signs of directionality in the transport of sorafenib, because there was no significant difference in the amount of sorafenib transported and the permeability between the A-to-B and B-to-A directions (Fig. 4). This indicated that sorafenib is not efficiently transported by P-gp in these cells. The in vitro studies suggest that although

Fig. 2. Directional transport of [^3H]sorafenib across MDCKII cell monolayers. A, amount of sorafenib transported across wild-type (●, A-to-B transport; ○, B-to-A transport) and *Bcrp1*-transfected (▲, A-to-B transport; △, B-to-A transport) cells. B, flux of sorafenib in *Bcrp1*-transfected cells in the presence (●, A-to-B transport; ○, B-to-A transport) and absence (▲, A-to-B transport; △, B-to-A transport) of the BCRP inhibitor Ko143. There was significant directionality in transport of sorafenib across *Bcrp1* monolayers. This directionality was abolished when cells were treated with 200 nM Ko143. The black arrows indicate the direction of change in sorafenib transport caused by BCRP (A) or addition of Ko143 (B). Mean $\pm$ S.D. ($n = 6$).

Fig. 3. Apparent permeability of [^3H]sorafenib across MDCKII wild-type and *Bcrp1*-transfected cell monolayers. In the *Bcrp1* transfects, the B-to-A permeability (gray bar) was significantly greater than the A-to-B permeability (black bar). Treatment with Ko143 decreased the B-to-A permeability. No difference in apparent permeability was observed in the wild-type cells. Mean $\pm$ S.D. ($n = 6$). *, $p < 0.05$.

sorafenib is a substrate for BCRP, it is not effectively transported by P-gp. We determined the apparent affinity constant (K_{mapp}) of BCRP for sorafenib by measuring the B-to-A permeability of sorafenib in the *Bcrp1*-transfected cells with varying concentrations of sorafenib applied to the donor (basolateral) side. The B-to-A permeability of sorafenib decreased with increasing sorafenib concentrations. An inhibitory E_{max} model fitted to the data estimated a K_{mapp} of 5.5 ± 1.2 nM, indicating that sorafenib is a high-affinity substrate for BCRP (Fig. 5).

Sorafenib as a P-gp or BCRP Inhibitor. The ability of sorafenib to inhibit P-gp and BCRP was evaluated by studying the intracellular accumulation of prototypical probe P-gp and BCRP substrates (vinblastine for P-gp, prazosin and mitoxantrone for BCRP) in the presence of varying concentrations of sorafenib. Treatment with sorafenib significantly increased intracellular accumulation of vinblastine in the *MDR1*-transfected cells (Fig. 6A). A sigmoidal E_{max} model was fit to the resulting data, and the IC_{50} of sorafenib for inhibition of P-gp was estimated to be 25 ± 6 μM . It is noteworthy that sorafenib treatment did not increase accumulation of mitoxantrone or prazosin in the *Bcrp1*-transfected cells (Fig. 6B). This suggests that although sorafenib is a high-affinity substrate for BCRP, it did not inhibit BCRP-mediated transport of the two common probe substrates. The fact that sorafenib is a substrate but does not inhibit BCRP in this particular case strongly suggests that sorafenib is transported via binding to a site that is different from that for prazosin or mitoxantrone.

Sorafenib Disposition in Plasma and Brain. We studied the brain and plasma pharmacokinetics of sorafenib in FVB wild-type mice. Sorafenib brain concentrations were significantly lower than the plasma concentrations at all the measured time points ($p < 0.05$; Fig. 7) with brain-to-plasma ratios lower than 0.15 at all time points. Noncompartmental analysis of the brain and plasma data yielded a terminal half-life of 1.6 h in plasma and 0.9 h in the brain (Table 1). The total body clearance was estimated to be 5.8 ml/min/kg. The area under the curve (AUC) in plasma was 1.6 mg $\cdot$ min/ml, whereas the AUC in brain was 0.11 mg $\cdot$ min/ml. The

Fig. 4. Directional transport of [³H]sorafenib across MDCKII cell monolayers. A, amount of sorafenib transported across wild-type (●, A-to-B transport; ○, B-to-A transport) and *MDR1*-transfected (▲, A-to-B transport; △, B-to-A transport) cells. B, permeability of [³H]sorafenib across MDCKII wild-type and *MDR1*-transfected cell monolayers. There was no difference in rate of sorafenib transported and apparent permeability of sorafenib between the A-to-B (black bar) and B-to-A (gray bar) directions, indicating a lack of P-gp mediated efflux. Mean $\pm$ S.D. ($n = 6$).

AUC_{brain} to AUC_{plasma} ratio was 0.06, suggesting that sorafenib has limited distribution to the brain.

Steady-State Brain and Plasma Pharmacokinetics of Sorafenib. The influence of active efflux transport by P-gp and BCRP on the brain distribution of sorafenib was examined by determining the steady-state brain and plasma concentrations in wild-type, *Mdr1a/b(-/-)*, *Bcrp1(-/-)*, and *Mdr1a/b(-/-)Bcrp1(-/-)* mice. Sorafenib was delivered at a constant rate of 2 mg/h/kg in the peritoneal cavity, and brain and plasma concentrations were determined at 48 h after the start of infusion. Given the plasma half-life (1.6 h), an infusion lasting 48 h was considered to be sufficient to attain steady state in both plasma and brain. The steady-state concentrations (C_{ss}) in plasma ranged from 1.5 to 3 μ g/ml in the four genotypes and were not statistically different from each other. Steady-state brain concentrations of sorafenib were significantly enhanced in the *Bcrp1(-/-)* and *Mdr1a/b(-/-)Bcrp1(-/-)* mice compared with wild-type mice ($p < 0.05$) (Table 2). There was no difference in the steady-state brain sorafenib concentrations between the wild-type and *Mdr1a/b(-/-)*

Fig. 5. Affinity of sorafenib for BCRP. The B-to-A permeability of sorafenib was determined in MDCKII-*Bcrp1* cells, with a range of concentrations (0.001 nM to 80 nM) applied to the donor side. A sigmoidal inhibitory $E_{\max}$ model was fitted to the data and the affinity constant ($K_{m_{app}}$) was determined to be 5 nM (●, observed data; dashed line, model predicted curve. Mean $\pm$ S.D. ($n = 3$); parameter estimate reported as mean $\pm$ S.D. (standard error of the estimate).

mice. The brain-to-plasma ratio at steady-state (B/P_{ss}) in the wild-type mice was $\sim 0.094 \pm 0.007$. This ratio was similar to the equilibrium distribution coefficient of 0.06 obtained from the intravenous study and indicates the limited CNS distribution of sorafenib. The B/P_{ss} ratio increased by approximately 4-fold in the *Bcrp1(-/-)* mice (0.36 ± 0.056) and 10-fold in the *Mdr1a/b(-/-)Bcrp1(-/-)* mice (0.91 ± 0.29) ($p < 0.05$; Fig. 8). This suggests that BCRP is the major transporter limiting brain penetration of sorafenib. The steady-state brain-to-plasma ratio in the wild-type mice was not statistically different from the ratio in the *Mdr1a/b(-/-)* mice (0.11 ± 0.021). This finding is similar to those by Lagas et al. (2010) and Gnoth et al. (2010), who also report a lack of P-gp mediated transport in vivo. The apparent plasma clearance (CL_{app}) of sorafenib between the wild-type, *Mdr1a/b(-/-)*, and *Mdr1a/b(-/-)Bcrp1(-/-)* mice were not significantly different from each other; however, the CL_{app} increased significantly in the *Bcrp1(-/-)* mice compared with wild-type ($p < 0.05$).

Influence of Elacridar on Brain Distribution of Sorafenib. The influence of the dual P-gp and BCRP inhibitor elacridar on the distribution of sorafenib to the brain was investigated. The brain-to-plasma ratios at 60 min were 0.083 ± 0.020 , 0.37 ± 0.035 , 0.16 ± 0.11 , and 0.86 ± 0.16 in the wild-type, *Bcrp1(-/-)*, *Mdr1a/b(-/-)*, and *Mdr1a/b(-/-)Bcrp1(-/-)* mice, respectively (Fig. 9). These values were similar to the steady-state brain-to-plasma ratios in the wild-type (0.094), *Bcrp1(-/-)* (0.36), *Mdr1a/b(-/-)* (0.11), and *Mdr1a/b(-/-)Bcrp1(-/-)* (0.91) mice (Table 1). The intravenous study indicated that around 60 min would represent a region where sorafenib is at a transient steady state in the brain. This is evident from the similarity in the brain-to-plasma ratios at 60 min and at steady state. Concurrent administration of the dual P-gp and BCRP inhibitor elacridar resulted in an enhancement in brain concentrations by 5-fold in the wild-type, 3-fold in the *Bcrp1(-/-)*, and 4-fold in the *Mdr1a/b(-/-)* mice, compared with the control group ($p < 0.05$; Table 3). Pretreatment with elacridar had no significant effect on the brain concentrations of sorafenib in the *Mdr1a/b(-/-)Bcrp1(-/-)* mice. Inhibition of P-gp and BCRP by

Fig. 6. Inhibition of P-gp and BCRP by sorafenib. A, intracellular accumulation of the P-gp substrate, vinblastine, in MDCKII-MDR1 cells in the presence of increasing sorafenib concentrations. Sorafenib inhibited the accumulation of vinblastine with an IC_{50} of 25 μM (●, observed data; dashed line, model predicted curve. Mean $\pm$ S.D. ($n = 4$); parameter estimate reported as mean $\pm$ S.D. (standard error of the estimate). B, intracellular accumulation of BCRP substrates, prazosin (●) and mitoxantrone (▲) in MDCKII-Bcrp1 cells in the presence of increasing sorafenib concentrations. Sorafenib has no effect on accumulation of the two prototypical BCRP substrates. Mean $\pm$ S.D. ($n = 4$).

elacridar increased the brain-to-plasma ratio to 0.76 ± 0.24 in wild-type, 1.03 ± 0.33 in the *Bcrp1*($-/-$), and 1.3 ± 0.29 in the *Mdr1a/b*($-/-$) mice ($p < 0.05$). Of interest was the fact that the B/P ratio in the *Bcrp1*($-/-$) mice also increased significantly upon treatment with elacridar. The brain-to-plasma ratios in the elacridar-treated cohort in the four groups were not statistically different from each other (Fig. 9). The brain-to-plasma ratios in the vehicle-treated cohort were not statistically different from that in the control cohort, the values being 0.13 ± 0.037 in the wild-type, 0.30 ± 0.082 in the *Bcrp1*($-/-$), 0.15 ± 0.045 in the *Mdr1a/b*($-/-$), and 0.69 ± 0.15 in the *Mdr1a/b*($-/-$) *Bcrp1*($-/-$) mice (Table 3). This confirmed that the enhanced brain penetration of sorafenib in the elacridar-treated cohort was caused by inhibition of P-gp and BCRP by elacridar. There was no significant difference in the plasma concentrations of sorafenib between the different mice groups except in the wild-type mice, where plasma levels were slightly higher compared with the other groups.

Fig. 7. Sorafenib brain and plasma concentrations after a single intravenous dose of 10 mg/kg in FVB wild-type mice. Brain concentrations were significantly lower than the plasma concentrations at all of the measured time points, suggesting limited delivery of sorafenib across the BBB. *, $p < 0.05$. Mean $\pm$ S.D. ($n = 4$).

TABLE 1

Plasma and brain pharmacokinetic parameters determined by noncompartmental analysis after the administration of a single intravenous bolus dose of sorafenib in wild-type mice

The ratio of AUC in the brain to that in plasma was estimated to be 0.06, signifying the restricted brain penetration of sorafenib.

Pharmacokinetic Parameter	Plasma	Brain
Terminal rate constant (h^{-1})	0.42	0.73
Half-life (h)	1.6	0.9
Clearance (ml/min/kg)	5.8	
Volume of distribution (ml/kg)	840	
$AUC_{0 \rightarrow \text{last}}$ ($\text{mg} \cdot \text{min/ml}$)	$1.5 \pm 0.1^*$	$0.11 \pm 0.008^*$
$AUC_{0 \rightarrow \text{inf}}$ ($\text{mg} \cdot \text{min/ml}$)	1.6	0.11
$AUC_{\text{brain}}/AUC_{\text{plasma}}$		0.06

* Mean $\pm$ S.D. (standard error of the estimate).

Discussion

Sorafenib is a potent inhibitor of several receptor tyrosine kinases and other regulators of tumor progression and tumor angiogenesis, e.g., vascular endothelial growth factor receptor, platelet-derived growth factor receptor, cKIT, and RAF (Wilhelm et al., 2006, 2008). This broad activity against critical targets makes sorafenib an attractive option for therapy in glioma, a tumor of the brain that is typically fatal (Wen and Kesari, 2008). Efflux transporters present at the BBB can limit drug delivery to the brain and therefore are one determinant of efficacy. This is especially relevant to an infiltrative disease such as glioma, where invasive tumor cells that may reside behind an intact BBB might be deprived of a drug that is effluxed by P-gp and BCRP. Several studies have attempted to explain the interaction of sorafenib with P-gp and BCRP, two critical efflux transporters at the BBB (Hu et al., 2009; Gnoth et al., 2010; Lagas et al., 2010). However, those studies failed to reach a consensus with respect to the role of the two proteins in limiting the CNS penetration of sorafenib. The current study investigated several questions that were hitherto unanswered with respect to transporter-mediated distribution of sorafenib to the brain. Our findings conclusively show that transport of sorafenib across the BBB is restricted by BCRP, and the data suggest some involvement of P-gp. Sorafenib differs from many other

TABLE 2

Steady-state plasma and brain concentrations of sorafenib in wild-type, *Mdr1a/b*(-/-), *Bcrp1*(-/-), and *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice after a constant intraperitoneal infusion of sorafenib at a constant rate of 2 mg/h/kg

Data presented as mean $\pm$ S.D., $P < 0.05$.

Genotype	Mice per group	Plasma C_{ss} $\mu\text{g/ml}$	Brain C_{ss} $\mu\text{g/g}$	Brain-to-Plasma Ratio	Apparent Plasma Clearance (Cl_{app}) ml/min/kg
FVB (wild-type)	4	2.5 ± 0.16	0.32 ± 0.18^a	0.094 ± 0.007	11.5 ± 4.01
<i>Bcrp1</i> (-/-)	4	1.5 ± 0.42^b	0.51 ± 0.074^a	0.36 ± 0.056^b	24.3 ± 5.71^b
<i>Mdr1a/b</i> (-/-)	4	3.07 ± 0.91	0.34 ± 0.16^a	0.11 ± 0.021	20.3 ± 7.07
<i>Mdr1a/b</i> (-/-) <i>Bcrp1</i> (-/-)	4	1.9 ± 0.17	1.7 ± 0.69^b	0.91 ± 0.29^b	17.3 ± 1.49

^a Significantly different compared with corresponding plasma concentration.

^b Significantly different compared with wild-type group.

Fig. 8. Steady-state brain distribution of sorafenib in wild-type, *Mdr1a/b*(-/-), *Bcrp1*(-/-), and *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice. Sorafenib was delivered at a constant rate of 2 mg/h/kg, and ratio of the brain and plasma concentrations at steady state (48 h) was determined. The B/P ratio in the *Bcrp1*(-/-) and *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice was significantly greater than that in wild-type mice, suggesting the role of BCRP in efflux of sorafenib at the BBB. *, $p < 0.05$, compared with wild type; †, $p < 0.05$, compared with *Bcrp1*(-/-) mice. Mean $\pm$ S.D. ($n = 4$).

Fig. 9. Influence of the dual inhibitor elacridar on the brain distribution of sorafenib. Elacridar (10 mg/kg i.v.) was given 30 min before the administration of 10 mg/kg sorafenib followed by the determination of brain and plasma concentrations at 60 min. Administration of elacridar increased the B/P ratio in wild-type, *Bcrp1*(-/-), and *Mdr1a/b*(-/-) mice such that they were not statistically different from the ratio in the *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice. *, $p < 0.05$, compared with wild-type control; †, $p < 0.05$, compared with corresponding control; #, no significant difference between the control and vehicle control groups of each genotype. Mean $\pm$ S.D. ($n = 4$).

tyrosine kinase inhibitors in that it is a higher-affinity substrate for BCRP than for P-gp, and as a result, irrespective of transporter capacity at the BBB, BCRP is the dominant transporter preventing sorafenib from entering the brain.

In vitro experiments showed that sorafenib is a high-affinity substrate for BCRP with an affinity constant (K_m) of 5 nM. Although it did not seem to be a substrate for P-gp from the in vitro data, sorafenib inhibited the transporter with an IC_{50} of 25 μM (Fig. 6A). These findings can be clinically relevant because sorafenib plasma concentrations in humans with the currently accepted treatment regimen (100–400 mg b.i.d) have been reported to be in the range of 1 to 15 μM (Richly et al., 2006; Duran et al., 2007). Inhibition of P-gp by sorafenib may alter tissue pharmacokinetics of concurrently administered drugs if the coadministered drug is a substrate for P-gp. Another interesting finding was that, although sorafenib inhibited the accumulation of vinblastine in the *MDR1*-transfected cells, it did not inhibit the accumulation of prazosin and mitoxantrone in the *Bcrp1*-transfected cells (Fig. 6B). We have previously shown that there are differences in the inhibition of BCRP depending on both the inhibitor used and the substrate under evaluation (Giri et al., 2009). In the same study we raised the possible outcomes of in vitro screening related to multiple binding sites on BCRP for transport of drugs. The finding that sorafenib is transported by BCRP, but does not inhibit it, suggests that sorafenib might interact with BCRP by binding to sites that do not overlap with the binding sites of either prazosin or mitoxantrone, prototypical substrates often used in drug screens.

The influence of P-gp and BCRP on transport of sorafenib across the BBB was examined by determining the steady-state B/P ratios in wild-type and transporter knockout mice (Table 2). The steady-state brain-to-plasma ratio of sorafenib in the wild-type mice was approximately 10%, indicating the restricted delivery of sorafenib across the BBB. Consistent with in vitro findings that sorafenib is a high-affinity substrate for efflux mediated by BCRP, the absence of BCRP resulted in a 4-fold increase in brain-to-plasma ratio of sorafenib in the *Bcrp1*(-/-) mice. This finding confirmed that sorafenib is actively effluxed by BCRP at the BBB, and this limits its brain penetration. It is noteworthy that the steady-state brain-to-plasma ratio in the *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice was 10-fold greater than the B/P ratio in the wild-type mice and approximately 2.5-fold greater than the ratio in *Bcrp1*(-/-) mice (Fig. 8; Table 2). The finding that the B/P ratio in the *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice was greater than the ratio in the *Bcrp1*(-/-) mice was unexpected, based on the fact that the B/P ratio was unchanged in the *Mdr1a/b*(-/-) mice and in vitro studies showed that sorafenib was not an avid P-gp substrate. This suggested that even though sorafenib does not seem to be transported by P-gp in vitro the presence of P-gp at the BBB is important to restrict brain penetration of sorafenib, particularly when BCRP is also absent. More-

TABLE 3

Plasma and brain concentrations of sorafenib at 60 min in wild-type, *Mdr1a/b*(-/-), *Bcrp1*(-/-), and *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice as influenced by treatment with the dual P-gp/BCRP inhibitor elacridar (elacridar was administered 30 min before the sorafenib dose)

Data are presented as mean $\pm$ S.D., $P < 0.05$.

Genotype and Cohort	Plasma Concentration	Brain Concentration	Brain-to-Plasma Ratio
	$\mu\text{g/ml}$	$\mu\text{g/g}$	
FVB (wild-type)			
Control	6.5 ± 1.5	0.52 ± 0.045^a	0.083 ± 0.020
Vehicle control	5.04 ± 0.87^b	$0.65 \pm 0.091^{a,b}$	0.13 ± 0.037^b
Elacridar pretreatment	4.04 ± 0.69^c	$2.9 \pm 0.46^{c,d,e}$	$0.76 \pm 0.24^{c,d,e}$
<i>Bcrp1</i> (-/-)			
Control	3.02 ± 1.03^c	1.1 ± 0.35^a	0.37 ± 0.035^c
Vehicle control	3.6 ± 0.11^b	$1.08 \pm 0.29^{a,b}$	0.30 ± 0.082^b
Elacridar pretreatment	3.3 ± 1.7^c	$3.6 \pm 1.9^{c,d,e}$	$1.03 \pm 0.33^{c,d,e}$
<i>Mdr1a/b</i> (-/-)			
Control	3.5 ± 2.1^c	0.71 ± 0.78^a	0.16 ± 0.11
Vehicle control	4.4 ± 0.36^b	$0.67 \pm 0.22^{a,b}$	0.15 ± 0.045^b
Elacridar pretreatment	2.4 ± 1.1^c	$3.01 \pm 1.09^{c,d,e}$	$1.3 \pm 0.29^{c,d,e}$
<i>Mdr1a/b</i> (-/-) <i>Bcrp1</i> (-/-)			
Control	4.7 ± 1.5^c	3.9 ± 0.79^c	0.86 ± 0.16^c
Vehicle control	3.07 ± 1.8^b	$2.08 \pm 1.6^{a,b}$	0.69 ± 0.15^b
Elacridar pretreatment	4.1 ± 3.04^c	$3.4 \pm 2.7^{c,e}$	$0.73 \pm 0.35^{c,e}$

^a Significantly different compared with corresponding plasma concentration.

^b No significant difference between the control and vehicle control groups of each genotype.

^c Significantly different compared with wild-type control group.

^d Significantly different compared with corresponding control group.

^e Not significant compared with *Mdr1a/b*(-/-)*Bcrp1*(-/-) control group.

over, the results clearly show that BCRP plays the leading role in restricting the delivery of sorafenib across the BBB. This finding is different from reports for other TKIs, where P-gp seems to be the dominant transporter keeping dual substrates out of the brain (Chen et al., 2009; Lagas et al., 2009; Polli et al., 2009; Agarwal et al., 2010; Kodaira et al., 2010).

The increased brain distribution of sorafenib when both P-gp and BCRP were absent in the *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice warranted further investigation into the potential role of P-gp in the efflux of sorafenib across the BBB. This was explored by administration of the dual P-gp and BCRP inhibitor in conjunction with sorafenib in the four genotypes. Coadministration of elacridar increased the brain concentrations of sorafenib in the wild-type, *Bcrp1*(-/-), and *Mdr1a/b*(-/-) mice such that they were not different from the concentrations in the *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice (Fig. 9; Table 3). This was a significant finding in light of the result that the steady-state brain concentrations of sorafenib did not increase when P-gp alone was absent in the *Mdr1a/b*(-/-) mice and confirmed that even though sorafenib is a weaker substrate for P-gp compared with BCRP P-gp may still be an influential determinant in the distribution of sorafenib to brain. Another intriguing possibility, yet to be confirmed, is that sorafenib brain distribution is limited by a different efflux system that is also inhibited by elacridar.

Our previous studies exploring the impact of drug efflux transporters on the delivery of TKIs to the brain have postulated that P-gp and BCRP cooperate at the BBB to limit brain distribution of gefitinib and dasatinib (Chen et al., 2009; Agarwal et al., 2010). This was first reported for the TKIs by Polli et al. (2009) where simultaneous absence of P-gp and BCRP in the *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice resulted in a greater than additive enhancement in the brain distribution of lapatinib. Similar findings have been reported for other TKIs such as imatinib and erlotinib (Breedveld et al., 2005; Kodaira et al., 2010). All of these studies have suggested that P-gp is the dominant transporter limiting

brain delivery of dual substrates. Sorafenib is different from these tyrosine kinase inhibitors in that BCRP plays the role of the dominant transporter in effluxing it at the BBB. Even so, it is similar to the other TKIs because absence/inhibition of both P-gp and BCRP is necessary to see a maximal improvement in brain penetration. An understanding of the possible mechanisms behind this cooperation can be gained by looking at the relative expression of P-gp and BCRP (capacities of the two transporters) at the BBB, as well as the relative affinity for substrate drugs. Warren et al. (2009) showed that the mRNA levels of P-gp in the brain vascular endothelial cells of mice was approximately 5-fold greater than the mRNA levels of BCRP. Likewise, Kamiie et al. (2008) quantified membrane transporter levels using LC-MS and reported approximately 5-fold higher levels of P-gp at the mouse BBB compared with BCRP. The significantly greater amount of P-gp at the BBB might be the reason behind it being dominant in the efflux of many dual substrates from the brain. The efflux mediated by BCRP can thus appear subdued and become apparent only when both transporters are absent or inhibited. This is of particularly important because most of the TKIs mentioned above demonstrate a significant BCRP mediated transport in vitro (Polli et al., 2008; Chen et al., 2009; Agarwal et al., 2010). The greater expression (capacity) of P-gp offsets the lower affinity, resulting in a pronounced P-gp effect on efflux of drugs at the BBB. However, for a drug such as sorafenib, which has a significantly higher affinity for BCRP compared with a moderate affinity for P-gp (not detectable in vitro), it is BCRP that plays the role of the dominant transporter in keeping it out of the brain. The effect of P-gp is noticeable only in the *Bcrp1*(-/-) and the *Mdr1a/b*(-/-)*Bcrp1*(-/-) mice. Kodaira et al. (2010) determined the net contribution of P-gp and BCRP to the overall efflux of various drugs at the BBB. This group showed that for many dual substrates the efflux clearance out of brain mediated by P-gp was greater than that mediated by BCRP. On the other hand, for dantrolene, the P-gp mediated efflux was 10-fold lower than the BCRP-me-

diated efflux at the BBB. Similar to sorafenib, the P-gp-mediated efflux of dantrolene was not detectable in vitro. Our study clearly shows that sorafenib is currently one of the select TKIs for which the role of BCRP in active efflux at the BBB is more than that of P-gp. It is also one of the few cases in which the in vitro BCRP-mediated transport correlates with limited brain penetration seen in vivo. Enokizono et al. (2008) and Zhao et al. (2009), in separate studies, have shown that this is true only for a handful of BCRP substrates such as dantrolene, prazosin, and alfuzosin.

In conclusion, we have shown that the brain distribution of sorafenib is restricted because of the active efflux transport at the BBB. BCRP plays a dominant role in limiting the distribution of sorafenib to the brain. Although we also present a possible mechanism behind the cooperative role of P-gp and BCRP at the BBB (simple compensation depending on relative expression and affinity), further studies in this direction are warranted. Finally, these results can be of clinical significance for therapy in glioma, because the ability of sorafenib to cross an intact BBB and achieve therapeutic concentrations in the tumor cells might determine efficacy against the invasive tumor cells that are out of the reach of surgical resection.

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Authorship Contributions

Participated in research design: Agarwal and Elmquist.

Conducted experiments: Agarwal and Sane.

Performed data analysis: Agarwal, Ohlfest, and Elmquist.

Wrote or contributed to the writing of the manuscript: Agarwal, Sane, Ohlfest, and Elmquist.

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