Role of ATP-Binding Cassette and Solute Carrier Transporters in Erlotinib CNS Penetration and Intracellular Accumulation

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Abstract

Purpose: To study the role of drug transporters in central nervous system (CNS) penetration and cellular accumulation of erlotinib and its metabolite, OSI-420.

Experimental Design: After oral erlotinib administration to wild-type and ATP-binding cassette (ABC) transporter-knockout mice (Mdr1a/b−/−, Abcg2−/−, Mdr1a/b−/−Abcg2−/−, and Abcc4−/−), plasma was collected and brain extracellular fluid (ECF) was sampled using intracerebral microdialysis. A pharmacokinetic model was fit to erlotinib and OSI-420 concentration–time data, and brain penetration ($P_{\text{brain}}$) was estimated by the ratio of ECF-to-unbound plasma area under concentration–time curves. Intracellular accumulation of erlotinib was assessed in cells overexpressing human ABC transporters or SLC22A solute carriers.

Results: $P_{\text{brain}}$ in wild-type mice was 0.27 ± 0.11 and 0.07 ± 0.02 (mean ± SD) for erlotinib and OSI-420, respectively. Erlotinib and OSI-420 $P_{\text{brain}}$ in Abcg2−/− and Mdr1a/b−/−Abcg2−/− mice were significantly higher than in wild-type mice. Mdr1a/b−/− mice showed similar brain ECF penetration as wild-type mice (0.49 ± 0.37 and 0.04 ± 0.02 for erlotinib and OSI-420, respectively). In vitro, erlotinib and OSI-420 accumulation was significantly lower in cells overexpressing breast cancer resistance protein (BCRP) than in control cells. Only OSI-420, not erlotinib, showed lower accumulation in cells overexpressing P-glycoprotein (P-gp) than in control cells. The P-gp/BCRP inhibitor elacridar increased erlotinib and OSI-420 accumulation in BCRP-overexpressing cells. Erlotinib uptake was higher in OAT3- and OCT2-transfected cells than in empty vector control cells.

Conclusion: Abcg2 is the main efflux transporter preventing erlotinib and OSI-420 penetration in mouse brain. Erlotinib and OSI-420 are substrates for SLC22A family members OAT3 and OCT2. Our findings provide a mechanistic basis for erlotinib CNS penetration, cellular uptake, and efflux mechanisms.

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Introduction

Erlotinib (Tarceva) is an orally administered epidermal growth factor receptor (EGFR) inhibitor approved for certain types of non–small cell lung cancer (NSCLC) and pancreatic cancer (1). In humans, erlotinib is extensively metabolized primarily by hepatic CYP3A4 and CYP3A5, with some metabolism by CYP1A2 and CYP2C8 (2). The metabolite OSI-420 retains EGFR tyrosine kinase inhibitory activity as well as in vivo tumor growth inhibitory activity (3). Clinical trials have evaluated the safety and activity of erlotinib in patients with primary and secondary central nervous system (CNS) tumors with frequent EGFR alterations, such as high-grade glioma and brain metastases of NSCLC (4, 5). In most of these studies, erlotinib failed to show clinically significant activity (1, 4, 6). A potential explanation for this lack of efficacy is that the blood–brain barrier (BBB) prevents the accumulation of effective erlotinib concentrations in the tumor (7, 8). This is supported by evidence from clinical studies showing low erlotinib and OSI-420 accumulation in high-grade glioma (9).

One mechanism by which the BBB limits erlotinib CNS penetration is the expression of efflux proteins of the ATP-binding cassette (ABC) transporter family at the brain endothelial cells (10). ABC transporters likely to impede erlotinib CNS distribution include P-glycoprotein (P-gp/Mdr1a/Mdr1b in mice), breast cancer resistance protein (Mdr1a/b in mice), and the multidrug resistance-associated protein 4 (Abcg2/Abcg2; Bcrp1/ Abcg2 in mice), and the multidrug resistance-associated protein 4 (Mrp4/Abcc4; Mrp4/Abcc4 in mice; refs. 11, 12). Expression of efflux transporters on tumor cell membranes would be an additional mechanism of drug resistance preventing the intracellular penetration of anticancer drugs (13).

In addition to ABC transporters, uptake transporters located at the BBB or at the blood–tumor barrier could also...
Translational Relevance

Drug transporters expressed at the blood–brain barrier or at tumor cell membranes may limit the accumulation of effective concentrations of erlotinib and its active metabolite OSI-420 within brain tumors. In this study, we identify specific efflux (BCRP) and uptake transporters (OCT2, OAT3) that affect accumulation of erlotinib and OSI-420, either within the brain extracellular fluid or within cells. This information contributes to our understanding of the factors that limit penetration of erlotinib into the brain and into tumor cells and thus may limit its effectiveness. In the future, BCRP inhibitors may be useful for increasing brain concentrations of erlotinib.

Materials and Methods

Animals

Animals used were wild-type FVB, Abcg2−/−, Mdr1a/b−/−, Abcc4−/−, and Mdr1a/b−/−Abcg2−/− mice. All mice were on a FVB genetic background, 3- to 4-month-old, female, and provided by Taconic (except Abcc4−/−, obtained from St. Jude Children’s Research Hospital). All animal studies were conducted using protocols and conditions approved by the Institutional Animal Care and Use Committee.

Chemicals and reagents

GF120918 was purchased from API. Erlotinib, OSI-420 analytical standards, and the internal standards for each compound used in the mass spectrometry assay were kindly provided by OSI Pharmaceuticals. 2-Hydroxypropyl-β-cyclodextrin (HPBCD) was purchased from CTD, Inc.

Cell lines

The porcine kidney epithelial LLC-PK1 cell line and the L-MDR1 cell lines stably expressing human ABCB1 were cultured in DMEM, 10% FBS, penicillin 100 U/mL, and streptomycin 100 μg/mL, all from Invitrogen. Saso2 cells containing pcDNA3.1 empty vector, ABCG2, or ABC4 were maintained in DMEM containing 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and G418 sulfate (500 μg/mL; Invitrogen). HEK-293 cells stably transfected with OCT1, OCT2 (25), OAT1, OAT2, OAT3 (26), OCTN1, and OCTN2 (27), along with pcDNA vector–transfected controls, were cultured in DMEM supplemented with 10% FBS and G418 sulfate (400–800 μg/mL).

Determination of erlotinib and OSI-420 protein binding in mouse plasma

Erlotinib and OSI-420 were added to mouse plasma (Hiltop) to make final erlotinib and OSI-420 concentrations of 1, 2, and 4 μg/mL. Two hundred microliters of plasma was added to each well of a 96-well equilibrium dialysis plate (Harvard Apparatus) and incubated on a rotator at 37°C. Samples were collected from plasma and PBS buffer chambers at several time points after starting the experiment. Samples were analyzed using a validated liquid chromatography/mass spectrometry (LC/MS) method (28). Unbound fraction (fu) of either erlotinib or OSI-420 was calculated as follows:

\[ fu = \frac{C_{PBS}}{C_{Plasma}} \]

where C PBS and C Plasma are the concentrations of erlotinib or OSI-420 in PBS and plasma, respectively. To compare the unbound fraction of erlotinib and OSI-420 in different strains, plasma was isolated from each strain and equilibrium dialysis was done as previously described.

Erlotinib and OSI-420 plasma pharmacokinetics

A single erlotinib dose was administered to all 5 strains of mice (n = 8–13 mice per group). To administer erlotinib, Tarceva (OSI Pharmaceuticals) tablets were pulverized and suspended in 0.2% carboxymethylcellulose and 0.05% Tween 20, for a final concentration of 5 mg/mL. The erlotinib suspension was administered at a dosage of 50 mg/kg by oral gavage. Plasma samples were taken by retro-orbital bleeding at 7 time points after drug administration (5 minutes, 0.5, 1, 2, 6, 12, and 24 hours). At least 4 different animals contributed to each time point. Blood samples were centrifuged at 3,000 rpm for 3 minutes. Plasma samples were immediately stored at –80°C till further analysis using LC MS/MS (24, 28).

Development of pharmacokinetic limited sampling models for erlotinib and OSI-420

Data from the plasma pharmacokinetic experiment were analyzed with nonlinear mixed-effects modeling by using
the importance sampling EM algorithm in NONMEM VII (29). A model with 1 compartment for erlotinib and 1 compartment for OSI-420 and first-order conversion from erlotinib to OSI-420 and first-order elimination of OSI-420 was used to fit the plasma concentration–time data. Models with first-order absorption, zero-order absorption, and sequential zero-order and first-order absorption were tested. The appropriate model was chosen on the basis of objective function value (OFV) and inspection of goodness-of-fit plots. The estimated parameters included the duration of zero-order infusion ($D_1$), first-order absorption rate constant ($k_a$), apparent oral clearance of erlotinib ($\text{CL}_{\text{ERL}}/F$), apparent volume of distribution of erlotinib ($V_{\text{ERL}}/F$), apparent clearance of OSI-420 ($\text{CL}_{\text{OSI}}/F$), and apparent volume of distribution of OSI-420 ($V_{\text{OSI}}/F$), where $F$ is the bioavailability and $FE$ is the fraction of erlotinib converted to OSI-420. Interindividual variability (IIV) terms were added to the $\text{CL}_{\text{ERL}}/F$, $V_{\text{ERL}}/F$, $\text{CL}_{\text{OSI}}/F$, and $D_1$ parameters. It was modeled as a log-normal distribution, and a proportional error model was used for residual variability. Data below the lower limit of quantitation of 1 ng/mL were included in the analysis with a likelihood-based approach using method M3 as previously described (30). Each strain of mice was analyzed separately, and the model parameters were used to develop a limited sampling strategy using the D-optimality algorithm in ADAPT 5 (31). The limited sampling strategy consisted of 3 time points at which plasma samples could be drawn in each microdialysis experiment to estimate the plasma exposure by using the population priors.

**In vitro probe recovery studies**

To study erlotinib and OSI-420 dialyzability as well as strategies required to enhance their relative recovery through microdialysis probes, a stock solution containing 1 μg/mL erlotinib and 0.5 μg/mL OSI-420 in artificial CSF (aCSF; ref. 32) was prepared. A 1-mm microdialysis probe (MBR-1-5 brain probe; BASi) was inserted into this solution, perfusates were pumped through the probe at 0.5 L/min and dialysates were obtained at different flow rates 0.2, 0.5, 1, and 4 μL/min. Dialysate concentrations were analyzed using LC MS/MS in GraphPad Prism (Version 5.0b) for Mac OS X (GraphPad Software) to fit the data using the following formula:

$$C_{\text{Dial}} = C_0 \times \exp(-rAX)$$

where $r$ (mass transport coefficient) and $A$ (surface area of the dialysis membrane) are constants, estimated using nonlinear regression, $C_0$ represents erlotinib or OSI-420 concentrations in the dialyzed tissue, $C_{\text{Dial}}$ is the concentration of erlotinib or OSI-420 in dialysates, and $X$ is the flow rate at which the dialysate was obtained at different flow rates. The microdialysis experiment was carried out at 0.5 μL/min perfusion rate, in vivo recovery was calculated as:

$$C_{\text{Dial at 0.5 μL/min}}/C_0 \approx 0.85$$

**Pharmacokinetic analysis of microdialysis studies**

A pharmacokinetic model was fit simultaneously to the plasma and brain ECF microdialysis data. To incorporate information from the previous plasma pharmacokinetic experiment, pharmacokinetic data from both experiments were pooled and analyzed together. Modeling was done with NONMEM VII by using the importance sampling EM algorithm. The model was based on the model described earlier for plasma data, with additional compartments for
erlotinib and OSI-420 in brain ECF. In addition to the previous parameters, this model included first-order rate constants describing the transfer of erlotinib in and out of the brain ECF \((k_{24} \text{ and } k_{32})\), first-order rate constants describing the transfer of OSI-420 in and out of the brain ECF \((k_{35} \text{ and } k_{53})\), and a single volume of distribution parameter for erlotinib and OSI-420 in the brain ECF \((V_{\text{brain}})\). Interindividual variability was included on \(D_{t}, CL_{\text{ERL}}/\text{F}, V_{\text{ERL}}/\text{F}, CL_{\text{OSI}}/\text{F}, V_{\text{OSI}}/\text{F},\) and intercompartment rate parameters.

Individual post hoc parameters were used to simulate the concentration–time curve from 0 to 24 hours in plasma and brain ECF for erlotinib and OSI-420 for each mouse from which the area under the concentration–time curves for plasma and brain (AUC\(_{0-24,\text{plasma}}\) and AUC\(_{0-24,\text{Brain}}\)) were calculated with the log-linear trapezoidal method. Unbound plasma AUC (AUC\(_{0-24,\text{uPlasma}}\)) for erlotinib and OSI-420 was obtained by multiplying AUC\(_{0-24,\text{plasma}}\) by the appropriate unbound fraction \(f_u\). The extent of brain penetration \(P_{\text{Brain}}\) for erlotinib and OSI-420 was calculated as the brain ECF-to-unbound plasma AUC ratio:

\[
P_{\text{Brain}} = \frac{\text{AUC}_{0-24,\text{Brain}}}{\text{AUC}_{0-24,\text{uPlasma}}}.
\]

Differences in \(P_{\text{Brain}}\) between wild-type mice and other mouse strains were assessed using the Mann–Whitney test.

**Intracellular drug accumulation studies**

To study the role of several efflux and influx transporters in erlotinib intracellular accumulation, we used an array of cell lines expressing specific efflux or influx transporters and compared the accumulation of erlotinib or OSI-420 in the cell lines overexpressing each transporter to that transfectected with an empty vector. Intracellular accumulation of erlotinib and OSI-420 was measured in Saos2 cells transfected with human BCRP or MRP4 and LLC-PK1 cells transfected with MDR1 in the presence or absence of the P-gp/BCRP inhibitor elidelact. Uptake experiments were done using HEK293 cells transfected with cDNAs coding for members of human OCT, OAT, and OCTN families. Briefly, \(5 \times 10^5\) cells of each cell line were plated in 6-well plates in triplicates. Cells were allowed to attach overnight. For cells expressing the OAT1, 2, and 3 and their vector control, sodium butyrate (5 \(\mu\)mol/L), was added to the medium for 24 hours to induce expression of the respective transporter genes (26). At the day of experiment, medium was removed and cells were incubated with media containing 0.5 \(\mu\)g/mL of either erlotinib or OSI-420 at 37°C. At a predetermined time interval, the experiment was terminated by removing the incubation medium and adding ice-cold PBS. Cells were washed twice with ice-cold PBS, gently scraped, collected, and centrifuged for 4 minutes at 3,000 rpm at 0°C. Subsequently, cells were resuspended in 100 \(\mu\)L of 5 mmol/L ammonium formate for cell lysis. The BCA assay was used to determine protein concentrations. Erlotinib and OSI-420 concentrations in the lysis supernatants were determined using LC MS/MS (28).

**Results**

**Erlotinib and OSI-420 protein binding in mouse plasma**

Using equilibrium dialysis, we assessed the extent of plasma protein binding for erlotinib and OSI-420. The range of concentrations used was similar to those expected after administering erlotinib orally to mice at 20 mg/kg. The time after which the dialysis reached equilibrium was 10 hours. Erlotinib, as well as its metabolite, was found to be highly protein bound in mouse plasma. Unbound fraction \(f_u\) of erlotinib was 4.8% ± 0.7% and for OSI-420 was 6.6% ± 0.7%. No significant differences were found in unbound fractions of erlotinib or OSI-420 between strains (Supplementary Table 2).

**Limited sampling models for erlotinib and OSI-420**

The erlotinib dosage used in preclinical studies has ranged from 5 to 150 mg/kg/d orally for up to 4 weeks (36, 37). In our studies, we sought to use an erlotinib dosage that achieves a systemic exposure similar to that observed in patients (4, 21). First, we conducted a plasma pharmacokinetic study of a single erlotinib dose administered orally at a dosage of 50 mg/kg in wild-type, Abcg2\(^{-/-}\), Mdr1a/b\(^{-/-}\), Mdr1a/b\(^{-/-}\)Abcg2\(^{-/-}\), and Abcc4\(^{-/-}\) mice. This single dose of erlotinib was tolerated well by all strains. Between the different absorption models, a sequential zero-order and first-order model described best the data on the basis of goodness-of-fit plots and lowered the OFV between 6 and 35 units for different strains compared with first-order absorption alone and lowered the OFV between 9 and 33 units for different strains for zero-order absorption alone. The final model is shown in Figure 1. Concentration–time plots for erlotinib and OSI-420 along with the model-predicted concentrations are shown in Figure 2 and Supplementary Figure 3. Pharmacokinetic parameters for all strains are listed in Supplementary Table 1. The AUC\(_{0-24,\text{plasma}}\) in each strain was compared with wild-type mice. Mean AUC\(_{0-24,\text{plasma}}\) for Abcg2\(^{-/-}\), Mdr1a/b\(^{-/-}\), Mdr1a/b\(^{-/-}\)Abcg2\(^{-/-}\), and Abcc4\(^{-/-}\) strains were 1.25×, 0.9×, 1.3×, and 0.9-fold compared with that of wild-type mice. Using the population parameters, we determined the optimal sampling time points to calculate the plasma AUC of erlotinib and OSI-420, constraining to 3 time points over 18 hours. The optimal time points were similar in all strains: 1 early time point after the administration (5 minutes), 1 coinciding with the plateau (range from 7.8 to 9 hours), and 1 late time point (18 hours). For the ease of conducting the experiments, we chose to obtain plasma samples at 0.08, 8, and 18 hours from all strains.

**In vitro microdialysis studies**

Because reproducible dialysis of lipophilic drugs can be difficult by using conventional microdialysis methods
We anticipated that the microdialysis of erlotinib would require the inclusion of an affinity-based trapping agent, such as HPBCD, in the perfusate. Such modifications in the perfusate have been used before to increase the recovery of poorly dialyzable drugs (40). Results of our preliminary in vitro microdialysis experiments supported that erlotinib and OSI-420 had relatively low relative recovery (1% and 2%, respectively), using aCSF as a perfusate. The addition of 10% HPBCD in aCSF improved the recovery of erlotinib and OSI-420 by ~8-fold (Supplementary Fig. 1A). Second, we tested the effect of different flow rates (0.3, 0.5, 1, and 2 mL/min) on relative recovery. Increasing the flow rate yielded lower relative recovery. Finally, we evaluated the effect of perfusing the microdialysis tubes with 4% BSA to prevent the binding of the drug to the tubes and thus improve the recovery. This strategy did not improve the relative recovery and added complexity due to tube blockage and flow resistance (data not shown). Thus, we decided to use 10% HPBCD in aCSF at 0.5 mL/min at room temperature for the in vivo microdialysis experiments to obtain sufficient recovery with adequate sample volume (30 μL/h). The in vitro recovery at that flow rate was 18% ± 2.5% and 20% ± 1.3% for erlotinib and OSI-420, respectively.

ZFR for estimation of in vivo recovery
To calculate erlotinib and OSI-420 concentrations in brain ECF, microdialysate concentrations were corrected for probe recovery in each mouse by using the ZFR method. This method is based on the fact that at slow perfusion rates and steady-state tissue concentrations of the analyte, extrapolation to a hypothetical flow rate value of zero will provide the absolute value of analyte in the dialyzed tissue (C_{ECF}; ref. 35). Probe recovery can then be calculated by dividing the concentration obtained at the flow rate at which the experiment is conducted (i.e., C_{Dial} at 0.5 mL/min) by C_{ECF}. First, we validated in vitro the use of the ZFR recovery method for recovery estimation. Dialysates were collected from a stock containing 1 mg/mL of each erlotinib and 0.5 mg/mL OSI-420 at different flow rates, and the concentrations were plotted against flow rate. The estimated value of C_{ECF} in vitro obtained from extrapolation...
to ZFR by using nonlinear regression was 96% ± 15% and 93% ± 12% for erlotinib and OSI-420, respectively (results from 6 in vitro experiments; Supplementary Fig. 1B). The ZFR requires that the concentration remain relatively constant while collecting the dialysates at different flow rates. Initial microdialysis experiments (n = 4) conducted under stable flow rate of 0.5 μL/min showed that brain ECF concentrations dropped by only 10% to 15% over the 5 to 8 hours period. Assuming relatively stable target tissue concentrations, we decided to carry out the recovery experiment after 5 hours from drug administration. After the recovery experiment, the flow rate was switched back to 0.5 μL/min and dialysates were collected up to 24 hours. The in vivo recovery for erlotinib was 39% ± 13% and for OSI-420 was 24% ± 13%.

**Increased ECF penetration of erlotinib and OSI-420 in Abcg2−/− and Mdr1a/b−/−Abcg2−/− mice**

Using microdialysis, we sampled brain ECF from wild-type, Abcg2−/−, Mdr1a/b−/−, Mdr1a/b−/−Abcg2−/−, and Abcc4−/− mouse strains after a single oral dose of erlotinib. Brain penetration ratios are shown in Figure 3A. In wild-type mice, P脑 (mean ± SD) for erlotinib and OSI-420 was 0.27 ± 0.11 and 0.07 ± 0.02, respectively. Abcg2−/− mice showed 5-fold higher erlotinib penetration (1.4 ± 0.9, P < 0.05, n = 5) and 7-fold higher OSI-420 penetration (0.51 ± 0.04, P < 0.05) than wild-type mice. However, neither erlotinib penetration (0.49 ± 0.3) nor OSI-420 penetration (0.04 ± 0.02) was enhanced in Mdr1a/b−/− mice (n = 4, P > 0.05 for both). In the Mdr1a/b−/−/Abcg2−/− mice, erlotinib brain penetration was 0.7 ± 0.26, ~3-fold higher than in wild-type mice (P < 0.01, n = 5) and OSI-420 brain penetration (0.34 ± 0.21) was 4-fold higher (P < 0.01). Brain penetration of Abcg2−/− mice was similar to wild-type mice for both erlotinib (0.32 ± 0.24, P > 0.05, n = 4) and OSI-420 (0.08 ± 0.06, P > 0.05). Histologic examination verified the localization of the probe track in the brain ECF and indicated no bleeding due to probe insertion (Supplementary Fig. 2).

**Intracellular accumulation experiments**

Intracellular accumulation of erlotinib and OSI-420 was significantly reduced in Saso2-BCRP cells as compared with Saso2-pCDNA3.1, indicating the significant role of BCRP in erlotinib transport. Accumulation of OSI-420, but not erlotinib, in LlcpK1-MDR1 cells was significantly reduced as compared with controls (LlcpK1 cells). Saso2-MRP4 cells did not show a significant difference in either erlotinib or OSI-420 accumulation (Fig. 4A). Preincubation for 30 minutes with the specific P-gp and BCRP inhibitor, elacridar significantly increased erlotinib and OSI-420 accumulation in Saso2-BCRP cells (P < 0.01) and OSI-420 accumulation in lLCPK1-MDR1 cells (P < 0.001; ref. Fig. 4B). Furthermore, we sought to determine whether erlotinib is a substrate for influx transporters using HEK-293 cells transfected with specific uptake transporters. Uptake of erlotinib and OSI-420 was significantly higher in the presence of organic anion transporter-3 (OAT3; P < 0.001) than in the HEK-293 cells transfected with empty vector. In addition, expression of the organic cation transporter-2 (OCT2) significantly increased erlotinib and OSI-420 uptake (P < 0.01; Fig. 5).

**Discussion**

Using microdialysis sampling, transporter-deficient mice, pharmacokinetic modeling, and cell lines transfected with efflux and uptake transporters, we elucidated the roles of transport mechanisms involved in erlotinib disposition in brain parenchyma. Our in vitro and in vivo data show that Bcrp1 is the primary efflux mechanism for erlotinib and OSI-420 at the murine BBB, whereas P-gp and Mrp4 have little or no effect on erlotinib CNS penetration. In vivo, intracellular accumulation of erlotinib and OSI-420...
Figure 4. Erlotinib and OSI-420 intracellular accumulation in vitro in cell lines expressing efflux transporters. Values are the percentage of the maximum accumulation (mean ± SD; n = 4–6) in control cells (Saos2-pcDNA for BCRP and MRP4 or LLC-PK1 for P-gp). A, time-course of drug accumulation in cell lines. Control, cells lines transfected with an empty vector control; transfected, cell lines transfected with a vector expressing the indicated transporter; transfected + elacridar, transporter-transfected cells treated with 4 μmol/L of the P-gp/BCRP inhibitor elacridar. B, intracellular accumulation data from 15 and 30 minutes time points combined. *, P < 0.001 as compared with accumulation in control cells (ANOVA with a post hoc Dunnett test).
showed that only OSI-420, not erlotinib, accumulation was reduced in cells overexpressing MDR1. This study also showed that erlotinib and OSI-420 are substrates for the human organic uptake transporter OAT3 and, to a lesser extent, OCT2.

Our technical approach of using microdialysis allowed the characterization of unbound (active) erlotinib concentrations in brain ECF. Our technique overcomes the limitations of (a) the CSF aspiration technique, which characterizes drug exposure in a very specialized CNS compartment (24), and (b) the whole brain homogenization technique, which does not discern between free and bound drug entities or between different CNS compartments (brain parenchyma, CSF, and brain vessels). Our study shows that the inclusion of HPBCD in the perfusate increases the recovery of erlotinib, probably by including the drug into the core of the HPBCD molecule (41).

Although HPBCD proved indispensable for the detection of erlotinib and OSI-420 in the dialysate, its presence provided a challenge as we carried out the recovery experiments. Because HPBCD forms inclusion complexes with erlotinib in the perfusate limiting drug movement to brain ECF, we could not use retrodialysis or no net flux to estimate in vivo recovery values. Instead, we chose to use the ZFR method to estimate recovery, realizing that as with all methods to calculate recovery, this approach has limitations. For example, the ZFR method should be applied when the target tissue (e.g., brain ECF) is at a steady state. Our initial microdialysis experiments \( n = 4 \) conducted under constant flow rate of 0.5 \( \mu \)L/min showed that brain ECF concentrations dropped by only \( \sim 12\% \) over the 5 to 8 hours period. Thus, we assessed recovery during a period 5 to 8 hours after the erlotinib dose was administered. Because we calculated the recovery during this time interval (i.e., 5–8 hours), we were able to collect only a single sample per flow rate. Although this short time frame may not have allowed adequate time to achieve stable recoveries at each flow rate, comparing in vitro recovery values obtained with either changing the flow rate or under constant (i.e., stable) flow rate yielded similar recovery values, suggesting that stable recoveries can be achieved with our experimental approach (Supplementary Table 3).

Our study has established that penetration of unbound erlotinib from plasma across the BBB of wild-type mice is limited (27%) and even lower for OSI-420 (7%). Thus, even though OSI-420 exhibits antitumor activity (3, 42), its low CNS penetration and systemic exposure (\( \sim 10\% - 20\% \) that of erlotinib in humans (2, 21)) suggest a minor role for OSI-420 in erlotinib clinical activity in brain tumors.

Our data show that Bcrp1 is the major efflux transporter limiting penetration of erlotinib and OSI-420 into murine brain ECF. In vitro studies showed reduced erlotinib and OSI-420 accumulation in Saos2-BCRP cells. Interestingly, a single nucleotide polymorphism in the ABCG2 promoter that correlates with lower BCRP expression was associated with higher erlotinib plasma exposure, indicating the important role of BCRP in erlotinib disposition (43). Our results contrast with the conclusions by Kodaira et al. stating that P-gp is the major efflux transporter for erlotinib at the murine BBB (44). This discrepancy can be explained by the different experimental designs used in each study. In the latter study, the authors administered erlotinib via the jugular vein for 2 hours and then analyzed erlotinib concentration in brain homogenates. They compared the erlotinib concentration in brain homogenates with that in plasma only at 1 time point (i.e., 2 hours) and did not account for erlotinib plasma protein binding. This study design has several limitations. First, the homogenate technique could provide different results as it describes drug accumulation in the whole brain rather than in specific compartments, and, second, relying on only 1 time point to determine the penetration ratio can mask the whole exposure profile for erlotinib in plasma as it provides only a “snapshot” of the penetration profile. In our study, we used the oral route for administering erlotinib to mimic the

Figure 5. Transport of erlotinib (A) and OSI-420 (B) by human organic ion transporters. Results are shown for drug accumulation in HEK293 cells after 5 minutes incubation in each cell line. Columns represent (mean ± SD) 8 to 18 observations per expressed transporter and are expressed as percentage of their respective control (white bar). Only 1 control bar is shown for clarity purposes. The contribution of each transporter toward erlotinib or OSI-420 uptake was established by comparing data obtained in HEK293 cells overexpressing the transporter and HEK293 cells transfected with an empty vector *, \( P < 0.05; **, P < 0.01; ***, P < 0.001 \) versus control, 1-way ANOVA was done followed by a post hoc Dunnett test.
clinical situation. Our studies also took into consideration the plasma protein binding of both erlotinib and OSI-420. Although intracellular accumulation experiments showed that OSI-420 is a P-gp substrate, OSI-420 brain penetration was not increased in Mdr1a/b−/− mice. This can be due to the compensatory upregulation of Bcrp1 in Mdr1a/b−/− mice, resulting in increased overall drug efflux from the brain. Our group previously used semi-quantitative immunohistochemical analysis to show that Bcrp1 is expressed at a higher level in the brain of Mdr1a/b−/− mice than in wild-type mice (24).

In vitro screening of uptake transporters using HEK-293 transfected cells identified erlotinib and OSI-420 as substrates for OAT3 and OCT2. The affinity of these transporters for both anionic and cationic molecules has been previously reported (45, 46). Although HEK-293/OCT2 cells showed significantly higher erlotinib uptake, OCT2 contribution toward erlotinib uptake should be cautiously interpreted given the ~40-fold higher expression of SLC22A2 gene, encoding OCT2, in these cells (47). Studying erlotinib disposition in OCT2-deficient mice will clarify the affinity of OCT2 toward erlotinib.

Our results could be clinically significant as Oat3 localized at the apical border of the choroid plexus can restrict the penetration of its substrates in the CSF (16). Our group previously showed that Bcrp1 and P-gp, localized apically at the choroid plexus, pump their substrate, topotecan, in the CSF (24, 33). Thus, Oat3 can oppose the transport direction of P-gp and Bcrp1 at the choroid plexus, decreasing CSF drug accumulation. In rodents, Oat3 is predominantly expressed at the basolateral border of brain endothelial cells where it has been implicated in the brain-to-blood transport of its substrates (17, 48). Furthermore, OCT2 is expressed at the apical border of the endothelial cells and was shown to increase the brain accumulation of their substrates (15). Thus, in absence of Bcrp1 (i.e., Abcg2−/− and Mdr1a/b−/− Abcg2−/− mice), the net erlotinib ECF accumulation would be determined by factors that favor brain ECF penetration (e.g., lack of Bcrp1, Oat3 at the choroid plexus, and OCT2 at the BBB) and those moving the drug from the brain to the blood or the CSF (e.g., Oat3 at the BBB, and P-gp at the choroid plexus) as schematized in Figure 6. This may explain the more than unity ECF accumulation for ABC transporters substrates in Abcg2−/− and Mdr1a/b−/− Abcg2−/− mice observed in our study and others (9, 18, 44). Another transporter of potential interest is the organic anion transporting polypeptide (OATP2), which is expressed on both the apical and basolateral membranes of brain endothelial cells and probably mediates the accumulation of its substrates across the intact BBB vessels (18, 19, 49). Further studies are warranted to identify the role of uptake transporters in erlotinib CNS penetration.

Regarding the implications of our results on erlotinib elimination, OCT2 and OAT3 transporters are abundantly expressed in the kidney (50). These transporters may explain the renal clearance component of erlotinib elimination, which accounts for ~10% of the overall elimination (2). However, transporters expressed in the liver such as OAT2 and OCT1 were not found to transport erlotinib, indicating the involvement of other transport mechanisms such as OATP1B1 and OAT1B3.

In conclusion, erlotinib is a Bcrp1 substrate whereas its metabolite is a substrate for both P-gp and Bcrp1. Erlotinib brain ECF accumulation was restricted mainly by Bcrp1. Intracellular accumulation studies confirmed the role of BCRP in erlotinib efflux and the dual P-gp/BCRP inhibitor elacridar increased erlotinib accumulation in cells in vitro. This study also indicates that erlotinib and OSI-420 are substrates for the uptake transporters OAT3 and OCT2. Further study is warranted to assess the role of these transporters in erlotinib brain accumulation. Our future experiments will focus on studying the effect of ABC transporter inhibitors on erlotinib penetration in high-grade glioma by using genetically engineered animal models that
recapitulate the genetics, the biology, and the histology of human tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Role of ATP-Binding Cassette and Solute Carrier Transporters in Erlotinib CNS Penetration and Intracellular Accumulation

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