

Use of the Cassette-Dosing Approach to Assess Brain Penetration in Drug Discovery

Xingrong Liu, Xiao Ding, Gauri Deshmukh, Bianca M. Liederer, and Cornelis E. C. A. Hop

Genentech, Inc., South San Francisco, California

Received December 23, 2011; accepted February 10, 2012

ABSTRACT:

The objective of the present study was to examine the cassette dosing method in determination of brain-to-plasma concentration ratio (area under the concentration-time profiles for plasma/area under the concentration-time profiles for brain, K_p). Eleven model compounds, amprenavir, citalopram, digoxin, elacridar, imatinib, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-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 (Ko143), loperamide, prazosin, quinidine, sulfasalazine, and verapamil, were selected to compare their K_p determined from discrete dosing in wild-type mice and their K_p from cassette dosing in wild-type, *Mdr1a/1b(-/-)*, *Bcrp1(-/-)*, and *Mdr1a/1b(-/-)/Bcrp1(-/-)* mice at 1 to 3 mg/kg. The mice brain and plasma were collected at 0.25, 1, and 3 h and were analyzed using high-performance liquid chromatography-tandem mass spectrometry methods. The K_p determined from discrete dosing versus cassette

dosing in the wild-type mice were within 2-fold for all the compounds except sulfasalazine and Ko143. The brain concentrations of sulfasalazine and Ko143 and the plasma concentrations of Ko143 were below the lower limit of quantitation. In addition, the K_p values estimated by mass spectrometry responses, namely the ratio of compound peak area to internal standard peak area, were within 2-fold of the K_p observed from the actual concentrations. Furthermore, the ratios of K_p in *Mdr1a/1b(-/-)*, *Bcrp1(-/-)*, and *Mdr1a/1b(-/-)/Bcrp1(-/-)* mice versus the K_p in the wild-type mice from cassette dosing were consistent with the ones reported in the literature where the compounds were dosed discretely. These results demonstrate that drug-drug interactions at the blood-brain barrier are unlikely at a subcutaneous dose of 1 to 3 mg/kg and support the use of the cassette dosing approach to assess brain penetration in drug discovery.

Introduction

The blood-brain barrier (BBB) consists of a continuous layer of endothelial cells joined by tight junctions at the cerebral vasculature. It represents a physical, enzymatic, and transporter barrier to restrict and regulate the penetration of compounds into and out of the brain (Davson and Segal, 1995). The main mechanisms limiting the delivery of drugs from blood into the brain are that the BBB exhibits very low paracellular permeability and expresses multiple drug transporters. Two efflux drug transporters, P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp) are the main efflux transporters expressed at the luminal side of the BBB, and their functional importance in limiting brain penetration of various compounds has been demonstrated (Schinkel et al., 1994; Chen et al., 2003; Breedveld et al., 2005; Enokizono et al., 2007; Polli et al., 2009; Zhou et al., 2009; Agarwal et al., 2011). In mice, P-gp is the product of *Mdr1a* (*Abcb1a*) and *Mdr1b* (*Abcb1b*) genes, and Bcrp is the product of *Bcrp1* (*Abcg2*) gene (Schinkel, 1999; Scherrmann, 2005).

A useful parameter to assess the efficiency of a drug to cross the BBB is the ratio of unbound brain concentration to unbound plasma

concentration ($K_{p,uu}$) (Liu et al., 2008; Hammarlund-Udenaes et al., 2009). The common method to estimate $K_{p,uu}$ is to determine in vivo plasma and brain concentrations (K_p) and in vitro unbound fraction in plasma and brain tissue (Maurer et al., 2005). K_p can be determined at steady state after intravenous infusion or from the area under the curve (AUC) of brain and plasma concentrations after a single dose. These experimental approaches are low throughput and resource-intensive. In the present study, we evaluated the cassette dosing approach to increase the throughput and reduce resource consumption in determination of K_p .

Traditionally pharmacokinetic parameters were generated by dosing compounds discretely. To increase the throughput of pharmacokinetic studies, cassette dosing (also called *N*-in-1 dosing) has been used in animal pharmacokinetic studies in drug discovery screening (Manitpisitkul and White, 2004). Extensive research work has been published to assess the cassette dosing approach for screening systemic pharmacokinetics in drug discovery. However, much less original research work has been published to evaluate cassette dosing to study brain penetration. Frick et al. (1998) briefly described in a review for the cassette dosing method to study a series of compounds in mice and observed good agreement between the

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

<http://dx.doi.org/10.1124/dmd.111.044420>.

ABBREVIATIONS: BBB, blood-brain barrier; P-gp, P-glycoprotein; Bcrp, breast cancer resistance protein; K_p , brain-to-plasma concentration ratio; $K_{p,uu}$, unbound brain-to-plasma concentration ratio; AUC, area under the curve; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-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; LLOQ, low limit of quantitation; AUC_{p,R}, area under the curve values calculated by using the mass spectrometer response for plasma; AUC_{b,R}, area under the curve values calculated by using the mass spectrometer response for brain; KO/WT ratio, K_p in knockout mice versus the K_p from wild-type mice; PF-407288, 2-(4-(2-(2-(4-methoxyphenyl)-5-methylloxazol-4-yl)ethoxy)benzyl)-tetrahydrofuran-2-carboxylic acid.

cassette dosing and discrete dosing. However, neither the compounds nor the methods were disclosed in the review. Zhang et al. (2004) reported use of cassette dosing with three to four compounds at 3 mg/kg for each compound in each cassette to determine K_p , but the authors did not compare the K_p between discrete and cassette dosing and did not disclose the compound structures or their transport characteristics.

Although it is plausible that drug transporter substrates and inhibitors may incidentally exist in one cassette and the brain penetration for the drug transporter substrates could be modified by the inhibitors, we hypothesized that if cassette dosing is conducted at a low dose such as at 1 to 3 mg/kg, the possibility of drug-drug interactions at the BBB may be low. To test this hypothesis, we selected a set of 11 compounds, namely amprenavir, citalopram, digoxin, elacridar, imatinib, (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 (Ko143), loperamide, prazosin, quinidine, sulfasalazine, and verapamil, and examined whether there is any difference for their K_p by dosing and analyzing the 11 compounds individually and by dosing and analyzing them as a cassette in mice. This set of compounds contains known potent P-gp and Bcrp inhibitors and typical P-gp and Bcrp substrates to create the "worst" scenario of potential drug-drug interactions at the BBB. We also examined whether we can estimate the K_p directly from mass spectrometer responses without using any standard curves to quantitate the actual plasma and brain concentrations. Furthermore, we assessed brain penetration of the 11 compounds in P-gp knockout mice [*Mdr1a/1b*(-/-)], Bcrp knockout mice [*Bcrp1*(-/-)], and P-gp and Bcrp knockout mice [*Mdr1a/1b*(-/-)/*Bcrp1*(-/-)] using the cassette dosing approach.

Materials and Methods

Chemicals. Citalopram, digoxin, loperamide, prazosin, quinidine, sulfasalazine, and verapamil were obtained from Sigma-Aldrich (St. Louis, MO). Amprenavir, amprenavir-D4, elacridar, and imatinib were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada). Ko143 was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY). All chemicals used in the experiments were of the highest available grade.

Animal Experiments. Male wild-type (FVB), P-gp knockout [*Mdr1a/1b*(-/-)], Bcrp1 knockout [*Bcrp1*(-/-)], and P-gp/Bcrp knockout mice [*Mdr1a/1b*(-/-)/*Bcrp1*(-/-)] of approximately 9 weeks of age, weighing 25 to 30 g, were obtained from Taconic Farms (Germantown, NY). Upon arrival, the mice were maintained for at least 5 days on a 12-h light/dark cycle in a temperature- and humidity-controlled environment with free access to food and water. In the discrete dosing study, FVB mice were administered a single subcutaneous dose ($n = 3$ /time point) of a single compound at 1 mg/kg for citalopram, elacridar, imatinib, loperamide, prazosin, and verapamil and at 3 mg/kg for amprenavir, quinidine, Ko143, digoxin, and sulfasalazine. In the cassette dosing study, FVB, P-gp, Bcrp, and P-gp/Bcrp knockout mice were administered a single subcutaneous dose ($n = 3$ /time point) of a mixture of 11 compounds at 1 mg/kg for citalopram, elacridar, imatinib, loperamide, prazosin, and verapamil and at 3 mg/kg for amprenavir, quinidine, Ko143, digoxin, and sulfasalazine. Dosing solutions of each drug were prepared in 100% *N*-methyl-2-pyrrolidone and were dosed at 1 ml/kg. Mice were euthanized in a CO₂ chamber at 0.25, 1, and 3 h postdose. Whole blood was collected by cardiac puncture into Costar tubes (Corning, Inc., Corning, NY) containing heparin and was stored on ice until centrifuged for the preparation of plasma. Whole brains were collected by decapitation, rinsed in phosphate-buffered saline, weighed, and immediately frozen on dry ice. All studies were conducted in accordance with approved Genentech Animal Care and Use Procedures.

Sample Analysis. Standard curves and quality control samples were prepared by spiking a known amount of a mixture of the 11 compounds into a blank mixed matrix of mouse plasma and brain homogenate (1:1 v/v). The brain tissue of each mouse was homogenized in 4 volumes (w/v) of water. Twenty-five microliters of plasma sample was mixed with 25 μ l of blank brain homogenate, and 25 μ l of brain homogenate sample was mixed with 25 μ l of

blank plasma. A total 50 μ l of samples, 50 μ l of calibration standards, or 50 μ l of quality controls was mixed with 15 μ l of internal standard (amprenavir-D4) and 150 μ l of acetonitrile. After vortexing and centrifugation at 1500g for 10 to 15 min, 150 μ l of supernatant was transferred to a 96-well plate and was diluted with 50 μ l of water before analysis by high-performance liquid chromatography combined with tandem mass spectrometry.

Samples were analyzed using two sets of standard curves and two sets of quality controls in each analytical run. The system consisted of an Accela pump (Thermo Fisher Scientific, Waltham, MA), an HTS-PAL autosampler (Leap Technologies, Chapel Hill, NC), and an AB Sciex API 5000 (AB Sciex, Foster City, CA) mass spectrometer with a turbo ion spray interface. A 20- μ l aliquot of each sample was injected onto a reverse-phase HALO C18 column. The lower limit of quantitation (LLOQ) for the compounds in the plasma and brain homogenate ranged from 0.122 to 7.80 ng/ml. The assay accuracy was between 75 and 125%.

Data Analysis. The total brain drug concentration was corrected for the residual blood in the brain vasculature by subtracting 1.03% of the plasma concentration determined in the corresponding samples (Fridén et al., 2010). The AUC values were calculated using the trapezoid rule from 0 to 3 h.

Results

Comparison of K_p from Discrete Dosing and Cassette Dosing in Wild-Type Mice. Plasma and brain concentrations of the 11 model compounds after a single subcutaneous administration at 1 or 3 mg/kg in either discrete or cassette dosing were above the detection limits except Ko143 and sulfasalazine. The plasma and brain concentrations of Ko143 were below its LLOQ (0.244 ng/ml for the plasma and 1.22 ng/g or ng/ml for the brain), although the chromatographic peaks were able to be identified in the brain samples. The plasma concentrations of sulfasalazine were above its LLOQ (7.8 ng/ml), but the brain concentrations were below its LLOQ (39 ng/g). The shape of the plasma and brain concentration-time profiles appeared similar for the discrete and cassette dosing (Fig. 1). The AUC values for plasma and brain from the discrete and cassette dosing are listed in Table 1. The K_p values from the discrete and cassette dosing are listed in Table 2 and are presented in Fig. 2. Although the plasma or brain AUC values were different between the discrete and cassette dosing groups, their K_p values were similar between the two groups (Tables 1 and 2). A good correlation was observed for the K_p from the discrete dosing and the K_p from cassette dosing with $R^2 = 0.99$ on a logarithmic scale. The K_p values from the cassette dosing were within 2-fold of the K_p from the discrete dosing for 9 of the 11 compounds. For sulfasalazine, its brain concentrations were below the LLOQ, so both cassette and discrete dosing demonstrated essentially no or low brain penetration, and K_p for this compound can be considered as similar in cassette and discrete dosing. The correlation for Ko143 K_p from cassette and discrete dosing cannot be assessed because its plasma and brain concentrations were below the LLOQ. The above results demonstrate that cassette dosing and discrete dosing generate similar K_p values.

Comparison of K_p Determined on the Basis of Concentration Versus Mass Spectrometer Response after Discrete or Cassette Dosing in Wild-Type Mice. We examined whether we can estimate the K_p without using standard curves to quantitate the actual brain and plasma concentrations and instead using the mass spectrometer response, namely peak area ratio between the compounds and the internal standard in the plasma and brain mixed matrix. The AUC values calculated by using the mass spectrometer response for plasma (AUC_{p,R}) and brain (AUC_{b,R}) in unit of response \cdot hour (R \cdot h) from the discrete and cassette dosing are listed in Table 1, and the corresponding K_p values are presented in Table 2. The K_p calculated from the concentrations versus the K_p calculated from the response were very similar, with correlation coefficient $R^2 = 0.98$ for discrete dosing

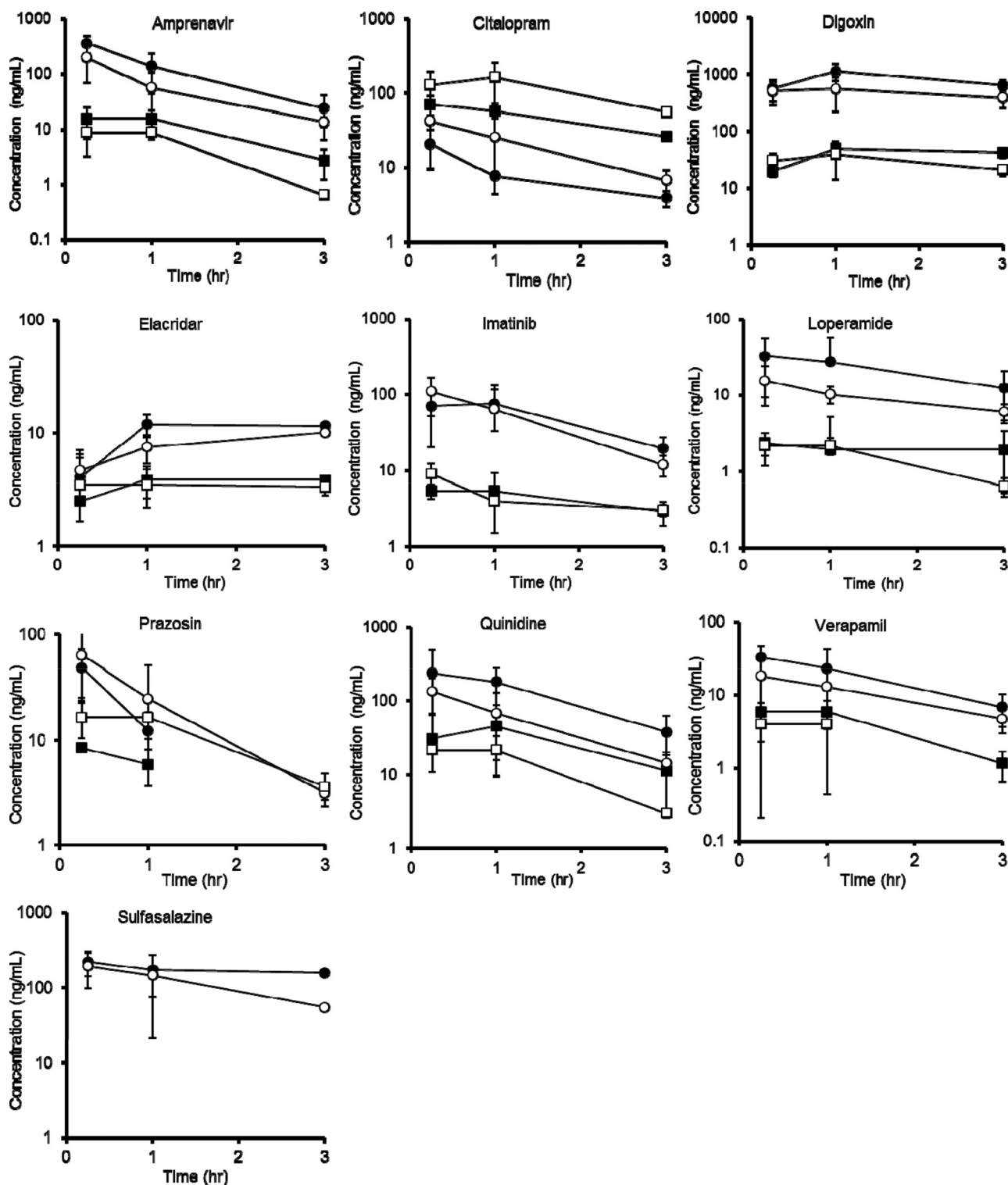


FIG. 1. Mouse plasma and brain concentration-time profiles of 10 compounds after a discrete or cassette dose of 1 to 3 mg/kg. The plasma and brain concentrations of Ko143 were below the LLOQ values. Circles and squares represent plasma and brain concentrations, respectively; closed and open symbols represent discrete and cassette dosing, respectively. Data points represent mean and S.D. from triplicate experiments.

(Fig. 3A) and $R^2 = 0.97$ for cassette dosing (Fig. 3B) on a logarithmic scale. The K_p values obtained from the response of mass spectrometry were within 2-fold of the K_p from the measured concentration. These results demonstrate that the K_p values estimated from the mass spectrometer response without using a standard curve are consistent with the K_p values determined from the actual concentrations.

Brain Penetration in P-gp, Bcrp, and P-gp/Bcrp Knockout Mice after Cassette Dosing. We examined brain penetration for the 11 compounds using the cassette dosing approach in P-gp, Bcrp, and P-gp/Bcrp knockout mice. The plasma and brain AUC values are shown in Table 1. The K_p values are listed in Table 2, and the correlation with the K_p from wild-type mice is presented in Fig. 4.

TABLE 1

Mouse brain and plasma $AUC_{(0-3\ h)}$ quantified as concentration or as mass spectrometer response following a discrete or cassette dose of the 11 compounds

Compound	Wild Type (Discrete)				Wild Type (Cassette)				P-gp (Cassette)		Bcrp (Cassette)		P-gp/Bcrp (Cassette)	
	$AUC_{p,R}$	$AUC_{b,R}$	AUC_p	AUC_b	$AUC_{p,R}$	$AUC_{b,R}$	AUC_p	AUC_b	AUC_p	AUC_b	AUC_p	AUC_b	AUC_p	AUC_b
	$R \cdot h$	$ng \cdot h/ml$			$R \cdot h$	$ng \cdot h/ml$			$ng \cdot h/ml$		$ng \cdot h/ml$		$ng \cdot h/ml$	
Amprenavir	0.168	0.00917	396	28.0	0.286	0.01512	190	15.4	288	157	244	8.09	202	114
Citalopram	1.94	10.2	25.1	140	0.929	5.04	63.4	344	70.1	583	50.4	103	59.3	403
Digoxin	0.0496	0.00147	2470	95.2	0.0279	0.00110	1400	76.5	5290	1780	2550	59.9	3130	1040
Elacridar	0.0191	0.00811	29.8	10.1	0.0882	0.0354	22.6	9.64	34.0	34.9	20.5	5.67	21.0	84.7
Imatinib	0.447	0.0392	159	11.5	0.293	0.0247	156	11.5	75.3	9.04	63.8	2.04	105	63.7
Ko143	BLLOQ	0.0126	BLLOQ	BLLOQ	BLLOQ	0.0113	BLLOQ	BLLOQ	BLLOQ	39.4	BLLOQ	12.1	BLLOQ	39.4
Loperamide	0.0778	0.00987	66.5	5.10	0.246	0.0481	28.0	4.44	46.0	99.7	36.1	BLLOQ	42.3	62.0
Prazosin	0.172	0.0481	41.1	11.9	0.183	0.0697	69.0	33.9	71.4	28.8	54.4	17.5	59.7	75.9
Quinidine	0.187	0.0429	409	85.5	0.138	0.0220	176	41.7	289	1430	235	27.7	215	1030
Sulfasalazine	0.238	BLLOQ	509	BLLOQ	0.163	BLLOQ	351	BLLOQ	645	BLLOQ	4050	BLLOQ	2640	BLLOQ
Verapamil	1.61	0.449	55.4	11.8	0.872	0.418	32.0	7.29	76.5	320	68.1	8.33	71.7	279

$R \cdot h$, mass spectrometer response (compound peak area to internal standard peak area ratio) \times hour; AUC_p , area under the curve values for plasma; AUC_b , area under the curve values for brain; BLLOQ, below the lower limit of quantitation.

The ratios of K_p in knockout mice versus the K_p from wild-type mice (KO/WT ratio) are presented in Table 3. For citalopram, the KO/WT ratios in all knockout mice were less than 2 (Table 3; Fig. 4). For Bcrp substrate, sulfasalazine, its plasma AUC increased 12- to 8-fold from 351 to 4050 $ng \cdot h/ml$ in Bcrp knockout mice and to 2640 $ng \cdot h/ml$ in P-gp/Bcrp knockout mice. However, its brain concentration was below the LLOQ in all strains of mice (Table 1). For P-gp substrates, amprenavir, digoxin, loperamide, quinidine, and verapamil, their KO/WT ratios in P-gp and P-gp/Bcrp knockout mice were similar and greater than 2 (Table 3; Fig. 4). For P-gp and Bcrp dual substrates elacridar, imatinib, and prazosin, their KO/WT ratios in P-gp/Bcrp knockout mice were greater than their KO/WT ratios in P-gp or Bcrp knockout mice (Table 3; Fig. 4). The plasma concentrations of Ko143 were below the LLOQ in all the samples. Very low amount in the wild-type mice brains was detected, but the concentrations were below the LLOQ (Table 1). However, its brain concentrations in knockout mice were above the LLOQ, and its brain concentrations in P-gp and P-gp/Bcrp mice were approximately 3-fold higher than that in Bcrp mice (Table 1). The KO/WT ratios in Bcrp knockout mice were lower or near 2 for all the compounds (Table 3; Fig. 4B).

Discussion

The results from the present study indicate that drug-drug interactions mediated by inhibition of efflux drug transporter P-gp or Bcrp at the BBB appear unlikely in cassette dosing at 1 to 3 mg/kg. In addition, the K_p may directly be estimated from the mass spectrometer response without using standard curves to quantitate the actual plasma and brain drug concentrations. Furthermore, the brain penetration in

P-gp, Bcrp, and P-gp/Bcrp knockout mice observed in cassette dosing is similar to the values reported in the literature.

Similar K_p from Discrete Dosing and from Cassette Dosing. We hypothesized that for cassette dosing at low doses such as subcutaneous injection of 1 to 3 mg/kg, the possibility of drug-drug interactions at the BBB is low even with compounds that are specifically designed to inhibit P-gp or Bcrp. To test this hypothesis, we selected 11 compounds including potent P-gp and Bcrp inhibitors and typical P-gp and Bcrp substrates to create the "worst" scenario of potential drug-drug interactions at the BBB. To classify the efflux effects of P-gp and Bcrp at the BBB, we used an arbitrary value 2-fold as the cutoff value (Table 4). A compound is considered a non-P-gp or Bcrp substrate if its KO/WT ratio in P-gp and Bcrp knockout mice is less than 2, a P-gp substrate if its KO/WT ratio is greater than 2 in P-gp knockout mice, a Bcrp substrate if its KO/WT ratio is greater than 2 in Bcrp knockout mice, or a P-gp and Bcrp substrate if its KO/WT ratio is greater than 2 in P-gp/Bcrp knockout mice. The 11 compounds in this study can be classified into five groups: 1) non-P-gp substrate: citalopram; 2) P-gp substrates: amprenavir, digoxin, loperamide, quinidine, and verapamil; 3) Bcrp substrate: sulfasalazine; 4) P-gp/Bcrp dual substrates: elacridar, imatinib, and prazosin; and 5) P-gp inhibitors: quinidine and verapamil; Bcrp inhibitor: Ko143; and P-gp/Bcrp dual inhibitor: elacridar.

The K_p values obtained from the discrete and cassette dosing are within 2-fold and showed a good correlation. These data demonstrate that inhibition of the drug efflux transporters such as P-gp and Bcrp is negligible at 1 to 3 mg/kg after a cassette dose of compounds containing potent P-gp and Bcrp inhibitors and specific substrates. Our

TABLE 2

Brain and plasma $AUC_{(0-3\ h)}$ ratio observed in wild-type, P-gp, Bcrp, or P-gp/Bcrp knockout mice

Compound	Wild-Type				P-gp	Bcrp	P-gp/Bcrp
	$K_{p,R}$ (Discrete)	K_p (Discrete)	$K_{p,R}$ (Cassette)	K_p (Cassette)	K_p (Cassette)	K_p (Cassette)	K_p (Cassette)
Amprenavir	0.0544	0.0706	0.0528	0.0808	0.546	0.0332	0.563
Citalopram	5.27	5.43	5.43	5.43	8.32	2.05	6.79
Digoxin	0.0297	0.0386	0.0393	0.0547	0.336	0.0235	0.332
Elacridar	0.424	0.340	0.401	0.426	1.03	0.276	4.04
Imatinib	0.088	0.0720	0.0841	0.0736	0.120	0.032	0.605
Ko143	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Loperamide	0.127	0.0768	0.196	0.159	2.17	N.A.	1.47
Prazosin	0.279	0.289	0.382	0.491	0.403	0.321	1.27
Quinidine	0.229	0.209	0.159	0.237	4.94	0.118	4.80
Sulfasalazine	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Verapamil	0.279	0.213	0.479	0.228	4.18	0.122	3.89

$K_{p,R}$, K_p calculated from the mass spectrometry response; N.A., not available.

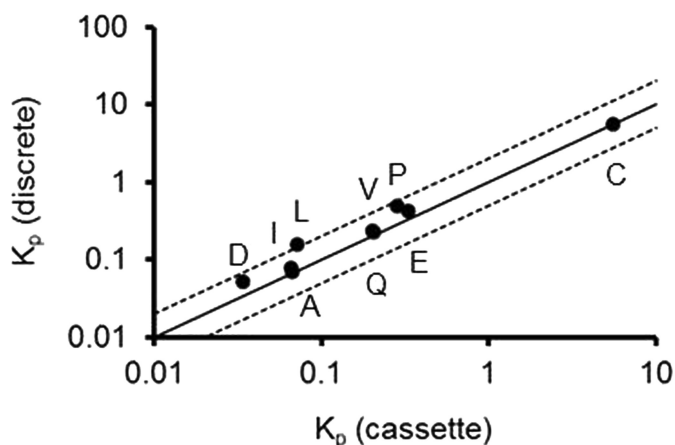


FIG. 2. Relationship between K_p determined from discrete dosing and cassette dosing of nine compounds. The solid and dotted lines represent unity and 2-fold of error. A, amprenavir; C, citalopram; D, digoxin; E, elacridar; I, imatinib; L, loperamide; P, prazosin; Q, quinidine; V, verapamil.

results are consistent with the results reported in the literature for two potent P-gp and Bcrp inhibitors: elacridar and Ko143. Elacridar is a third-generation P-gp inhibitor with K_i of 1.6 nM for P-gp inhibition and is also a potent Bcrp inhibitor with in vitro EC_{90} of 51 to 61 nM (Allen et al., 2002; Sugimoto et al., 2011). Its in vivo mouse and rat plasma IC_{50} for inhibition of P-gp at the BBB ranged from 114 to 282

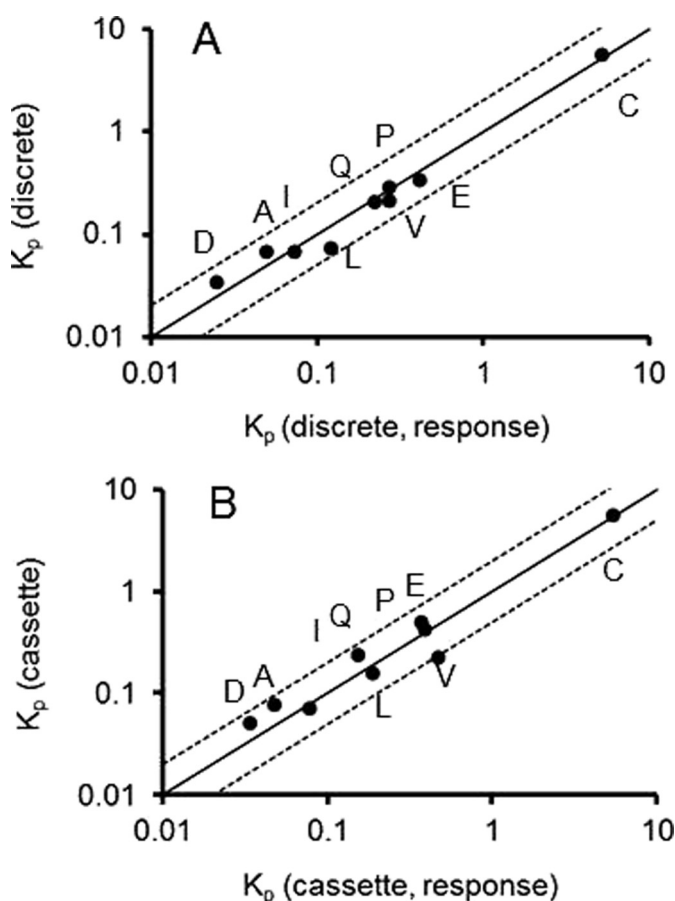


FIG. 3. Relationship between the K_p determined from concentration, and the K_p determined from mass spectrometer response [$K_{p(response)}$] of nine compounds from discrete dosing (A) and cassette dosing (B). The solid and dotted lines represent unity and 2-fold of error. A, amprenavir; C, citalopram; D, digoxin; E, elacridar; I, imatinib; L, loperamide; P, prazosin; Q, quinidine; V, verapamil.

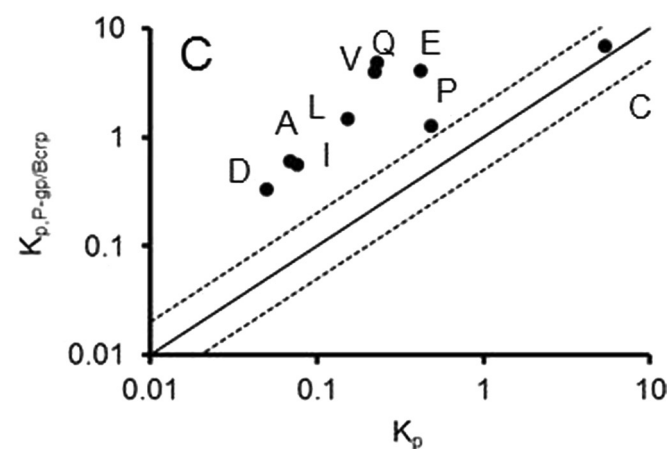
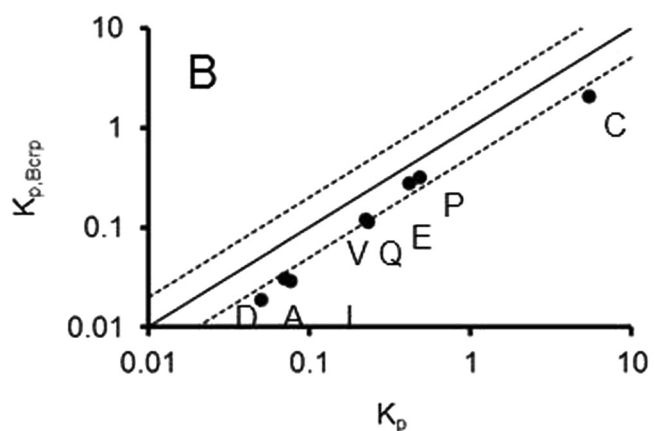
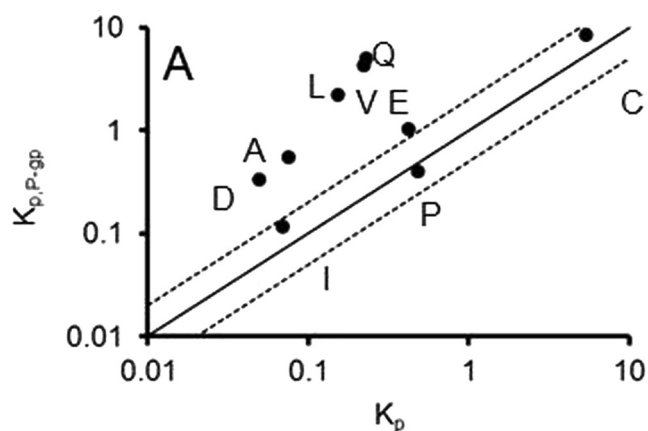


FIG. 4. A, relationship between the K_p in P-gp knockout mice ($K_{p,P-gp}$) versus K_p in wild-type mice (K_p). B, the K_p in Bcrp knockout mice ($K_{p,Bcrp}$) versus K_p . C, the K_p in P-gp and Bcrp knockout mice ($K_{p,P-gp/Bcrp}$) versus K_p . The solid and dotted lines represent unity and 2-fold of error, respectively. A, amprenavir; C, citalopram; D, digoxin; E, elacridar; I, imatinib; L, loperamide; P, prazosin; Q, quinidine; V, verapamil.

ng/ml, and its IC_{50} for inhibition of Bcrp was 790 ng/ml determined in a brain perfusion study (Cutler et al., 2006; Bihorel et al., 2007; Oostendorp et al., 2009; Kuntner et al., 2010; Sugimoto et al., 2011). Thus, with 2-fold as a cutoff value for the effects of elacridar on K_p , no change for the K_p values of P-gp and/or Bcrp substrates occurs when elacridar plasma concentrations are below 114 ng/ml. In the present study, the mouse plasma concentrations for elacridar were less than 15 ng/ml from time 0 to 3 h, so it is expected no much increase

TABLE 3

Ratio of K_p in P-gp, Bcrp, and P-gp/Bcrp knockout mice versus K_p in wild-type mice

Compound	P-gp	Bcrp	Pgp/Bcrp
	KO/WT	KO/WT	KO/WT
Amprenavir	6.8	0.41	7.0
Citalopram	1.5	0.38	1.3
Digoxin	6.2	0.43	6.1
Elacridar	2.4	0.65	9.5
Imatinib	1.6	0.44	8.2
Ko143	N.A.	N.A.	N.A.
Loperamide	14	N.A.	9.3
Prazosin	0.82	0.65	2.6
Quinidine	21	0.50	20
Sulfasalazine	N.A.	N.A.	N.A.
Verapamil	18	0.54	17

N.A., not available.

for the K_p values for P-gp substrates. Ko143 is a potent and selective Bcrp inhibitor. Its cell EC_{50} is 23 to 26 nM (Allen et al., 2002), and its effects on the oral absorption of topotecan have been demonstrated after a 10 mg/kg oral dose. However, no studies have been reported to examine the in vivo effects of Ko143 in enhancement of the brain penetration for Bcrp substrates.

Estimation of K_p Directly from Mass Spectrometer Response.

The main objective in brain penetration study is to determine K_p and not the absolute brain and plasma concentrations. In the present study, we examined a method to estimate the K_p by using the compound chromatographic peak area relative to an internal standard peak area without determining the actual plasma and brain concentrations. This method significantly reduced the bioanalytical work because it is not required to prepare and analyze the standards. The issue for this method is that plasma and brain homogenate may have different matrix effects on the mass spectrometer responses, and therefore, the K_p determined from the responses cannot represent the K_p determined from the actual concentration. To eliminate the different matrix effects, we mixed the brain homogenate with blank plasma and mixed plasma with blank brain homogenate so the matrices were identical for the brain homogenate and plasma samples. The K_p values calculated from the instrument response were almost identical to the ones from the measured concentration, supporting the use of this simplified bioanalytical method in drug discovery. A similar analytical approach has been used in the determination of unbound fractions in brain

homogenate or slices where the absolute concentrations are not critical but the concentration ratios are important (Fridén et al., 2011).

Similar Brain Penetration after Discrete or Cassette Dosing in P-gp, Bcrp, and P-gp/Bcrp Knockout Mice. Our data from the cassette dosing method are consistent with literature results in classifying whether the brain penetration of a compound is impaired by P-gp or Bcrp or both P-gp and Bcrp. The observed KO/WT ratio of citalopram in P-gp knockout mice was 1.9 (Doran et al., 2005). In the present study, the KO/WT ratio of citalopram in P-gp, Bcrp, or P-gp/Bcrp was less than 2, indicating P-gp and Bcrp have negligible effects on the brain penetration of citalopram. For P-gp substrates, amprenavir, digoxin, loperamide, quinidine, and verapamil, whose KO/WT ratio was reported in the range of 8.3 to 36 in P-gp knockout mice, showed a KO/WT ratio of 5.3 to 20 in the present study. No data are reported for their KO/WT ratios in Bcrp or P-gp/Bcrp knockout mice, except quinidine, which has a KO/WT ratio of unity in Bcrp knockout mice. Our data demonstrated similar KO/WT ratios in P-gp and P-gp/Bcrp mice for these compounds, and the KO/WT ratios in Bcrp mice were less than 2, indicating mainly P-gp at the BBB impairs the brain penetration for these compounds. For Bcrp substrate sulfasalazine, a 13-fold increase in plasma exposure in Bcrp knockout mice over the wild-type mice after an intravenous dose was observed (Zaher et al., 2006). In our study, plasma exposure increased 8- to 12-fold in Bcrp and P-gp/Bcrp knockout mice. Sulfasalazine was not detected in the brains in all strains of mice, suggesting P-gp and Bcrp may not be important in impairing Bcrp substrate sulfasalazine brain penetration. For P-gp and Bcrp dual substrates elacridar, imatinib, and prazosin, the reported KO/WT ratios in P-gp/Bcrp were 5.5 to 28 with less increase in P-gp or Bcrp knockout mice. In the present study, their KO/WT ratios increased 2.6 to 9.6 fold with less increase for their KO/WT ratios in P-gp or Bcrp knockout mice, confirming that removal of either P-gp or Bcrp from the BBB has a limited effect on the brain penetration, but removal of both P-gp and Bcrp has a profound effect on the brain penetration (Kusuhara and Sugiyama, 2009; Kodaira et al., 2010).

The available data support that P-gp is the main efflux protein at the BBB and that Bcrp at the BBB may play an important role for P-gp and Bcrp dual substrates. However, our data were not able to demonstrate the importance of Bcrp in impairing brain penetration for selective Bcrp substrate sulfasalazine. A similar observation was reported in the literature for a selective Bcrp substrate 2-(4-(2-(4-

TABLE 4

The ratio of K_p in in vivo transport activities of 11 compounds

Compound	P-gp		Bcrp		P-gp/Bcrp	
	KO/WT	Reference	KO/WT	Reference	KO/WT	Reference
Amprenavir	21	Polli et al., 1999	N.A.		N.A.	
Citalopram	1.9	Doran et al., 2005	N.A.		N.A.	
Digoxin	28	Mayer et al., 1997	N.A.		N.A.	
Elacridar	3	Kawamura et al., 2011	1	Kawamura et al., 2011	9	Kawamura et al., 2011
Imatinib	5	Bihorel et al., 2007	2	Breedveld, et al., 2005		
	2.3	Oostendorp et al., 2008	1	Bihorel, et al., 2007	13	Oostendorp et al., 2008
	4.4	Zhou et al., 2009	0.5	Oostendorp et al., 2008; Zhou et al., 2009	28	Zhou et al., 2009
			0.9			
Ko143	N.A.	N.A.	N.A.		N.A.	
Loperamide	9.3	Doran et al., 2005	N.A.		N.A.	
Prazosin	1.8	Zhou et al., 2009	1.5	Enokizono et al., 2008	5.5	Zhou et al., 2008
			1.3	Zhou et al., 2009		
Quinidine	36	Doran et al., 2005	1	Kodaira et al., 2010	10	Kodaira et al., 2010
	10	Kodaira et al., 2010				
Sulphasalazine	N.A.		N.A.		N.A.	
Verapamil	17	Doran et al., 2005	N.A.		N.A.	

N.A., not available.

methoxyphenyl)-5-methyloxazol-4-yl)ethoxy)benzyl)-tetrahydrofuran-2-carboxylic acid (PF-407288), whose KO/WT ratio was close to unity in Bcrp knockout mice (Zhou et al., 2009). The underlying mechanism for the lack of enhancement of brain penetration for selective Bcrp substrates in Bcrp knockout mice or both P-gp and Bcrp knockout mice may be due to up-regulation of other efflux.

Ko143 is a potent *in vitro* and *in vivo* Bcrp inhibitor, but no data have been reported for the *in vivo* disposition of Ko143. In the present study, the plasma concentration was below the LLOQ in all mouse strains after a 3 mg/kg subcutaneous dose. However, its brain concentrations were below the LLOQ in wild-type mice but were above its LLOQ in P-gp, Bcrp, and P-gp/Bcrp knockout mouse. Its brain concentrations in P-gp and P-gp/Bcrp mouse brains were approximately 3-fold higher than that in Bcrp mouse brains, indicating that Ko143 is a P-gp and Bcrp dual substrate and P-gp may be more important than Bcrp in limiting its brain penetration.

In summary, the present study demonstrates that inhibition of P-gp or Bcrp at the BBB is unlikely in cassette dosing for the compounds dosed at 1 to 3 mg/kg. Furthermore, the K_p can be estimated from the mass spectrometer response without using standard curves. Finally, use of the cassette dosing approach can correctly identify the impairing effects of P-gp or Bcrp or both P-gp and Bcrp on brain penetration. The results from the present study support the use of the cassette dosing approach to screen brain penetration in drug discovery. It may also be used to study drug transport at the BBB when the number of available animals such as transgenic mice is limited.

Acknowledgments

We thank Kirsten Messick, Linda Bao, Po-Chang Chiang, Jason Boggs, and Brian Dean for their contributions to this work.

Authorship Contributions

Participated in research design: Liu, Ding, Deshmukh, Liederer, and Hop.
Conducted experiments: Ding and Deshmukh.
Contributed new reagents or analytic tools: Ding.
Performed data analysis: Liu and Deshmukh.
Wrote or contributed to the writing of the manuscript: Liu, Ding, Deshmukh, Liederer, and Hop.

References

Agarwal S, Sane R, Ohlfest JR, and Elmquist WF (2011) The role of the breast cancer resistance protein (ABCG2) in the distribution of sorafenib to the brain. *J Pharmacol Exp Ther* **336**:223–233.

Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Koomen GJ, and Schinkel AH (2002) Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* **1**:417–425.

Bihorel S, Camenisch G, Lemaire M, and Scherrmann JM (2007) Influence of breast cancer resistance protein (Abcg2) and p-glycoprotein (Abcb1a) on the transport of imatinib mesylate (Gleevec) across the mouse blood-brain barrier. *J Neurochem* **102**:1749–1757.

Breedveld P, Pluim D, Cipriani G, Wielinga P, van Tellingen O, Schinkel AH, and Schellens JH (2005) The effect of Bcrp1 (Abcg2) on the *in vivo* pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer Res* **65**:2577–2582.

Chen C, Liu X, and Smith BJ (2003) Utility of Mdr1-gene deficient mice in assessing the impact of P-glycoprotein on pharmacokinetics and pharmacodynamics in drug discovery and development. *Curr Drug Metab* **4**:272–291.

Cutler L, Howes C, Deeks NJ, Buck TL, and Jeffrey P (2006) Development of a P-glycoprotein knockout model in rodents to define species differences in its functional effect at the blood-brain barrier. *J Pharm Sci* **95**:1944–1953.

Davson H and Segal MB (1995) *Physiology of the CSF and Blood-Brain Barriers*, CRC Press, Inc., Boca Raton.

Doran A, Obach RS, Smith BJ, Hosea NA, Becker S, Callegari E, Chen C, Chen X, Choo E, Cianfroga J, et al. (2005) The impact of P-glycoprotein on the disposition of drugs targeted for indications of the central nervous system: evaluation using the MDR1A/1B knockout mouse model. *Drug Metab Dispos* **33**:165–174.

Enokizono J, Kusahara H, and Sugiyama Y (2007) Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens. *Mol Pharmacol* **72**:967–975.

Frick LW, Adkison KK, Wells-Knecht KJ, Woollard P, and Higton DM (1998) Cassette dosing: rapid *in vivo* assessment of pharmacokinetics. *Pharm Sci Technol Today* **1**:12–18.

Fridén M, Bergström F, Wan H, Rehngren M, Ahlin G, Hammarlund-Udenaes M, and Bredberg U (2011) Measurement of unbound drug exposure in brain: modeling of pH partitioning explains diverging results between the brain slice and brain homogenate methods. *Drug Metab Dispos* **39**:353–362.

Fridén M, Ljungqvist H, Middleton B, Bredberg U, and Hammarlund-Udenaes M (2010) Improved measurement of drug exposure in the brain using drug-specific correction for residual blood. *J Cereb Blood Flow Metab* **30**:150–161.

Hammarlund-Udenaes M, Bredberg U, and Fridén M (2009) Methodologies to assess brain drug delivery in lead optimization. *Curr Top Med Chem* **9**:148–162.

Kawamura K, Yamasaki T, Konno F, Yui J, Hatori A, Yanamoto K, Wakizaka H, Takei M, Kimura Y, Fukumura T, et al. (2011) Evaluation of limiting brain penetration related to P-glycoprotein and breast cancer resistance protein using [(11)C]GF120918 by PET in mice. *Mol Imaging Biol* **13**:152–160.

Kodaira H, Kusahara H, Ushiki J, Fuse E, and Sugiyama Y (2010) Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. *J Pharmacol Exp Ther* **333**:788–796.

Kuntner C, Bankstahl JP, Bankstahl M, Stanek J, Wanek T, Stundner G, Karch R, Brauner R, Meier M, Ding X, et al. (2010) Dose-response assessment of tariquidar and elacridar and regional quantification of P-glycoprotein inhibition at the rat blood-brain barrier using (R)-[(11)C]verapamil PET. *Eur J Nucl Med Mol Imaging* **37**:942–953.

Kusahara H and Sugiyama Y (2009) *In vitro-in vivo* extrapolation of transporter-mediated clearance in the liver and kidney. *Drug Metab Pharmacokinet* **24**:37–52.

Liu X, Chen C, and Smith BJ (2008) Progress in brain penetration evaluation in drug discovery and development. *J Pharmacol Exp Ther* **325**:349–356.

Manitpisitkul P and White RE (2004) Whatever happened to cassette-dosing pharmacokinetics? *Drug Discov Today* **9**:652–658.

Maurer TS, DeBartolo DB, Tess DA, and Scott DO (2005) Relationship between exposure and nonspecific binding of thirty-three central nervous system drugs in mice. *Drug Metab Dispos* **33**:175–181.

Mayer U, Wagenaar E, Dorobek B, Beijnen JH, Borst P, and Schinkel AH (1997) Full blockade of intestinal P-glycoprotein and extensive inhibition of blood-brain barrier P-glycoprotein by oral treatment of mice with PSC833. *J Clin Invest* **100**:2430–2436.

Oostendorp RL, Buckle T, Beijnen JH, van Tellingen O, and Schellens JH (2009) The effect of P-gp (Mdr1a/1b), BCRP (Bcrp1) and P-gp/BCRP inhibitors on the *in vivo* absorption, distribution, metabolism and excretion of imatinib. *Invest New Drugs* **27**:31–40.

Pollí JW, Jarrett JL, Studenberg SD, Humphreys JE, Dennis SW, Brouwer KR, and Woolley JL (1999) Role of P-glycoprotein on the CNS disposition of amprevir (141W94), an HIV protease inhibitor. *Pharm Res* **16**:1206–1212.

Pollí JW, Olson KL, Chism JP, John-Williams LS, Yeager RL, Woodard SM, Otto V, Castellino S, and Demby VE (2009) An unexpected synergist role of P-glycoprotein and breast cancer resistance protein on the central nervous system penetration of the tyrosine kinase inhibitor lapatinib (N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({(2-methylsulfonyl)ethyl}amino)methyl]-2-furyl]-4-quinazolinamine; GW572016). *Drug Metab Dispos* **37**:439–442.

Scherrmann JM (2005) Expression and function of multidrug resistance transporters at the blood-brain barriers. *Expert Opin Drug Metab Toxicol* **1**:233–246.

Schinkel AH (1999) P-Glycoprotein, a gatekeeper in the blood-brain barrier. *Adv Drug Deliv Rev* **36**:179–194.

Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, and te Riele HP (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**:491–502.

Sugimoto H, Hirabayashi H, Kimura Y, Furuta A, Amano N, and Moriwaki T (2011) Quantitative investigation of the impact of P-glycoprotein inhibition on drug transport across blood-brain barrier in rats. *Drug Metab Dispos* **39**:8–14.

Zaher H, Khan AA, Palandra J, Brayman TG, Yu L, and Ware JA (2006) Breast cancer resistance protein (Bcrp/abcg2) is a major determinant of sulfasalazine absorption and elimination in the mouse. *Mol Pharm* **3**:55–61.

Zhang MY, Kerns E, McConnell O, Sonnenberg-Reines J, Zaleska MM, Steven Jacobsen J, Butera J, and Kreft A (2004) Brain and plasma exposure profiling in early drug discovery using cassette administration and fast liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal* **34**:359–368.

Zhou L, Schmidt K, Nelson FR, Zelesky V, Troutman MD, and Feng B (2009) The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic acid (PF-407288) in mice. *Drug Metab Dispos* **37**:946–955.

Address correspondence to: Dr. Xingrong Liu, Genentech, Inc., MS 41-2A, 1 DNA Way, South San Francisco, CA 94080. E-mail: liu.xingrong@gene.com