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Research Paper

Assessment of Blood–Brain Barrier Permeability Using the *In Situ* Mouse Brain Perfusion Technique

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Purpose. To assess the blood–brain barrier (BBB) permeability of 12 clinically-used drugs in *mdr1a*(+/+) and *mdr1a*(−/−) mice, and investigate the influence of lipophilicity, nonspecific brain tissue binding, and P-gp-mediated efflux on the rate of brain uptake.
Methods. The BBB partition coefficient (PS) was determined using the *in situ* mouse brain perfusion technique. The net brain uptake for 12 compounds, and the time course of brain uptake for selected compounds ranging in BBB equilibration kinetics from rapidly-equilibrating (e.g., alfentanil, sufentanil) to slowly-equilibrating (fexofenadine), was determined and compared.
Results. There was a sigmoidal relationship in *mdr1a*(−/−) mice between the log-PS and $clogD_{7.4}$ in the range of 0–5. The brain uptake clearance was a function of both permeability and blood flow rate. The brain unbound fraction was inversely proportional to lipophilicity. Alfentanil achieved brain equilibrium approximately 4,000-fold faster than fexofenadine, based on the magnitude of PS×*fu,brain*.
Conclusions. *In situ* brain perfusion is a useful technique to determine BBB permeability. Lipophilicity, ionization state, molecular weight and polar surface area are all important determinants for brain penetration. The time to blood-to-brain equilibrium varies widely for different compounds, and is determined by a multiplicity of pharmacokinetic factors.

KEY WORDS: blood–brain barrier; permeability; protein binding; time to equilibrium.

INTRODUCTION

The blood–brain barrier (BBB) is a continuous layer of brain capillary endothelial cells, connected by highly-developed tight junctions, that expresses numerous efflux transporters and metabolizing enzymes. The BBB represents a formidable barrier to the access of therapeutic targets for agents intended to have central nervous system (CNS) activity (1–4). Physicochemical properties (e.g., molecular weight, pKa, lipophilicity, polar surface area, and the number of hydrogen bonds) as well as biological factors (e.g., plasma and brain tissue binding, affinity to transporter systems at the BBB) are known determinants of substrate flux across the luminal and abluminal membranes of brain capillary endothelial cells (5–8). Whereas the role of other efflux transporters at the BBB remains unclear, P-glycoprotein (P-gp)-mediated efflux of many therapeutic agents has been widely accepted (4,9). P-gp is a 170-kDa membrane protein encoded by multidrug resistance gene *MDR1* in human and *mdr1a* and *mdr1b* in rodents. P-gp-mediated reduction in brain exposure has been linked to decreased CNS pharmacologic or toxic effects (9,10).

The logarithm of the brain-to-blood (or brain-to-plasma) concentration ratio (logBB) often is used to characterize and predict BBB permeation. However, it is recognized that the unbound brain concentration is the relevant driving force for most pharmacologic events in the CNS. Because logBB is a hybrid parameter that is determined by permeability, plasma and brain tissue binding, and active transport mechanisms, it may provide misleading information regarding potential *in vivo* pharmacologic response, thereby excluding potentially useful drug candidates from further consideration (11,12). The log of the permeability-surface area coefficient (logPS), in contrast, measures the ability of a drug to cross the BBB and move into brain tissue over time, and is a more relevant metric of the rate of BBB penetration (11–13).

PS can be measured using an *in vivo* or *in situ* short-duration vascular perfusion method, e.g., brain uptake index, indicator dilution, or *in situ* brain perfusion (14). *In situ* brain perfusion was developed to provide a simpler and more accurate method to determine BBB permeability compared

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ABBREVIATIONS: BBB, blood–brain barrier; $clogD_{7.4}$, calculated octanol–water partition coefficient at pH 7.4; clogP, calculated octanol–water partition coefficient in unionized state; Cl_{up}, uptake clearance; CNS, central nervous system; $f_{u,brain}$, brain unbound fraction; P-gp, P-glycoprotein; PS, permeability-surface area product; RSD, relative standard deviation.

to other techniques (15). Furthermore, changes in perfusate composition or use of gene knockout animals enable study of variables such as protein binding and efflux transporter mechanisms, and the resultant influence of these variables on CNS distributional kinetics. This method has been applied to study the CNS uptake of compounds (16–19), and the data generated were used successfully to develop computational models to predict the BBB permeation (13).

Nonspecific brain tissue binding not only influences measures of drug exposure in the brain (7), but also impacts the rate of brain uptake (20). Mathematical modeling of this system showed that the time to reach brain equilibrium is determined by the product of PS and $f_{u,brain}$ (PS×$f_{u,brain}$). The intrinsic equilibrium half-life calculated based on PS×$f_{u,brain}$ was found to be significantly correlated with the experimentally measured distributional half-life (20).

CNS drug discovery and development have been relatively unsuccessful due to the complexity of the BBB and the difficulties associated with the assessment and prediction of CNS penetration (21). Understanding the parameters favorable for CNS penetration is critically important for CNS drug design and optimization, and ultimately for understanding and treating CNS diseases. In the present study, the BBB permeation of five compounds with CNS activity, and seven non-CNS-active drugs, representing a small compound set with substantial structural diversity covering various therapeutic categories and a wide range of physicochemical characteristics, was investigated with the *in situ* mouse brain perfusion technique. The effect of P-gp on net brain uptake, the relationship between the brain permeability coefficient (PS), the unbound drug fraction in mouse brain tissue ($f_{u,brain}$), PS×$f_{u,brain}$ and the calculated octanol-water partition coefficient at pH 7.4 ($clogD_{7.4}$) were examined. In addition, alfentanil and sufentanil are known to reach plasma-biophase equilibration in minutes (22), while fexofenadine is poorly- and slowly-permeable at the BBB (23,24). The time course of equilibration for these rapidly- and slowly-equilibrating compounds was determined, and the influence of PS and $f_{u,brain}$ on the rate of brain uptake of these compounds was compared.

MATERIALS AND METHODS

Adult CF-1 *mdr1a*(+/+) and *mdr1a*(−/−) mice (30–40 g, 6–8 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA, USA). All mice were maintained on a 12-h light/dark cycle with access to water and food *ad libitum*. All experimental procedures were performed under full anesthesia induced with ketamine/xylazine (100/10 mg/kg, i.p.), were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill, and were conducted in accordance with "Principles with Laboratory Animal Care" (NIH publication No. 85-23, revised in 1985).

^{3}H-cimetidine (25.0 Ci/mmol) and ^{14}C-diazepam (56.0 mCi/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK), ^{14}C-inulin (2.1 mCi/g) from American Radiolabeled Chemical Inc. (St. Louis, MO, USA), ^{3}H-inulin (180.0 mCi/g), ^{3}H-colchicine (80.4 Ci/mmol), ^{3}H-verapamil (74.2 Ci/mmol) and ^{3}H-phenytoin (53.1 mCi/mmol) from PerkinElmer (Waltham, MA, USA), ^{3}H-quinidine (20 Ci/mmol) from American Radiolabeled Chemicals Inc. (St. Louis, MO), and ^{3}H-valproic acid (55 Ci/mmol) from Moravek Biochemicals Inc. (Brea, CA, USA). Alfentanil, sufentanil, cimetidine, terfenadine, fexofenadine, loperamide, phenytoin and valproic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were commercially available and of reagent grade or better.

IN SITU MOUSE BRAIN PERFUSION

The details of the *in situ* mouse brain perfusion have been described elsewhere (18). Briefly, CF-1 *mdr1a*(+/+) and *mdr1a*(−/−) mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.). The perfusion buffer (Krebs-bicarbonate buffer, with 9 mM of D-glucose, pH 7.4) was oxygenated with 95% O_2 and 5% CO_2 and maintained at 37°C. ^{14}C-diazepam and ^{3}H- or ^{14}C-inulin were used as blood flow rate and vascular space markers, respectively. Test compounds (2 μM) with or without radiolabeled tracer (0.1–1.6 μCi/ml) were added to the perfusate. The right common carotid artery was cannulated with polyethylene tubing (0.30-mm inner diameter×0.70-mm outer diameter; Biotrol Diagnostic, Chennevières-les-Louvres, France) following ligation of the external carotid artery. The cardiac ventricles were severed immediately before perfusion at 2.5 ml/min for 60 s (all compounds except alfentanil, sufentanil and fexofenadine) via a syringe pump (Harvard Apparatus, Holliston, MA, USA). Alfentanil and sufentanil (2 μM) were perfused for 10 (alfentanil only), 20, 40 and 60 s, and fexofenadine for 60, 90, and 120 s in *mdr1a*(+/+) and *mdr1a*(−/−) mice, respectively. For additional groups of *mdr1a*(+/+) and *mdr1a*(−/−) mice, fexofenadine was perfused for a single time-point at 60 s, followed by an additional 15-s drug-free saline perfusion to wash out the remaining fexofenadine in the capillary space. All of the experiments were terminated by decapitation, and the right cerebral hemisphere was collected. Aliquots of perfusate were collected through the syringe and tubing for determination of perfusate concentrations. When radiotracers were used, brain samples were digested with 0.7 ml Solvable® (Packard, Boston, MA, USA) at 50°C overnight. Five ml UltimaGold® scintillation cocktail (PerkinElmer, Wellesley, MA, USA) was added and mixed by vortex. Total radioactivity (^{3}H and/or ^{14}C) was determined in a Packard Tri-carb TR 1900 liquid scintillation analyzer (Packard, Boston, MA, USA). For non-radioactive samples, the hemisphere was homogenized with two volumes of distilled water by brief probe sonication. The perfusate and brain samples were stored at −20°C until analysis by HPLC/MS/MS.

Parameters related to the *in situ* brain perfusion, i.e., the cerebral vascular volume (milliliter per 100 g brain), was calculated using the equation:

$$V_{VASC} = \frac{X_{inulin}}{C_{inulin}}.$$

At any single time point, the initial brain uptake clearance (Cl_{up}, milliliter per minute per 100 g brain) was calculated as:

$$Cl_{up} = \frac{X_{brain}/T}{C_{perf}},$$

and the apparent distributional volume (V_d, ml/100 g brain) was calculated as:

$$V_D = \frac{X_{brain}}{C_{perf}}$$

where the amount of substrate in brain, X_{brain}, was corrected for blood vessel contamination by subtraction of vascular volume or a final 15-s washout with drug-free saline. For compounds that were perfused for several different times, the V_d values were plotted against perfusion time, and Cl_{up} was calculated as the slope of the initial linear phase (14,18). Efflux ratio ($Cl_{up}^{(-/-)}/Cl_{up}^{(+/+)}$) was calculated as the ratio of Cl_{up} in P-gp-deficient to P-gp-competent mice. The permeability surface product (PS) values were calculated based on Crone-Renkin equation (15):

$$PS = -F_{Pf} \times 1n\left(1 - Cl_{up}/F_{pf}\right).$$

where F_{pf} is the perfusion flow rate measured using ^{14}C-diazepam as the flow rate marker.

EQUILIBRIUM DIALYSIS

Plasma and brain unbound fractions were determined in a 96-well equilibrium dialysis apparatus (HTDialysis, Gales Ferry, CT, USA) using a previously reported method (7). Briefly, fresh CF-1 mouse plasma and brain tissue were obtained the same day of the study. Spectra-Por 2 membranes obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA) were conditioned in HPLC-grade water for 15 min, followed by 30% ethanol for 15 min, and 100 mM sodium phosphate buffer (pH 7.4) for 15 min. Brain tissue was diluted threefold with 100 mM sodium phosphate buffer (pH 7.4) and homogenized with a sonic probe. The drug of interest was added to brain homogenate to achieve a final concentration of 3 and 1 μM, respectively; 150-μl aliquots (n=6) were loaded into the 96-well equilibrium dialysis apparatus and dialyzed against an equal volume of 100 mM sodium phosphate buffer (pH 7.4). The 96-well equilibrium dialysis apparatus was incubated for 4.5 h in a 155-rpm shaking incubator maintained at 37°C. For non-radioactive samples, after 4.5 h 10 μl of brain homogenate sample and 50 μl of buffer sample were removed from the apparatus and added directly to HPLC vials containing 100 μl of methanol with an appropriate internal standard. A 50-μl aliquot of control buffer was added to the brain homogenate sample, and either a 10-μl aliquot of control brain homogenate was added to the buffer samples to yield identical sample composition between buffer and non-buffer samples. The samples were vortex-mixed, centrifuged, and the supernatant was analyzed by HPLC/MS/MS. For radioactive samples, 5 ml UltimaGold® scintillation cocktail (PerkinElmer, Wellesley, MA, USA) was added and vortex-mixed. Total radioactivity (^{3}H and/or ^{14}C) was determined in a Packard Tri-carb TR 1900 liquid scintillation analyzer (Packard, Boston, MA, USA). The brain unbound fraction was calculated according to the equation:

$$\text{Undiluted } f_{u,brain} = \frac{1/D}{\left(\left(1/f_{u,measured}\right) - 1\right) + 1/D}$$

where D represents the fold dilution of brain tissue, and $f_{u,measured}$ was the ratio of concentrations determined from the buffer and brain homogenate samples.

HPLC/MS/MS ASSAY

Non-radioactive samples were analyzed by HPLC/MS/MS (API 4000 triple quadrupole with TurboIonSpray interface; Applied Biosystems/MDS Sciex, Concord, ON, Canada). A 25-μl aliquot of the cerebral hemisphere homogenate was transferred to an HPLC vial, protein was precipitated with 100 μl of methanol containing internal standard (10 ng/ml loperamide), and a 25-μl aliquot of DMSO was added. The sample was vortex-mixed and centrifuged. Standard solutions ranged from 0.5 to 5,000 nM and were prepared in a similar way, i.e., 25 μl of blank plasma or brain matrix, 100 μl of methanol containing internal standard, and 25 μl of serially-diluted standard solution were mixed and centrifuged. A 3-μl aliquot of supernatant was injected into autosampler (Leap, Carrboro, NC, USA). Test compounds and the internal standard were eluted from an Aquasil C18 column (2.1× 50 mm, d_p=5 μm; Thermo Electron Corporation, Waltham, MA, USA) using a mobile phase gradient (A, 0.1% formic acid in water; B, 0.1% formic acid in methanol; 0–0.70-min hold at 0% B, 0.70–3.12-min linear gradient to 90% B, 3.12–4.10 min hold at 90% B, 4.10–4.20-min linear gradient to 0% B, 4.20–4.90-min hold at 0% B; solvent delivery system (Shimadzu, at a flow rate of 0.75 ml/min; from 0.8 to 4 min, flow was directed to the mass spectrometer) and were detected in positive ion mode using multiple reaction monitoring: alfentanil: 417.3 → 268.3 m/z, fexofenadine: 502.4 → 466.4 m/z, ritonavir: 721.5 → 296.4 m/z, sufentanil: 387.2 → 238.4 m/z, terfenadine, 472.3 → 436.5 m/z, loperamide, 477.4 → 266.0 m/z. All analytes were quantified with standard curves (0.05–5,000 ng/ml) prepared in the appropriate matrix. The lower limit of detection was 0.5 ng/ml; inter- and intra-day RSDs were <15% for each of the analytes examined in this study.

STATISTICAL ANALYSIS

Data are reported as mean±SD for three mice per condition. One-way or two-way ANOVA, where appropriate, was used to determine the statistical significance of differences among two or more groups. The level of significance was corrected for multiple comparisons (e.g., Bonferroni test) or adjusted for unequal variance when necessary. In all cases, $p<0.05$ was considered to be statistically significant.

RESULTS

^{14}C- or ^{3}H-inulin was used as the cerebral capillary space marker (V_{vasc}) to measure BBB physical integrity. V_{vasc} was 1.69±0.10 and 1.68±0.11 ml/100 g in *mdr1a*(+/+) and *mdr1a*(−/−) mice, respectively (two-tailed Student's t-test, $p>0.05$). Functional perfusion rate (^{14}C-diazepam Cl_{up}) was 250±41 ml/min/100 g brain in both *mdr1a*(+/+) and *mdr1a*(−/−) mice.

The octanol–water partition coefficients in the unionized state or at pH 7.4 (clogP or $clogD_{7.4}$) for each compound were calculated using Marvin and calculator plug-in freeware (www.chemaxon.com, ChemAxon Kft, Budapest, Hungary). The calculated clogP, $clogD_{7.4}$, initial brain uptake clearance Cl_{up} in *mdr1a*(+/+) and *mdr1a*(−/−) mice, calculated efflux ratio ($Cl_{up}^{(-/-)}/Cl_{up}^{(+/+)}$), PS×$f_{u,brain}$ and the unbound

Table I. Calculated clogP, clogD$_{7.4}$, uptake clearance in *mdr1a*(+/+) and *mdr1a*(−/−) mice, efflux ratio ($Cl_{up}^{(-/-)}/Cl_{up}^{(+/+)}$) and brain unbound fraction ($f_{u,brain}$) for selected compounds

Name	clogP	clogD7.4	Cl_{up}(+/+)	Cl_{up}(−/−)	Efflux ratio	PS× $f_{u,brain}$	$f_{u,brain}$ (reference)
Alfentanil	3.55	3.20	194±38	229±46	1.4	196	0.32 (8)
Colchicine	1.08	1.08	0.9±0.1	1.9±0.1	2.1	0.05	0.024 (8)
Cimetidine	0.24	0.12	0.7±0.2	1.1±0.4	1.5	0.6	0.53 (8)
Diazepam	3.01	3.01	250±41	250±41	1.0	–	0.05 (25)
Fexofenadine	5.68	0.92	0.3±0.1	1.3±0.4	4.4	0.1	0.077 (8)
Phenytoin	2.28	2.27	33.4±1.2	34.7±9.9	1.0	3.1	0.082
Quinidine	2.32	1.46	3.4±0.4	54.1±12.2	13.6	1.6	0.037 (8)
Ritonavir	5.04	5.04	2.3±0.7	8.0±2.7	3.5	0.1	0.0106 (8)
Sufentanil	3.17	1.69	34.0±6.6	29.5±9.0	0.9	1.1	0.034 (8)
Terfenadine	6.45	4.82	174±52	202±85	1.2	0.5	0.0011
Valproate	2.61	0.37	24.3±0.7	18.1±4.5	0.7	18.8	1.0
Verapamil	4.30	2.56	31.5±3.0	137±23	5.0	7.7	0.033 (8)

fractions in mouse brain for all compounds determined using equilibrium dialysis or obtained from the literature (8,25) are summarized in Table I. Based on comparisons of Cl_{up} in *mdr1a*(+/+) and *mdr1a*(−/−) mice, P-gp-mediated efflux decreased the initial rate of brain uptake by up to 14-fold for quinidine in *mdr1a*(+/+) mice. Fig. 1 demonstrates a sigmoidal relationship between logPS in *mdr1a*(−/−) mice and clogD$_{7.4}$ in the range of 0–5. Valproic acid and ritonavir were excluded from the regression because the uptake of valproic acid at the BBB is known to be facilitated by a medium-chain fatty acid transporter (26), and ritonavir is extensively bound and sequestrated in the brain capillary endothelium (27). The log of the unbound fraction in mouse brain for these 12 compounds is inversely correlated to clogD$_{7.4}$ (Fig. 2).

The relationship between PS×$f_{u,brain}$ and clogD$_{7.4}$ is shown in Fig. 3. The PS×$f_{u,brain}$ was calculated based on the regression in Figs. 1 and 2. This relationship suggests that the rate of brain-to-blood equilibration is not a simple function of lipophilicity, permeability or brain unbound fraction. In addition, the PS×$f_{u,brain}$ varied widely among the compounds studied, with the highest value of 196 for alfentanil and lowest value of 0.05 for colchicine.

The time course of sufentanil and alfentanil brain uptake is presented in Fig. 4. The apparent distributional volume (V_d) for sufentanil was linear over all of the perfusion times evaluated in both *mdr1a*(+/+) and *mdr1a*(−/−) mice (Fig. 4A). Alfentanil brain uptake was linear over 20 s in both *mdr1a*(+/+) and *mdr1a*(−/−) mice (Fig. 4C). The calculated uptake clearance (Cl_{up}) at each time point is shown in Fig. 4B, D. Cl_{up} values for sufentanil were constant over the perfusion period and were comparable in *mdr1a*(+/+) and *mdr1a*(−/−) mice (two-way ANOVA, $p>0.05$). Cl_{up} values for alfentanil were constant for the first 20 s and then decreased significantly at 60 s from ~229 ml/min/100 g to ~102 ml/min/100 g. Cl_{up} in *mdr1a*(−/−) mice was about 1.4-fold higher than that in *mdr1a*(+/+) mice (two-way ANOVA, $p<0.05$).

Fexofenadine brain uptake was linear over 120 s in *mdr1a*(+/+) and *mdr1a*(−/−) mice (Fig. 5A). Initial uptake clearance of fexofenadine, determined with *versus* without the final washout step, did not differ in either *mdr1a*(+/+) or *mdr1a*(−/−) mice. Brain uptake was 4.4- and 4.6-fold higher in *mdr1a*(−/−) mice than in *mdr1a*(+/+) mice in the absence and presence of final washout step, respectively (Fig. 5B, two-way ANOVA, $p<0.05$).

DISCUSSION

Previous studies have suggested that lipophilicity is one of the most important determinants of brain penetration (28,29), and that logPS in rat brain is related linearly with

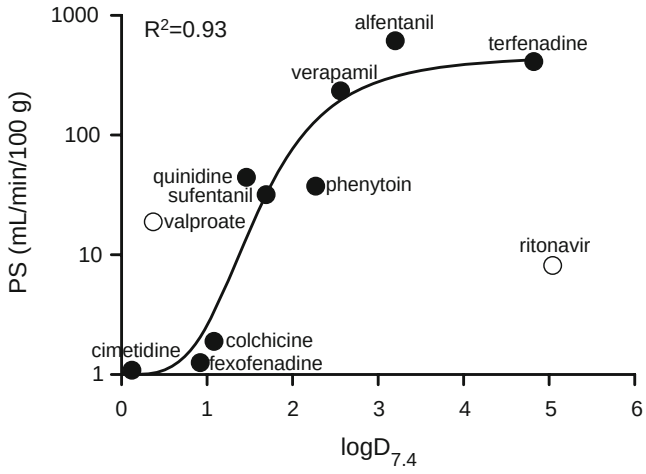

Fig. 1. Relationship between PS and clogD$_{7.4}$.

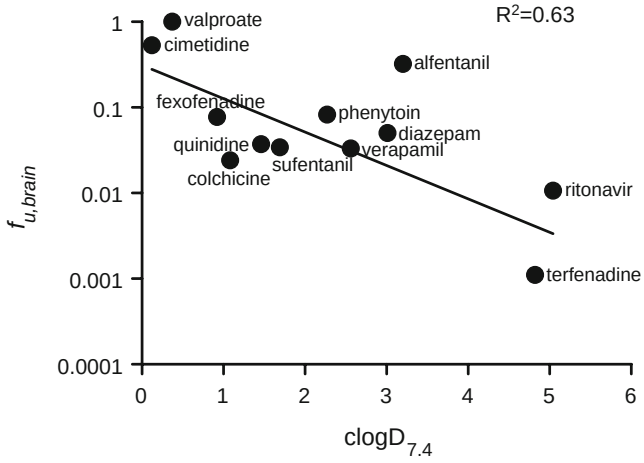

Fig. 2. Relationship between brain unbound fraction $f_{u,brain}$ and clogD$_{7.4}$.

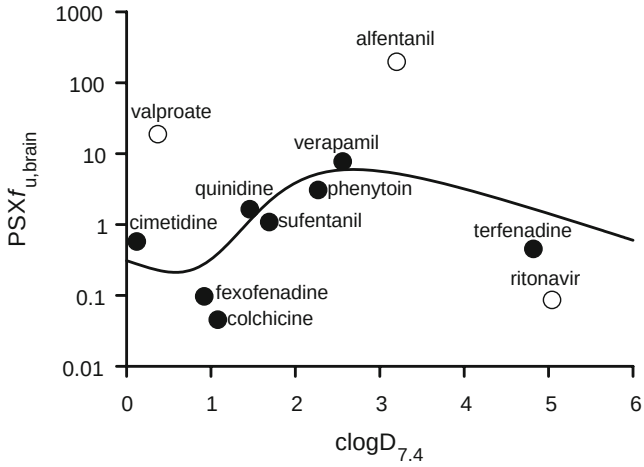

Fig. 3. Simulated relationship between PS×$f_{u,brain}$ and clogD$_{7.4}$. The line was constructed based on the regressions of PS on clogD$_{7.4}$ (Fig. 1) and $f_{u,brain}$ on clogD$_{7.4}$ (Fig. 2).

cLogP (for octanol:water) over six orders of magnitude (15,29). However, the compounds studied previously are, for the most part, non-CNS-therapeutic agents with limited presentation to brain tissue. In the present study, BBB penetration of 12 clinically-used drugs in several therapeutic classes, covering a clogD$_{7.4}$ range of 0–5, was investigated. PS ranged nearly 600-fold, and $f_{u,brain}$ ranged almost 1,000-fold, among these compounds. Thus, the selected compound set contained sufficient physicochemical diversity to allow dissection of the contributors to net brain uptake.

The relationship between the logPS in *mdr1a*(−/−) mice and clogD$_{7.4}$ was generally sigmoidal (Fig. 1). The logPS for valproic acid fell above this relationship, consistent with the observation that its brain uptake is facilitated by a medium-chain fatty acid transporter (26). Ritonavir brain uptake is slow, although it is highly lipophilic and not ionized at pH 7.4. This slow uptake is explained by the molecular weight of 720.9, and polar surface area of 202 Å^2, whereas a molecular weight less than 450 and polar surface area less than 90 Å^2 are typically considered to be favorable for brain penetration (30). In addition, capillary depletion techniques have been used to demonstrate that ritonavir is extensively bound to or sequestrated inside the brain capillary endothelium after crossing the luminal membrane (27). The regression of logPS *versus* clogP showed a similar relationship (data not shown), with fexofenadine as an outlier. Fexofenadine has a high lipophilicity (clogP=5.68) when unionized. However, it is both positively- (due to the tertiary amine group) and negatively- (due to its carboxylic acid group) charged at pH 7.4 (clogD$_{7.4}$= 0.92), which explains the very low brain penetration of fexofenadine at the BBB, consistent with lipophilicity and ionization state as important factors for brain penetration (28). LogPS is limited by permeability for more hydrophilic compounds (cimetidine, fexofenadine and colchicine) and increases with clogD$_{7.4}$ when clogD$_{7.4}$ is in the range of 1–3 (quinidine, sufentanil, quinidine and verapamil). When clogD$_{7.4}$ exceeds a value of 3 (alfentanil, diazepam and

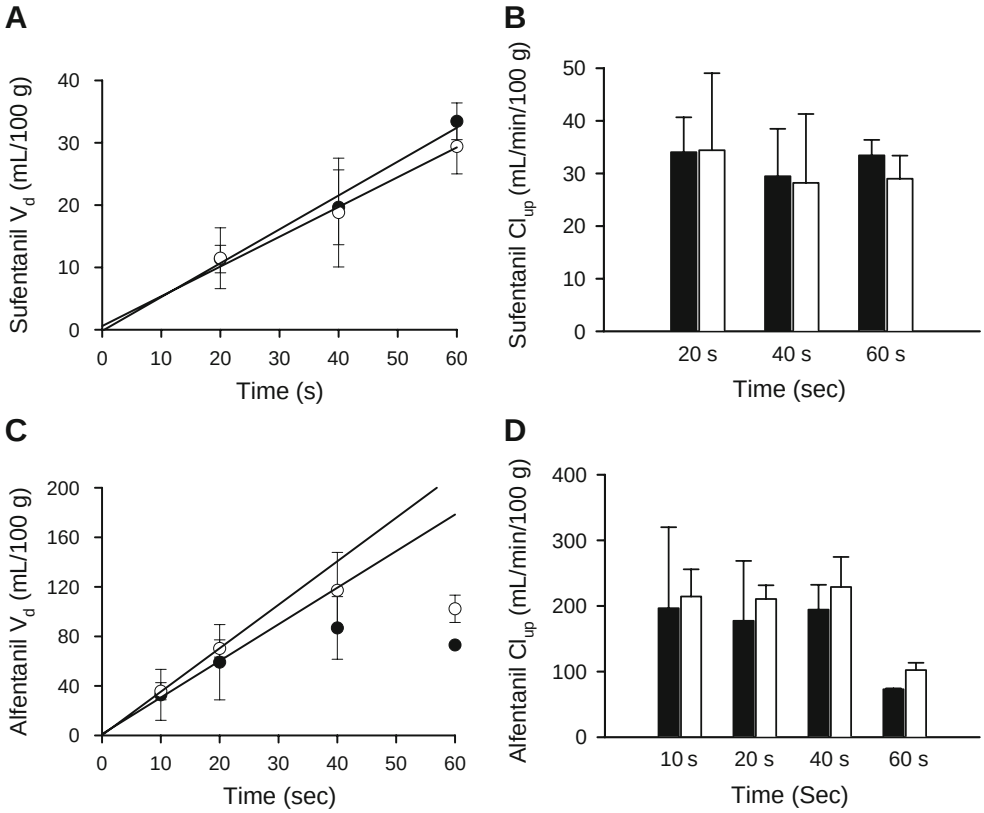

Fig. 4. The time course of apparent brain distributional volume of (**A**) sufentanil and (**C**) alfentanil, and the initial brain uptake clearance of (**B**) sufentanil and (**D**) alfentanil, in *mdr1a*(+/+) (*solid symbol*) and *mdr1a*(−/−) (*open symbol*) mice following brain perfusion at 2.5 ml/min (n=3).

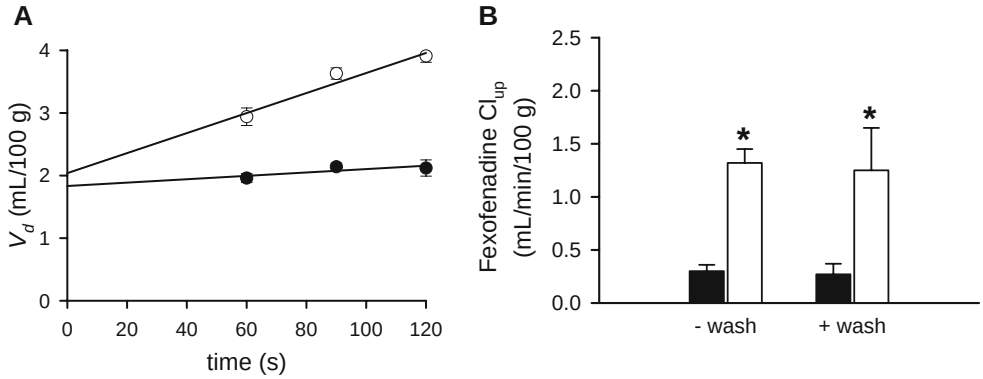

Fig. 5. **A** The apparent distributional volume at 60-, 90-, and 120-s of perfusion for fexofenadine in *mdr1a*(+/+) (*solid circle*) and *mdr1a*(−/−) (*open circle*) mice; and (**B**), initial brain uptake clearance of fexofenadine in *mdr1a*(+/+) (*solid bar*) and *mdr1a*(−/−) (*open bar*) mice in the absence (−wash) or presence (+wash) of additional 15-s drug-free saline washout following brain perfusion at 2.5 ml/min. Fexofenadine brain uptake increased approximately fivefold in *mdr1a*(−/−) mice than in *mdr1a*(+/+) mice (*, $p<0.05$).

terfenadine), BBB permeability does not increase further and becomes limited by capillary perfusion rate.

Interestingly, in the dataset generated for drugs active against CNS targets (CNS^+) *in vivo* drugs (i.e., diazepam, phenytoin, alfentanil, sufentanil, and valproate), the mean $clogD_{7.4}$ was 2.11, and the mean Cl_{up} values were 93.0 and 101.0 in *mdr1a*(+/+) and *mdr1a*(−/−) mice, respectively. For non-CNS therapeutic (CNS^-) agents (i.e., cimetidine, colchicine, fexofenadine, quinidine, ritonavir, and verapamil), the mean $clogD_{7.4}$ was 1.86, and the mean Cl_{up} values were 6.2 and 34.1 in *mdr1a*(+/+) and *mdr1a*(−/−) mice, respectively. Terfenadine was not included in the CNS^- group because it is known to be a CNS penetrant (23,31) and it is metabolized rapidly systemically and in the brain (32). This comparison clearly shows that CNS^+ drugs tend to have higher Cl_{up} with minimal or no P-gp-mediated efflux. In contrast, CNS^- drugs have lower Cl_{up} in the absence of P-gp, with Cl_{up} decreased further by P-gp-mediated efflux at the BBB. For these P-gp substrates, P-gp-mediated efflux decreased brain uptake by 1.5-, 2.1-, 3.5-, 4.4-, 5.0-, and 13.6-fold for cimetidine, colchicine, ritonavir, fexofenadine, verapamil, and quinidine, respectively. A similar observation, i.e., that the passive permeability and P-gp-mediated efflux could differentiate the CNS and non-CNS drugs, also was made using *in vitro* cell-based models (33).

No statistical differences were detected between *mdr1a*(+/+) and *mdr1a*(−/−) mice for sufentanil, and alfentanil brain uptake was only 1.4-fold higher in *mdr1a*(−/−) mice than in *mdr1a*(+/+) mice. Sufentanil and alfentanil were reported previously as weak P-gp substrates, with *in vivo* P-gp efflux ratios of 3.0 and 2.8, respectively (34,35). Previous work has also demonstrated that the P-gp efflux ratio measured *in situ* correlates well with that determined *in vivo*, but with lower apparent P-gp effects at the BBB using the *in situ* brain perfusion technique (19). This result has also been supported by mathematical analysis recently: a smaller ER_B (*in situ* P-gp efflux ratio) compared to ERα (*in vivo* P-gp efflux ratio) is expected for the same experimental system (i.e., same substrate and same animal species) (36). For other P-gp substrates in the present compound set, P-gp-mediated efflux decreased the brain uptake by 1.5- to 13.6-fold, suggesting that the *in situ* brain perfusion technique is a sensitive method to test the efflux transporter kinetics at the BBB.

In the absence of active transport, the rate of brain equilibration is a function of both PS and unbound brain fraction ($f_{u,brain}$), and is directly proportional to the PS×$f_{u,brain}$ product (5,20). Lipophilic compounds tend to cross the BBB (high PS; Fig. 1) and bind to brain tissue more extensively (low $f_{u,brain}$; Fig. 2). The apparent inverse relationship between PS and $f_{u,brain}$ leads to the common assumption that the rate of equilibration across the BBB might be similar for most drugs (37). However, *in vivo* experiments and mathematical modeling indicate that the time to brain equilibrium varied widely among compounds (20). A mathematical simulation based on the sigmoidal relationship between PS and $clogD_{7.4}$ and the linear relationship between $f_{u,brain}$ and $clogD_{7.4}$ showed the trend of the rate to brain equilibrium (PS×$f_{u,brain}$) increases with the lipophilicity of compounds up to $clogD_{7.4}$ of 3, and then decreases after the compounds become too lipophilic (Fig. 3). The compounds in the $clogD_{7.4}$ range of 2–4 evidenced the most rapid brain penetration. Alfentanil reached an equilibrium condition about 200-fold faster than sufentanil, and about 2,000-fold faster than fexofenadine and ritonavir. This difference is consistent with the clinically-observed onset of alfentanil anaesthetic effect, which is more rapid than that observed for sufentanil (22,34). In contrast, ritonavir brain uptake has been shown previously to be quite slow, failing to reach equilibrium within 30 min during perfusion in guinea pigs (27).

The *in situ* brain perfusion technique measures the initial rate of brain uptake during a time period when there is no significant back flux from the brain to the perfusate. Care should be taken when a single-time-point method is used with this technique. In many cases, a single time-point at 30- or 60-s can provide an accurate estimate of Cl_{up} (17,38). However, a curvilinear relationship between net brain uptake and time has been demonstrated for some compounds such as iodoantipyrine (16) that equilibrate rapidly (less than 60 s) across the BBB. In the present study, a similar result was observed for alfentanil, which shares some physicochemical characteristics with iodoantipyrine (high PS and high $f_{u,brain}$). Alfentanil achieved equilibrium in 20 s in both *mdr1a*(+/+) and

mdr1a(−/−) mice. By 60 s, V_d had reached a plateau due to significant back flux from the brain to the perfusate. The calculated Cl_{up} values therefore decreased about 50–60% at 60 s compared to other time points. Only Cl_{up} calculated based on unidirectional flux from perfusate to brain will reflect the actual rate of alfentanil brain uptake (Fig. 4C and D). Sufentanil Cl_{up} was ~8-fold lower than that for alfentanil, corresponding to a lower $clogD_{7.4}$ value. Sufentanil binds to brain tissue ~10-fold more extensively than alfentanil, limiting the back-flux of sufentanil from the brain to the perfusate during perfusion (essentially creating a sink condition with respect to unbound substrate in brain tissue available for back-diffusion). Sufentanil brain uptake therefore is linear over the entire 60 s perfusion, and Cl_{up} is time-independent.

Fexofenadine brain uptake was unidirectional over 120 s (Fig. 5). Fexofenadine is a very low-permeability compound and has high nonspecific binding in brain tissue. Combined with a 15-s drug-free saline washout at the end of the perfusion, the single-time-point method for calculating Cl_{up} yielded results similar to those obtained from the three time-point linear phase slope method. Because the variability in vascular volume might appreciably affect the Cl_{up} calculation, particularly for compounds with poor BBB permeability, the washout step increased the accuracy of measurement of the brain permeability when using a single-time-point method (14,39).

The *in situ* brain perfusion technique is a useful method to determine the BBB permeability. However, caution needs to be taken when using a single-time-point method. The time point should be in the initial linear (unidirectional) phase of brain uptake and a final washout step might increase the accuracy of measuring brain permeability for poorly-permeable compounds. The kinetics of BBB permeation is a balance between the intrinsic permeability and nonspecific brain tissue binding. Other variables, i.e., physicochemical properties, and P-gp-mediated efflux also determine the rate and extent of brain penetration. Highly lipophilic compounds tend to have high permeability and high brain tissue binding, which causes the $PS \times f_{u,brain}$ product to vary across a wide range. This variability is apparent when comparing a compound such as alfentanil, which reaches equilibrium very quickly, to a compound such as fexofenadine, which reaches equilibrium very slowly.

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