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Regional differences in capillary density, perfusion rate, and P-glycoprotein activity: A quantitative analysis of regional drug exposure in the brain

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ABSTRACT

The *in situ* brain perfusion technique was used to assess the impact of local capillary density, blood flow rate and P-gp-mediated efflux activity on regional drug exposure for the P-gp substrates colchicine, quinidine, verapamil, and loperamide, the perfusion flow rate marker diazepam, and the vascular volume marker inulin, in *mdr1a*(+/+) and *mdr1a*(−/−) mice. Regional perfusion flow rate varied 7.5-fold, and capillary density (based on vascular volume) varied 3.7-fold, across the 13 brain regions examined. The rate of regional flow, as well as P-gp-mediated colchicine efflux activity, was directly proportional to local capillary density. A decrease in perfusion rate attenuated verapamil brain uptake and had significant effect on P-gp-mediated efflux activity for this substrate in brain regions with lower capillary density. Regional brain uptake and calculated log *D* at pH 7.4 (clog $D_{7.4}$) were well-related in P-gp-deficient mice, indicating that in the absence of P-gp-mediated efflux, physicochemical properties of the compound (i.e., lipophilicity) serve as the primary determinant of regional brain uptake. Loperamide regional brain uptake and P-gp effect during a 60-s brain perfusion or at 30 min after subcutaneous administration were significantly correlated with local capillary density. The highest P-gp-mediated efflux activity was consistently observed in cerebral cortex and midbrain regions for loperamide following short-term brain perfusion and at all time points following subcutaneous administration. These results in intact animal emphasize that the regionality of substrate exposure in brain as measured by the *in situ* brain perfusion technique is actually biologically relevant.

1. Introduction

The blood–brain barrier (BBB) is the primary interface between the brain and peripheral circulation. The BBB is composed of brain capillary endothelial cells, which are characterized by highly developed tight junctions and a paucity of fenestrae and pinocytotic vesicles [1]. P-gp, a member of the ATP-binding cassette (ABC) superfamily of transport proteins, is a 170-kDa membrane protein encoded by multidrug resistance gene (*ABCB1* in human, *Abcb1a* and *Abcb1b* in rodents). P-gp is expressed at the luminal side of the brain capillary endothelium and mediates the flux of various endogenous compounds and xenobiotics in the brain-to-blood direction, thereby serving as an important barrier to the entry of P-gp substrates into brain [2–5].

The mammalian brain is a highly specialized organ from both the structural and functional perspective [6–8]. Most of the targets for treatment of brain disorders, e.g., neurodegenative disorders (dopamine receptors), epilepsy (e.g., GABA receptors) and neuropathic pain (μ-opioid receptors) are not homogeneously distributed in the brain [8]. The rate of local cerebral blood flow under physiologic conditions varies about 18-fold among brain areas in the rat [9]. In addition, clinical practice has shown variations in sensitivity to the therapeutic effect or CNS side effects of drugs associated with different brain regions [10–12]. Previous studies in our laboratory demonstrated that P-glycoprotein (P-gp)-mediated efflux at the BBB influences the distribution of ^{3}H-verapamil within the brain following intranasal administration to P-gp-competent and -deficient mice [13]. Pharmacokinetic modeling of these data revealed that the apparent P-gp effect on verapamil exposure was negligible in some regions, and produced nearly a 100-fold attenuation of exposure in others [13]. The regional cerebral vascular volume and blood flow rate, as well as regional P-gp-mediated efflux activity at the BBB differ between brain regions; these differences may result in regional variations in drug exposure.

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; MTT, mean transit time; P-gp, P-glycoprotein.

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Thus knowledge of the regional drug exposure is of particular interest with regard to targeted treatment for cerebral diseases.

In situ brain perfusion was developed to evaluate BBB permeability in mice [14,15]. This technique allows examination of single-pass permeability of molecules at the BBB without systemic contamination, with complete control of the composition and flow of perfusion buffer, and without other pharmacokinetic events (e.g., hepatic metabolism) confounding the experimental results. In the present study, the *in situ* brain perfusion technique was utilized to study the impact of local cerebral perfusion flow rate, vascular volume, and P-gp efflux activity on regional drug exposure. ^{14}C-diazepam and ^{3}H-inulin, selected as markers of functional perfusion flow rate and vascular volume, respectively, were perfused simultaneously, followed by microdissection of the brain to assess regional flow rate and vascular space. Subsequent experiments were directed towards examining regional brain exposure of the P-gp substrates colchicine, quinidine, loperamide and verapamil during perfusion in P-gp-competent and -deficient mice, allowing assessment of regional P-gp-mediated efflux activity in P-gp-expressing animals. The *in situ* perfusion experiments were complemented by an *in vivo* evaluation of P-gp-mediated efflux activity over 12 h following subcutaneous administration of loperamide to assess the potential time-dependency of efflux activity estimates.

2. Materials and methods

2.1. Animals and reagents

Adult CF-1 [*mdr1a*(+/+) and *mdr1a*(−/−)] mice (6–8 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA). 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.). All procedures 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).

^{14}C-diazepam (56.0 mCi/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). ^{14}C-inulin (2.1 mCi/g) and ^{3}H-quinidine (20 Ci/mmol) were obtained from American Radiolabeled Chemical Inc. (St. Louis, MO). ^{3}H-inulin (180.0 mCi/g), ^{3}H-colchicine (80.4 Ci/mmol) and ^{3}H-verapamil (74.2 Ci/mmol) were purchased from PerkinElmer (Waltham, MA). Loperamide, fexofenadine, D-glucose, and Krebs-bicarbonate buffer were purchased from Sigma–Aldrich (St. Louis, MO). All of the other chemicals were commercially available and of reagent grade.

2.2. In situ mouse brain perfusion procedure

In situ mouse brain perfusions were conducted as described in Ref. [14]. Briefly, P-gp-competent [*mdr1a*(+/+)] and P-gp-deficient [*mdr1a*(−/−)] mice ($n = 3$) 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. The right common carotid artery was cannulated with PE-10 tubing (Braintree Scientific Inc., Braintree, MA) following ligation of the external carotid artery. The cardiac ventricles were severed immediately before starting perfusion for 60 s at 2.5 mL/min via a syringe pump (Harvard Apparatus, Holliston, MA). The experiment was terminated by decapitation immediately following perfusion. The right cerebral hemisphere was removed carefully and placed on cold saline-soaked filter paper and dissected on ice. Approximately 150 mg of perfusate was collected through the syringe and tubing for determination of the concentration.

2.3. Brain perfusion experimental design

In order to determine the regional cerebral capillary volume and functional perfusion flow rate, ^{14}C-inulin (at 1.6 μCi/mL) and ^{14}C-diazepam at (0.4 μCi/mL) as vascular volume and blood flow rate markers, respectively, were perfused simultaneously for 60 s in *mdr1a*(+/+) and *mdr1a*(−/−) mice ($n = 3$). In separate studies, ^{3}H-colchicine (at 1.6 μCi/mL), ^{3}H-verapamil (at 0.4 μCi/mL), ^{3}H-quinidine (at 1 μCi/mL), or unlabeled loperamide (at 2 μM) was perfused for 60 s, followed by 15 s of cold saline washout to remove remaining perfusate in the vasculature.

Among the P-gp substrates evaluated, verapamil is the most permeable at the BBB, with a brain uptake clearance in most brain regions approaching perfusion flow rate in *mdr1a*(−/−) mice. Verapamil brain uptake is expected to be sensitive to changes in perfusion flow rate. For this reason, verapamil was selected as a model compound to study the impact of perfusion flow on brain uptake and P-gp effect. Additional groups of *mdr1a*(+/+) and *mdr1a*(−/−) mice ($n = 3$) were perfused with ^{3}H-verapamil at 1 mL/min to investigate the potential influence of changes in flow rate on apparent regional P-gp efflux activity.

2.4. Loperamide subcutaneous administration

Loperamide was administered to *mdr1a*(+/+) mice (30 mg/kg, s.c.) and *mdr1a*(−/−) mice (1 mg/kg, s.c.), and animals ($n = 3$ per group) were sacrificed by decapitation at 0.5, 1, 2, 4, 6 and 12 h post-dose. The doses were selected to produce approximately equivalent brain tissue concentrations in the two mouse strains. Trunk blood was collected and centrifuged at 3000 rpm for 5 min. The plasma was transferred to a fresh microcentrifuge tube and stored at −20 °C until analysis by HPLC/MS/MS. The brain was carefully removed from the skull, cleaned of meninges and choroid plexus, dissected on ice, and stored at −20 °C pending analysis.

2.5. Mouse brain microdissection

To ensure the reproducibility of the dissection technique, bregma coordinates and anatomic boundaries defining each region were established based on the Franklin and Paxinos mouse brain atlas [7]. The microdissection procedure was conducted according to photographs and step-by-step instructions downloaded from a publicly accessible relational database (www.barlow-lockhartbrainmap-nimhgrant.org). For studies involving radiolabeled substrate, the perfused brain hemisphere was dissected into 13 regions (olfactory bulb, striatum, hippocampus, frontal cortex, parietal cortex, occipital cortex, superior and inferior colliculi, hypothalamus, thalamus, midbrain, pons, medulla, and cerebellum). Larger brain regions were required for accurate loperamide analysis. Accordingly, the perfused hemisphere for loperamide *in situ* studies, or brain tissue after *in vivo* administration of loperamide, was dissected into 7 regions [olfactory bulb, striatum, hippocampus, cortex, midbrain (including midbrain, thalamus and hypothalamus), brainstem (including pons and medulla) and cerebellum].

2.6. Radioactive sample quantitation

Samples containing radiolabeled substrate were digested with 0.3 mL of Solvable® (Packard, Boston, MA) at 50 °C for 4 h. UltimaGold® scintillation cocktail (PerkinElmer, Wellesley, MA) was added (5 mL) 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).

2.7. Loperamide sample preparation and HPLC/MS/MS assay

Distilled water was added to brain samples (2:1, v/w) and homogenized with brief probe sonication. Plasma (for *in vivo* studies), brain homogenate, and perfusate (for *in situ* studies) 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 brain hemisphere homogenate or plasma was transferred to an HPLC vial, and protein was precipitated with 100 μL of methanol containing internal standard (10 ng/mL fexofenadine), followed by a 25-μL aliquot of DMSO. The sample was vortex-mixed and centrifuged. Standard solutions, ranging from 0.5 to 5000 nM were prepared similarly, 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 was mixed and centrifuged. Supernatant (3 μL) was injected by autosampler (Leap, Carrboro, NC). Loperamide and the internal standard, fexofenadine, were eluted from an Aquasil C18 column (2.1 mm × 50 mm, d_p = 5 μm; Thermo Electron Corporation, Waltham, MA) 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); flow rate = 0.75 mL/min; 0.8–4 min directed to mass spectrometer) and were detected in positive ion mode using multiple reaction monitoring: fexofenadine: 502.4 → 466.4 *m/z*, loperamide, 477.4 → 266.0 *m/z*. The lower limit of detection was 0.5 ng/mL; inter- and intraday RSDs were <15%.

2.8. Parameter calculation

The regional vasculature volume was calculated using the equation: $V_{vasc} = X_{inulin}/C_{inulin}$, where X_{inulin} and C_{inulin} are the amount of ^{3}H-inulin radioactivity in the brain region and concentration in the perfusate, respectively. Regional ^{14}C-diazepam brain uptake clearance (Cl_{up}), which is flow rate-limited and therefore serves as a surrogate marker of functional perfusate flow, co-perfused with ^{3}H-inulin, was calculated using the equation: $Cl_{up} = (X_{brain} - X_{inulin})/(C_{perf} \times T)$ for each brain region [14], where X_{brain} is the amount of ^{14}C-diazepam radioactivity in the brain region and T is the perfusion time. For each of the test compounds, regional Cl_{up} was calculated as $Cl_{up} = X_{brain}/(C_{perf} \times T)$, where C_{perf} is the test substrate concentration in the perfusate. No correction was made for substrate mass contained in residual because the vasculature substrate content was cleared via the 15-s cold saline washout at the end of the perfusion period. P-gp efflux ratio was calculated as the ratio of Cl_{up} in each brain region in P-gp-deficient to P-gp-competent mice [$Cl_{up}(-/-)/Cl_{up}(+/+)$].

2.9. Statistical analysis

Data are reported as mean ± SD for 3 mice per condition. One-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.

3. Results

3.1. Regional cerebral vascular volume and flow rate

During *in situ* mouse brain perfusion, the regional vascular volume and perfusion flow rate in *mdr1a*(+/+) and *mdr1a*(−/−) mice were comparable (one-way ANOVA, $p > 0.05$ for each brain region), so the data were pooled for each structure, as summarized in Table 1 (n = 6, mean ± SD). The regional vascular volume ranged from 0.62 mL/100 g in pons and medulla to 2.21 mL/100 g in colliculi. The perfusion flow rate ranged from 38.1 mL/min/100 g in medulla to 285 mL/min/100 g in colliculi. An orthogonal linear least-squares regression analysis between the local perfusion flow rate and vascular volume, with a correlation coefficient of 0.955, is illustrated in Fig. 1. The mean transit time (MTT), calculated as the ratio of vascular volume to perfusion flow rate in each brain structure, was 0.4–0.6 s among most of the regions. A notable exception was medulla, where the mean transit time was 1.0 s (Table 1).

Table 1
Regional cerebral vascular volume (V_{vasc}), perfusion flow rate and mean transit time (MTT) determined by *in situ* brain perfusion at 2.5 mL/min for 60 s in *mdr1a*(+/+) and *mdr1a*(−/−) mice (n = 3 per strain).

Region	V_{vasc} (mL/100 g)	Flow rate (mL/min/100 g)	MTT (s)
Olfactory bulb	1.1 ± 0.3	155 ± 52	0.4
Striatum	1.5 ± 0.5	180 ± 20	0.5
Hippocampus	1.6 ± 0.3	200 ± 4	0.5
Frontal cortex	1.5 ± 0.3	167 ± 21	0.5
Parietal cortex	1.7 ± 0.3	223 ± 11	0.5
Occipital cortex	1.7 ± 0.4	215 ± 24	0.5
Colliculi	2.2 ± 0.5	285 ± 49	0.5
Hypothalamus	1.5 ± 0.4	214 ± 21	0.4
Thalamus	1.9 ± 0.4	238 ± 39	0.5
Midbrain	1.6 ± 0.3	198 ± 20	0.5
Pons	0.6 ± 0.1	65 ± 45	0.6
Medulla	0.6 ± 0.2	38 ± 32	1.0
Cerebellum	1.1 ± 0.2	122 ± 40	0.6

There is no statistical difference between these two strains (one-way ANOVA, $p > 0.05$ for each brain region). The data in *mdr1a*(+/+) and *mdr1a*(−/−) mice are pooled and presented below (n = 6). The V_{vasc} and flow rate cross-different brain regions are statistically different (one-way ANOVA, $p < 0.05$).

3.2. Regional P-gp activity in mouse brain

The regionality of brain uptake of ^{3}H-colchicine, ^{3}H-quinidine and ^{3}H-verapamil in *mdr1a*(+/+) and *mdr1a*(−/−) mice is shown in Fig. 2. Colchicine, quinidine and verapamil brain uptake were significantly higher in most of the brain regions studied in *mdr1a*(−/−) mice compared to wild-type animals, consistent with P-gp-associated impairment of brain uptake. In addition, a stronger relationship between Cl_{up} and regional vascular volume was observed in *mdr1a*(−/−) vs. *mdr1a*(+/+) mice. The uptake clearance in *mdr1a*(−/−) mice [$Cl_{up}(-/-)$] for colchicine, quinidine, and verapamil correlated well with the local capillary density, with

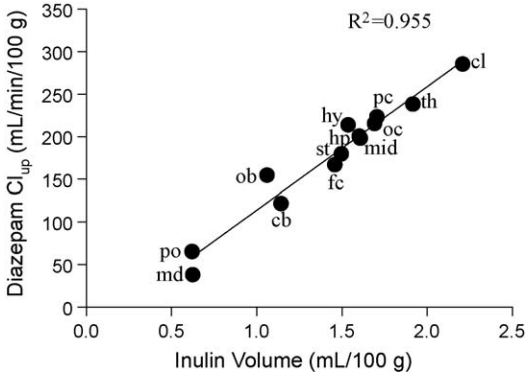

Fig. 1. Orthoganol linear least-squares regression (R^2 = 0.955) between regional flow rate (^{14}C-diazepam Cl_{up}, mL/min/100 g brain tissue) and regional cerebral vascular volume (^{3}H-inulin volume, mL/100 g brain tissue). ob, olfactory bulb; st, striatum; hp, hippocampus; fc, frontal cortex; pc, parietal cortex; oc, occipital cortex; cl, colliculi; hy, hypothalamus; th, thalamus; mid, midbrain; po, pons; md, medulla; cb, cerebellum.

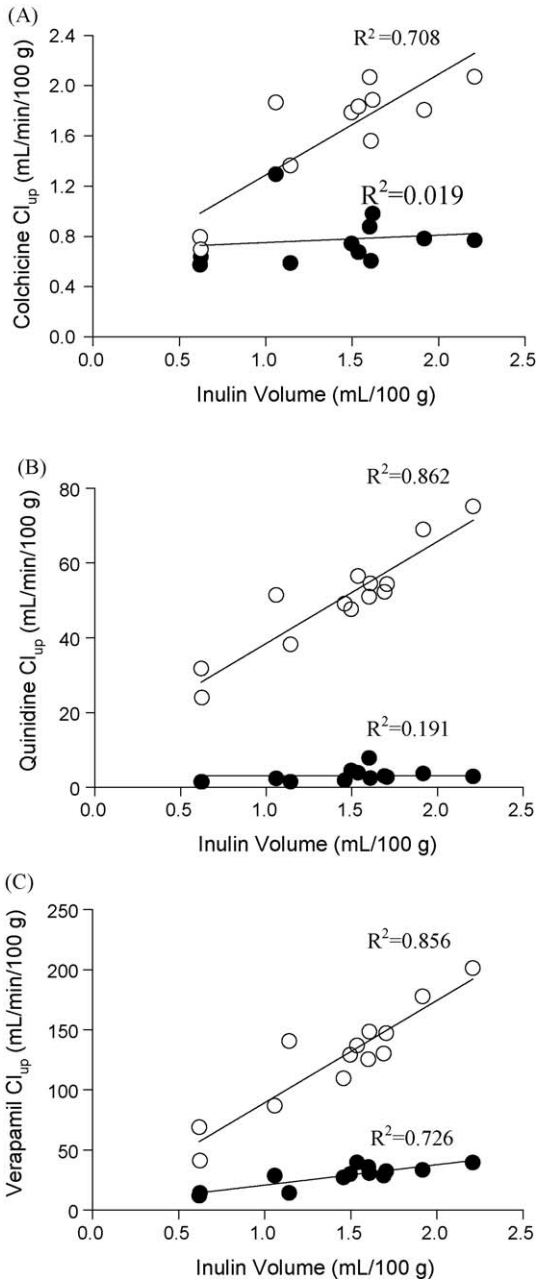

Fig. 2. The regional Cl_{up} (mL/min/100 g brain tissue) in *mdr1a*(+/+) (solid symbol) and *mdr1a*(−/−) (open symbol) mice vs. regional cerebral vascular volume (inulin volume, mL/100 g brain tissue) for (A) colchicine, (B) quinidine, and (C) verapamil. The solid lines are orthogonal linear regression lines.

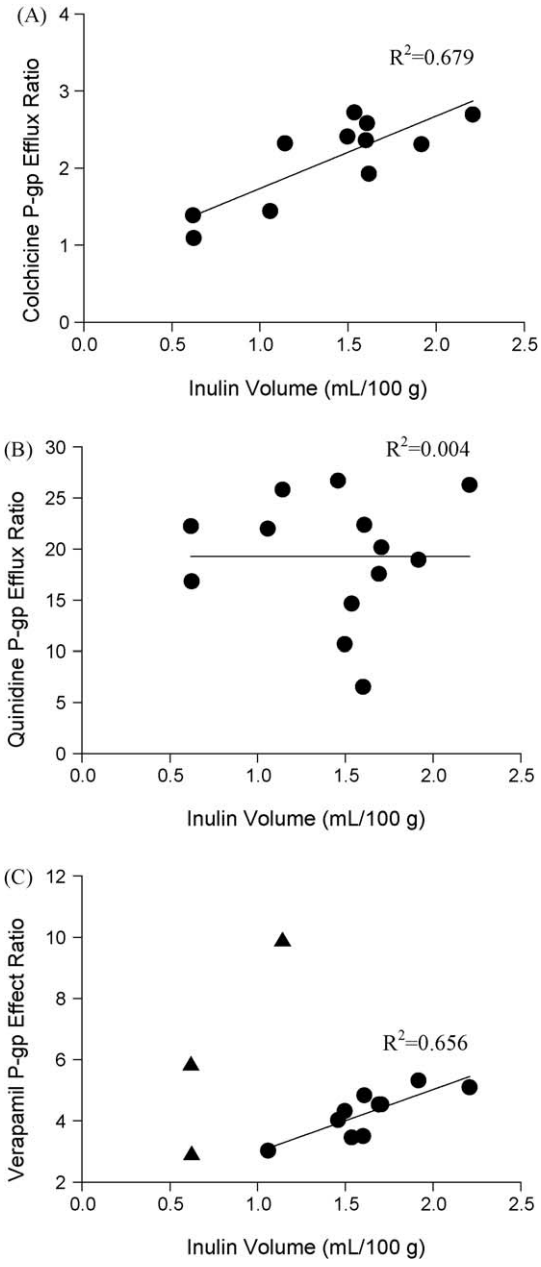

Fig. 3. The observed relationship of regional P-gp efflux ratio and cerebral vascular volume (inulin volume, mL/100 g brain tissue) determined by *in situ* brain perfusion at 2.5 mL/min for 60 s in *mdr1a*(+/+) and *mdr1a*(−/−) mice for (A) colchicine, (B) quinidine, and (C) verapamil. The orthogonal regression was carried out for colchicine and quinidine using all 13 brain regions examined, and for verapamil using only brain regions (circles) excluding pons, medulla and cerebellum (triangles).

R^2 values of 0.708, 0.862, and 0.856, respectively, suggesting that the vascular volume in the regional BBB is very important for brain uptake (Fig. 2). However, there was practically no such relationship in wild-type mice for colchicine (R^2 = 0.019) and quinidine (R^2 = 0.191).

The actual regional P-gp efflux ratios were calculated as the ratios of Cl_{up} values in *mdr1a*(−/−) mice to wild-type mice for each compound, and plotted against regional cerebral vascular volume (Fig. 3). Approximately 67.9% of the variance in colchicine regional P-gp efflux ratio can be explained by the local cerebral vascular volume (Fig. 3A). However, there is essentially no relationship between regional P-gp activity and vascular volume for quinidine or verapamil (Fig. 3B and C, less than 1% of the variance in P-gp efflux ratio can be explained by the local vascular volume). The regression excluding pons, medulla and cerebellum (triangles in Fig. 3C), which have the lowest perfusion flow rate, for verapamil evidences a reasonable correlation between regional P-gp efflux ratio and vascular volume (R^2 = 0.656).

Three-dimensional illustrations of the brain distribution and P-gp effect for colchicine, quinidine, and verapamil in each brain region vs. clog $D_{7.4}$ (the calculated logarithm of the octanol water partition coefficient at pH 7.4) are provided in Fig. 4. The value of clog $D_{7.4}$ for each compound (1.08 for colchicine, 1.46 for quinidine, and 2.56 for verapamil) was estimated using Marvin and calculator plugin freeware (www.chemaxon.com, ChemAxon

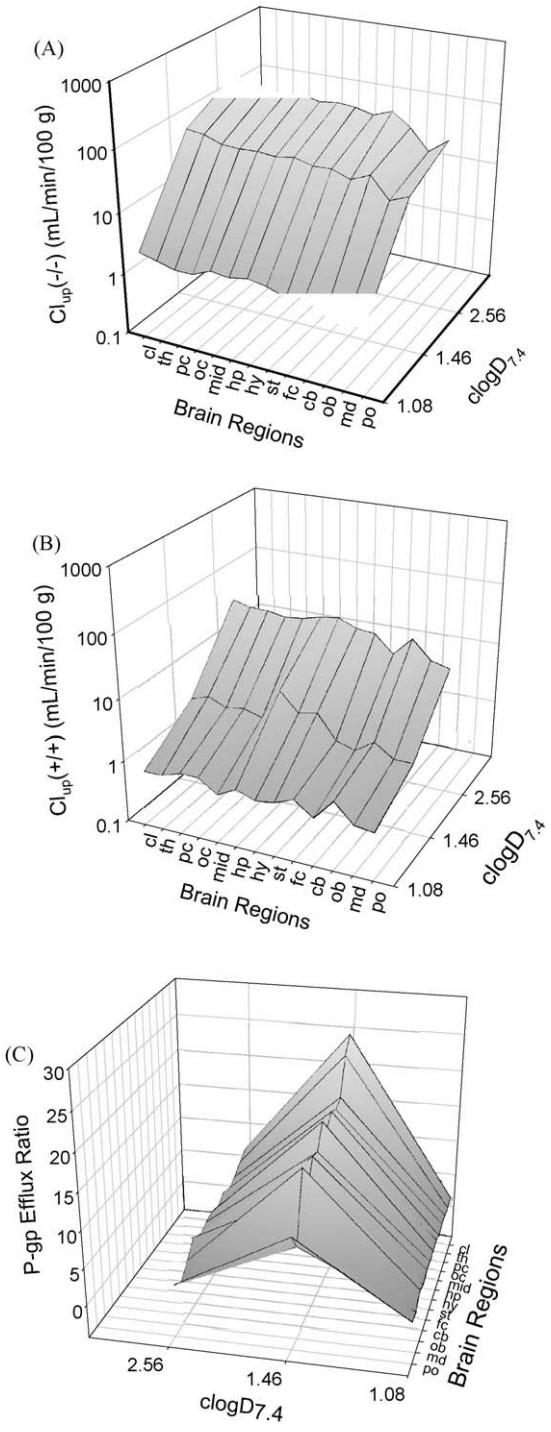

Fig. 4. The relationship of brain uptake of colchicine, quinidine and verapamil and diazepam (A) in *mdr1a*(−/−) mice [Cl_{up}(−/−)], (B) *mdr1a*(+/+) mice [Cl_{up}(+/+)] and (C) P-gp efflux ratio and the logarithm of octanol water patition coefficient at pH 7.4 (clog $D_{7.4}$) in each brain region. The brain structures are arranged in the order of the magnitude of capillary volume.

Kft, Budapest, Hungary). The initial rate of brain uptake in *mdr1a*(−/−) mice [Cl_{up}(−/−), mL/min/100 g] was a function of lipophilicity (clog $D_{7.4}$). Cl_{up}(−/−) increased with increasing clog $D_{7.4}$ until Cl_{up}(−/−) approached the regional perfusion flow rate (for verapamil) (Fig. 4A). In the presence of P-gp, the initial rate of colchicine, quinidine and verapamil brain uptake was mitigated by P-gp-mediated efflux (Fig. 4B). The P-gp efflux ratio was not a function of lipophilicity (Fig. 4C).

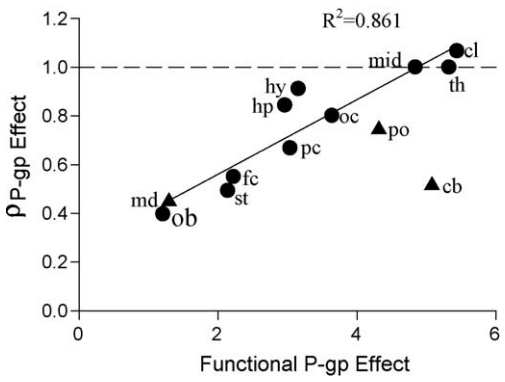

Fig. 5. The influence of perfusion flow rate perturbation in the regional verapamil P-gp efflux ratio. $\rho_{\text{P-gp effect}}$ is defined as the ratio of P-gp efflux ratios at 1 mL/min to that at 2.5 mL/min; the abscissa is the functional P-gp effect expressed as the regional P-gp efflux ratio at perfusion flow rate of 2.5 mL/min. The orthogonal least-squares linear regression (R^2 = 0.861) was conducted with brain regions (circles) excluding pons, medulla and cerebellum (triangles).

3.3. Effect of flow rate perturbation on brain distribution

As expected, lower brain uptake was observed for verapamil at 1 mL/min compared to 2.5 mL/min (data not shown). The resulting change in P-gp efflux ratio ($\rho_{\text{P-gp effect}}$, defined as the ratio of P-gp efflux ratios at 1 mL/min to that at standard flow rate) for verapamil is plotted against the functional P-gp effect, expressed as P-gp efflux ratio at the standard flow rate of 2.5 mL/min (Fig. 5). Pons, medulla and cerebellum were again excluded from the regression for the same reason mentioned above. A linear relationship (R^2 = 0.861) was observed between the flow-related change in P-gp effect ($\rho_{\text{P-gp effect}}$) and local functional P-gp activity expressed as the regional efflux ratio at the standard flow rate. In the brain regions that showed significant P-gp activity (such as thalamus and colliculi), changes in perfusion flow had little influence on P-gp activity. In regions with limited P-gp activity, a change in perfusion flow rate had a more substantial impact on P-gp activity (Fig. 5).

3.4. Regional P-gp transport of loperamide

Fig. 6 illustrates the regional P-gp effect following subcutaneous administration of loperamide, with the P-gp efflux ratio at 0.5, 1, 2, 4, 6, and 12 h plotted for each region. The regional P-gp efflux ratio was statistically (R^2 = 0.775) related to the regional vascular volume only at the earliest time point (0.5 h). P-gp efflux ratios were always the highest in cerebral cortex and the midbrain regions at all time points (data not shown). Regional P-gp-mediated efflux of loperamide correlated well with regional vascular volume during *in situ* brain perfusion for 60 s in mice (R^2 = 0.639; data not shown).

4. Discussion

The current study demonstrated that regional drug exposure in the brain is a function of many drug- and region-specific factors, including physicochemical characteristics (lipophilicity), drug concentration in blood/perfusate, local cerebral perfusion flow rate, brain capillary volume, regional capillary transit time, and the flux of drugs across the BBB that may include both passive and active (transport-mediated) components. Generally, brain structures such as colliculi, thalamus and parietal cortex have the highest vascular volume and functional flow rate, while the brainstem (pons and medulla) and cerebellum have the lowest vascular volume and functional flow rate (Fig. 1). Colchicine Cl_{up}

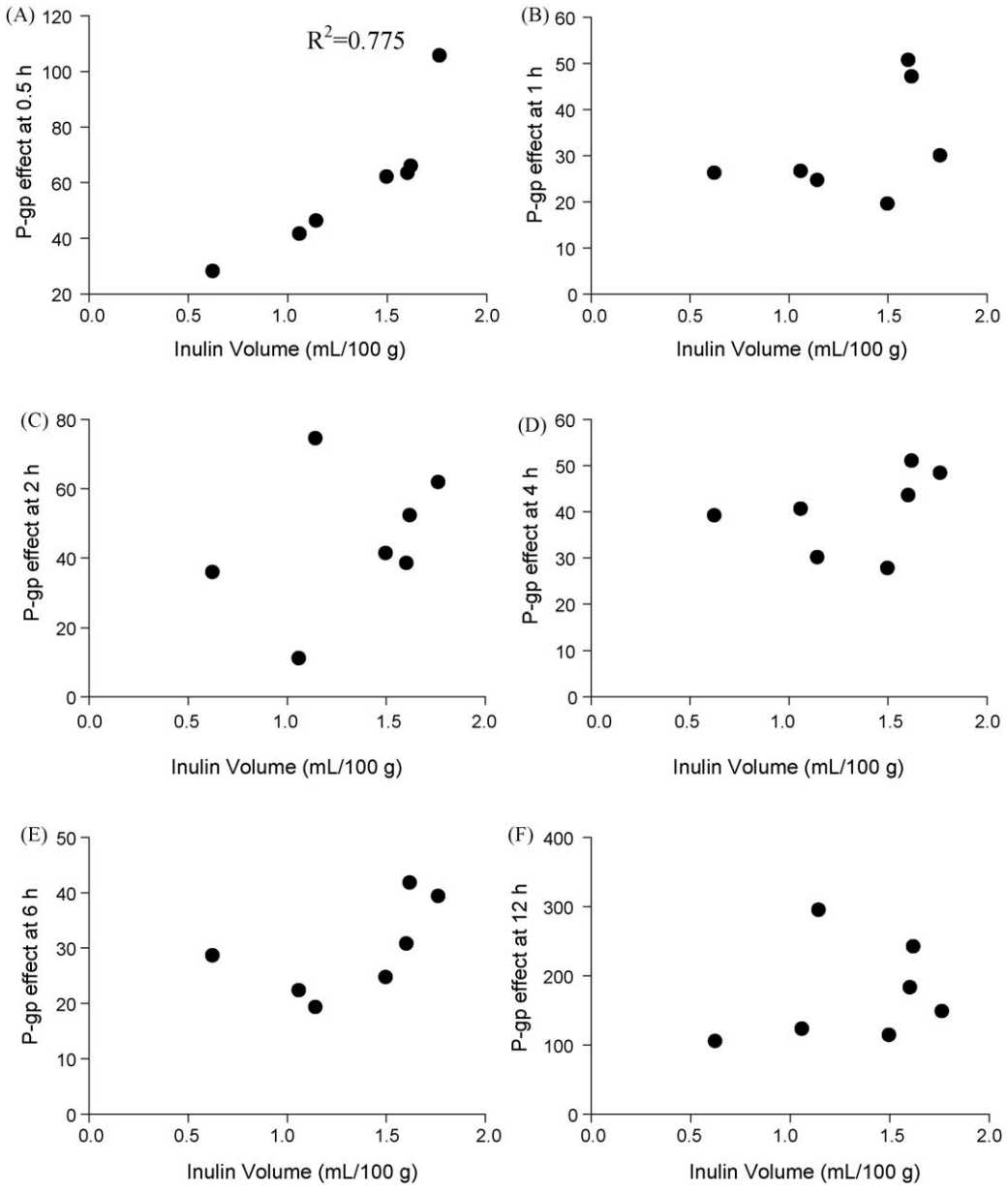

Fig. 6. The relationship of regional P-gp efflux ratio of loperamide and regional cerebral vascular volume at (A) 0.5 h, (B) 1 h, (C) 2 h, (D) 4 h, (E) 6 h, and (F) 12 h following subcutaneous administration of 30 and 1 mg/kg of loperamide in *mdr1a*(+/+) and *mdr1a*(−/−) mice, respectively.

ranged from 0.57 to 2.07 mL/min/100 g, with the lowest exposure in pons and medulla, and the highest exposure in hippocampus, olfactory bulb and colliculi, areas that have been reported to be selectively vulnerable to colchicine toxicity [10,11]. In addition, the exposures of colchicine in olfactory bulb lie significantly above the regression lines in both P-gp-competent and -deficient mice, suggesting possible specific binding in this area (Fig. 2A). The mean transit times determined in the current study are similar to the values (0.3–0.6 s) reported for various rat brain areas, as determined by an autoradiography technique following intravenous administration of ^{14}C-iodoantipyrine to intact animals [9].

The P-gp efflux ratio determined using *in situ* brain perfusion has been shown to be directly proportional to P-gp efflux activity, using a mathematical three-compartment model in which P-gp efflux activity was directly proportional to functional protein expression level [16]. Fig. 3A illustrates that the colchicine P-gp efflux ratio correlated with capillary density. Gjedde and Diemer [17] demonstrated that in brain regions with higher blood flow, there is a correspondingly larger capillary vascular volume, i.e., larger capillary density and total surface area. Thus cerebral capillary diameters are considered standard across the brain and do not vary between different brain structures. The present results with colchicine suggest that the expression level of P-gp, a membrane-bound protein expressed in the brain capillary endothelium, is related to local capillary density. Thus, it is likely that the amount of P-gp per unit capillary surface area is the same in all regions and functions similarly. This is also consistent with the observations that P-gp is expressed in endothelial cells, with little or no expression in other brain cells (i.e., astrocytes and neurons) under normal conditions [18,19]. Among the three P-gp substrates, the P-gp efflux activities in cerebellum were significantly higher than that predicted by the orthogonal linear regression line comparing to other brain regions. This might be explained by a greater-than-proportional (-to-capillary density) expression of P-gp in cerebellum than in other brain regions, as indicated in rat brain [20]. Verapamil P-gp efflux ratio correlated

well with capillary volume when excluding the pons, medulla and cerebellum (R^2 = 0.656, Fig. 3C), where greater P-gp efflux ratios were consistently observed. This is related to increased interaction of substrate with P-gp due to increased residence time as a result of diminished perfusion rate under the current perfusion condition. Regional P-gp efflux ratios following intranasal administration of verapamil also showed that the most substantial regional P-gp effects were in parietal cortex and the thalamas [13], corresponding to the areas that have the highest capillary density and, by analogy, P-gp expression level. Surprisingly, there was no such correlation between P-gp effect and capillary density for quinidine. Since quinidine has proved to be a good P-gp substrate in the body of literature as well as the current studies [5], a speculation is that there might be more than one P-gp binding sites for quinidine (unpublished data). Further investigations are warranted to understand such kinetics.

The molecular mechanisms of P-gp-mediated efflux, and even the substrate interaction site(s) of P-gp, are still unknown. Access to the binding site that resides inside the plasma membrane can be the rate-limiting step for less lipophilic compounds, and transport efficiency might be influenced by residence time at or near the binding site [21,22]. The relationship between regional P-gp efflux ratio and clog $D_{7.4}$ (Fig. 4C) suggested that lipophilicity also influences the interaction between P-gp and substrates. For less lipophilic compounds such as colchicine, substrate presentation to the binding site is a limiting factor; while lipophilic compounds such as verapamil, substrate presentation to P-gp binding site is only limited by perfusion flow rate, but the short residence time limits substrate interaction with P-gp. For compounds with intermediate lipophilicity such as quinidine, adequate substrate presentation to the binding site(s) as well as residence time in the binding site(s) maximize the P-gp-mediated efflux activity. Other variables such as the number of binding sites, or affinity towards the binding sites and transport capacity influence the nature of substrate interaction with P-gp.

A decrease in perfusion rate leads to decreased rate of substrate presentation to P-gp binding site(s), as well as increased residence time to interact with P-gp. The balance of these two countereffect will determine the magnitude of P-gp activity. Verapamil P-gp efflux ratios decreased, to differing extents based on brain region, at the lower perfusion rate. Changes in flow appeared to have a more substantial impact in areas that had a weaker P-gp activity, but little or no effect in areas with stronger P-gp activity (i.e., areas that have larger capillary beds) (Fig. 5). The change in P-gp efflux ratio in response to a decrease in perfusion flow was found to be correlated with the basal (i.e., at 2.5 mL/min) regional P-gp effect or regional cerebral volumes, when excluding pons, medulla and cerebellum regions for reasons described above. How the change of flow rate influences other P-gp substrates (e.g., with different lipophilicities) interaction with P-gp needs more investigation.

The *in situ* brain perfusion is an oversimplified system that only approximates intact systems. In the *in vivo* situation, factors such as plasma protein binding and non-specific binding in brain tissue, hemodynamic parameters such as pCO_2, pO_2, *p*H, and electrolyte concentrations influence the vascular volume, blood flow rate, and BBB permeability. In intact animals, at the earliest time point (0.5 h) following subcutaneous administration, loperamide P-gp efflux ratio correlated with local capillary density, a result anticipated based upon the preceding *in situ* perfusion experiments. At all later time points, the apparent P-gp effect lost regionality, most likely due to inter-area diffusion and exchange, including distribution across the regional BBB. This explanation is kinetically sound because it is expected that the short-term perfusion in mouse brain represents *in vivo* situation prior to attainment of distribution equilibrium (i.e., the initial phase of brain uptake, when the brain behaves as an approximate sink for substrate uptake). In contrast, later time points during *in vivo* distribution (i.e., when no net flux occurs between blood and brain tissue) brain-to-blood partitioning is determined primarily by relative protein binding in blood and brain tissue, not BBB permeability in isolation. A previous study resulted in a similar observation: short-term (up to 30 s) intravenous infusion of ^{3}H-nicotine might be used to estimate local cerebral blood flow rate (initial rate of brain uptake determined by flow rate) and long-term (up to 4 min) infusion could be used to estimate density of cells and nerve endings, which was determined by the tissue binding of nicotine [23]. These results demonstrate that the *in situ* brain perfusion technique has realistic biological relevance in studying BBB drug transport.

In summary, the current study using an *in situ* mouse brain perfusion technique demonstrated that the perfusion flow rate and functional P-gp activity appeared to correlate well with the regional vascular volume. Thus lipophic molecules tend to distribute to the highly perfused/vascularized areas when P-gp-mediated efflux mechanism is missing. However, P-gp substrates will be subject to more tense efflux in these regions. The regionality of drug exposure in the brain might have pharmacologic consequences when the target receptor evidences regional expression.

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