Effect of *mdr1a* P-Glycoprotein Gene Disruption, Gender, and Substrate Concentration on Brain Uptake of Selected Compounds

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Purpose. This study assessed the influence of *mdr1a* P-glycoprotein (P-gp) gene disruption, gender and concentration on initial brain uptake clearance (Cl_{up}) of morphine, quinidine and verapamil.

Methods. Cl_{up} of radiolabeled substrates was determined in P-gp-competent and deficient [*mdr1a*(–/–)] mice by *in situ* brain perfusion. Brain:plasma distribution of substrates after i.v. administration was determined in both strains.

Results. Genetic disruption of *mdr1a* P-gp resulted in 1.3-, 6.6- and 14-fold increases in Cl_{up} for morphine, verapamil and quinidine, respectively. With the exception of small differences for verapamil, gender did not affect Cl_{up}. Saturable transport of verapamil and quinidine was observed only in P-gp-competent mice, with apparent IC_{50}values for efflux of 8.6 ± 2.3 μM and 36 ± 2 μM, respectively. Verapamil Cl_{up} was ~50% higher in *mdr1a*(+/–) *vs. mdr1a*(+/+) mice; no such difference was observed for quinidine. In P-gp-competent mice, uptake of verapamil and quinidine was unaffected by organic vehicles. Plasma decreased VER Cl_{up} to a greater extent in the presence of P-gp. The influence of P-gp *in situ* was lower than, but correlated with, the effect *in vivo*.

Conclusions. P-gp decreases Cl_{up} of morphine, verapamil and quinidine *in situ* with little or no influence of gender, but this effect cannot fully account for the effects of P-gp *in vivo*. P-gp is the only saturable transport mechanism for verapamil and quinidine at the murine blood-brain barrier. The influence of protein binding on Cl_{up} may be enhanced by P-gp-mediated efflux.

KEY WORDS: P-glycoprotein; *mdr1a*(–/–) mice; morphine; verapamil; quinidine; blood-brain barrier; plasma protein binding.

INTRODUCTION

The efflux transporter P-glycoprotein (P-gp) is an integral component of the blood-brain barrier (BBB), where it serves a protective function (1). Accumulation of substrates in the brain of transgenic and mutant mice lacking *mdr1a* P-gp is increased significantly as compared to wild-type animals, resulting in enhanced sensitivity to the pharmacologic and toxic effects of several centrally-active compounds (1,2). In humans, the *MDR1* gene encodes P-gp capable of transporting a wide variety of drugs and endogenous substances, whereas the *mdr1a* and *mdr1b* isoforms are expressed in rodents. Only *mdr1a* P-gp is expressed at the luminal surface of capillary endothelial cells of the murine BBB (1).

In situ brain perfusion techniques are particularly powerful for mechanistic transport studies across the intact BBB (3). Complete control over perfusion conditions, such as flow and perfusate composition, eliminates the confounding factors of systemic disposition, and makes the technique suitable for determination of carrier-mediated transport parameters. For instance, brain perfusion applied to various rodent species has been used to characterize the saturable uptake of amino acids, endogenous peptides and glucose (3,4). On the other hand, only a limited number of studies have examined efflux processes at the BBB with *in situ* brain perfusion (5–8).

Recently, *in vivo* experiments utilizing transgenic mice and/or chemical inhibition of transport have shown that morphine, quinidine and verapamil interact with P-gp in the BBB (9–12). The influence of P-gp on morphine uptake appears to be modest compared to that for quinidine and verapamil. Thus, it is possible to construct a limited compound set, consisting of these three compounds, that displays a range of P-gp-mediated influence on brain uptake.

A very limited number of reports have established or suggested gender differences in BBB transport. For instance, estradiol administered to ovariectomized female rats was shown to increase the expression of glucose transporter (GLUT1) in the BBB (13). Interestingly, the expression of P-gp in the secretory epithelium of the uterus is regulated by steroid hormones (14). P-gp expression in the BBB also may be under gender-specific hormonal influence.

We recently developed an *in situ* brain perfusion model to exploit the availability of transgenic or mutant murine models such as the *mdr1a*(–/–) mice (4). A limited number of P-gp substrates has been examined with this model to date (15). The purpose of the present study was to assess the influence of *mdr1a* P-gp gene disruption and gender on initial brain uptake of selected substrates, and to determine transport parameters for P-gp-mediated efflux at the BBB. The effect of vehicles and/or plasma on the BBB transport of verapamil and quinidine also was investigated to further characterize the *in situ* brain perfusion model.

METHODS

Materials

Probe substrates were obtained from the following sources: [^{14}C]-inulin (2.21 mCi/g) and [^{3}H]-(±)-verapamil (85 Ci/mmol) [NEN Life Science Products, Boston, MA]; [^{3}H]-morphine (80 Ci/mmol) and [^{3}H]-quinidine (20 Ci/mmol) [American Radiolabeled Chemicals, St. Louis, MO]; quinidine sulfate, (±)-verapamil hydrochloride and (±)-methoxyverapamil hydrochloride [Sigma-Aldrich, St. Louis, MO]; morphine sulfate [BDH, Toronto, ON]; naloxone hydrochloride [Research Biochemicals Inc., Natick, MA]. Dexverapamil HCl [R-(+)-verapamil, LU 33925] was a gift from Knoll AG (Ludwigshafen, Germany). All other reagents used in this study were of the highest grade available from commercial sources.

Animals

Adult CF-1 mice [*mdr1a*(+/+) and *mdr1a*(–/–), 30-40 g, 6–8 weeks of age] were purchased from Charles River Labo-

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ratories (Wilmington, MA) and maintained in a breeding colony in the School of Pharmacy, The University of North Carolina at Chapel Hill. Male and female mice were housed separately (groups of 4–5) in wire-mesh cages in a temperature- and humidity-controlled room with a 12-h dark/12-h light cycle, and had free access to food and water. The experimental protocols were approved by the institutional animal care and use committees of The University of North Carolina at Chapel Hill and AstraZeneca R&D Montréal; all procedures were conducted according to the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, D.C., 1996).

In Situ Brain Perfusion

The details of the *in situ* mouse brain perfusion have been described elsewhere (4). Briefly, mice were anesthetized with ketamine/xylazine (140/8 mg/kg i.p.) ~15 min following administration of atropine (0.05 mg/kg s.c.). The right common carotid artery was catheterized following ligation of the external branch, and the brain was perfused (20–120 sec) with Krebs-bicarbonate buffer via a syringe pump (2.5 mL · min^{-1}, pH 7.4 with 95% O_2 and 5% CO_2, 37°C). This flow rate yields an estimated brain perfusion of 255 mL · min^{-1} · 100 g^{-1} tissue in the right hemisphere (4). The cardiac ventricles were severed immediately before the start of perfusion. Radiolabeled substrates (≤0.3 μCi/mL; corresponding to 15 nM or less of tritium labels, and ~ 27 μM [^{14}C]-inulin) were added to the perfusate to obtain ≥1000 dpm per tissue sample. Aqueous solutions of unlabeled substrates were diluted 20-fold in perfusion buffer to the desired concentrations (1, 5, 15, 30, 100 and 300 μM dexverapamil; 2, 20, 40 and 200 μM quinidine). Vehicles [ethanol, dimethylsulfoxide (DMSO) or 4:1 PEG 400:ethanol] were added to the perfusate at a concentration of 2% v/v. The perfusion was terminated by decapitation. The brain was removed from the skull and dissected on ice to isolate the right hemisphere. Samples were collected and weighed in tared 8-ml glass scintillation vials. Perfusate (~100 mg) was obtained from the tip of the catheter by activation of the pump. The right hemisphere (~150 mg) was digested with 0.7 mL Solvable (Packard, Meriden, CT) at 50°C. Samples were mixed with 5 mL Ultima Gold (Packard). Total ^{14}C and ^{3}H were determined simultaneously in a Packard 1600TR liquid scintillation analyzer.

In Vivo Determination of Brain-to-Plasma Distribution Ratios

Morphine, verapamil and quinidine were dissolved in saline and administered to mice by tail vein injection (4 mL · kg^{-1}) at a dose of 2 μmol · kg^{-1} (morphine and quinidine) or 1 μmol · kg^{-1} (verapamil). Verapamil was used at a lower dose to avoid cardiac toxicity. The animals were sacrificed by decapitation at 30 min. Trunk blood (~ 0.4–0.8 mL) was collected in 1.5-mL polypropylene tubes containing 20 U of heparin and centrifuged for 15 min at 6000 rpm (4°C). The brain was removed from the skull and dissected on ice to isolate the two hemispheres. Samples were frozen at –80°C until analysis by HPLC-MS. Brain tissue concentration was corrected for vascular contamination (2.5 mL · 100g^{-1}) (4).

HPLC-MS Analysis

Non-radioactive samples were analyzed by HPLC-MS on an 1100 Series Liquid Chromatograph/Mass Selective Detector (LC/MSD; Agilent Technologies, Kirkland, QC) with an atmospheric pressure ionization-electrospray (API-ES) chamber. Mass detection of morphine (m/z 286.2), verapamil (m/z 455.4) and quinidine (m/z 325.1) was performed in positive single ion monitoring mode. Methoxyverapamil (m/z 485.5) served as the internal standard for verapamil and quinidine, and naloxone (m/z 328.1) was used for morphine.

Internal standard (50 μL; 125 nM in 1% acetic acid) was added to plasma samples (100 μL) and proteins were precipitated with two volumes of ice-cold acidified acetonitrile (0.1% acetic acid). A brain hemisphere was weighed in a tared 5-mL polypropylene tube and homogenized with 400 μL internal standard solution, followed by ultrasonic cell disruption. Proteins were precipitated with 1.0 mL ice-cold acidified acetonitrile. Plasma and brain suspensions were mixed by vortex (45 sec) and centrifuged (14000 rpm, 5 min). Supernatant (300 μL) was transferred to a clean polypropylene tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of 20% (quinidine) or 40% (verapamil) acetonitrile in 0.04% formic acid, or 0.04% formic acid (morphine).

For verapamil and quinidine, aliquots (30 μL) were injected on a reversed-phase 75 mm × 4.6 mm Luna column (3 μm C-18; Phenomenex, Torrance, CA) at 40°C . The mobile phase (1 mL/min) consisted of a mixture of 0.04% formic acid (solvent A) and acetonitrile (solvent B) as follows: 100% A (0–2 min), linear gradient 0–75% B (2–10 min), 75% B (10–12 min) and equilibration (4 min) with 100% A. For morphine, aliquots (30 μL) were injected on a reverse-phase 150 mm × 4.6 mm YMC column (3 μm C-18; Chromatographic Sciences Company, Montréal, QC) at 40°C. The mobile phase (0.7 mL/min) consisted of a mixture of 0.04% formic acid (solvent A), methanol (solvent B) and acetonitrile (solvent C) as follows: linear gradient 0–50% B and 0–1.5% C (0 to 10 min), and equilibration (3.5 min) with 100% A. Calibration curves were constructed with plasma and brain collected from naïve mice under the same conditions as used in the experimental animals, to which known amounts of analytes were added. Typically, the calibration curves were linear from 10 to 1000 nM in plasma, and 3 to 300 pmol per brain hemisphere. The limit of quantitation was defined as a signal-to-noise ratio ≥4.

Calculation of Apparent Brain Distributional Volume and Initial Uptake Clearance

In all perfusion experiments, [^{14}C]-inulin was coperfused with the tritium-labeled substrates to estimate vascular volume (V_{vasc}, mL · 100 g^{-1}) as:

$$V_{vasc} = X^*/C^* \quad (1)$$

where X^* and C^* represent [^{14}C]-inulin in the brain (dpm · 100 g^{-1}) and perfusate (dpm · mL^{-1}), respectively. Apparent brain distributional volumes of substrates (V_{brain}, mL · 100 g^{-1}) were calculated as:

$$V_{brain} = X_{brain}/C_{perf} \quad (2)$$

where X_{brain} is substrate in brain (dpm · 100 g^{-1} or nmol · 100 g^{-1}) corrected for vascular contamination ($X_{total} - V_{vasc} \cdot C_{perf}$)

and C_{perf} is substrate in perfusate (dpm · mL^{-1} or nmol · mL^{-1}). Initial uptake clearances of substrates (Cl_{up}, mL · 100 g^{-1} · min^{-1}) were calculated from:

$$Cl_{up} = (X_{brain}/T)/C_{perf} \quad (3)$$

where T is the perfusion time (min). The P-gp effect on brain uptake was defined as the ratio of uptake clearance in P-gp deficient mice to that in P-gp competent mice.

Determination of Verapamil Plasma Protein Binding by Ultrafiltration

[^{3}H]-Verapamil (0.3 μCi/mL) was pre-incubated in 5 mM phosphate-buffered saline (pH 7.4) or human plasma for 30 min at 37°C in the presence and absence of 30 μM dexverapamil (n = 4 per condition). Samples (1 mL) were loaded in Centrifree devices (Millipore Corporation, Bedford, MA) and centrifuged at 1000g for 3.5 min at 37°C . Radioactivity was determined in aliquots (50–100 μL) from the loading compartment and collecting vessel.

Data Analysis

Data are presented as mean ± SD for 3–5 mice per group and are reported for male animals unless specified otherwise. One- and two-way analysis of variance (ANOVA) techniques or two-tailed Student *t* tests, where appropriate, were used to determine the statistical significance of differences between experimental groups. The level of significance was corrected for multiple comparisons (e.g., Bonferroni *t* tests) or adjusted for unequal variance when necessary. Statistical significance was defined as $P < 0.05$. The concentration-dependent brain uptake data for verapamil and quinidine were analyzed by nonlinear least-squares regression (WinNonlin 1.1, Pharsight, Cary, NC):

$$V_{brain} = V_{brain,0} + (V_{brain,\max} - V_{brain,0})\left(\frac{C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}}\right) \quad (4)$$

where $V_{brain,0}$ and $V_{brain,max}$ are the apparent brain distributional volumes at tracer amount (C → 0) and at saturation, respectively, C is the perfusate concentration, IC_{50} is the apparent half-inhibitory constant for efflux and γ is a sigmoidicity factor. The SD of ratios was derived from the SD of the numerator (x) and denominator (y) (16):

$$SD_{x/y} = \frac{x}{y}\sqrt{\left(\frac{SD_x}{x}\right)^2 + \left(\frac{SD_y}{y}\right)^2} \quad (5)$$

RESULTS

Time Course of Brain Uptake

The kinetics of verapamil and quinidine uptake into brain in P-gp- competent and deficient mice, expressed as apparent brain distributional space (V_{brain}), are shown in Fig. 1. For each compound, uptake increased with time through the maximum duration examined (100 sec) and was significantly higher in the absence of *mdr1a* P-gp. A perfusion time of 60 sec provided sufficient sensitivity while retaining linear uptake conditions, and was selected for subsequent experiments. Since the BBB permeability of morphine is low (17), a single perfusion time of 120 s was selected to provide accurate and reproducible determinations of uptake (i.e., ensuring at least an equal amount of substrate in brain tissue relative to vascular contamination).

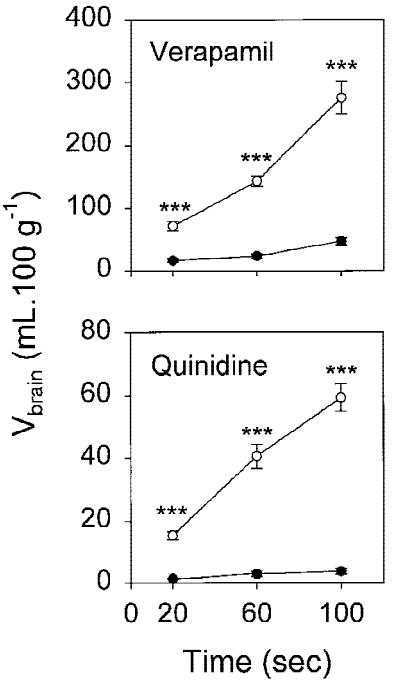

Fig. 1. Time-dependent uptake of trace amounts of verapamil (top) and quinidine (bottom) in the right hemisphere (expressed as apparent brain distributional volume, V_{brain}) of P-gp-competent [*mdr1a*(+/+); solid] and deficient [*mdr1a*(–/–); open] mice. Data are presented as mean ± SD (n = 4 per point). ***$P < 0.001$ vs. P-gp-competent mice at individual time points.

Effect of *mdr1a* P-gp Gene Disruption and Gender on the Brain Uptake of Morphine, Quinidine, and Verapamil

The initial brain uptake clearance (Cl_{up}) of substrates in male and female mice, and in the presence and absence of *mdr1a* P-gp, is presented in Table I. Regardless of gender, brain uptake was higher in P-gp-deficient mice for each compound. The influence of P-gp varied among the substrates, with quinidine evidencing the highest P-gp effect. At 60 sec, the brain uptake of verapamil was ~ 50% higher in male heterozygous *mdr1a*(+/–) mice as compared to homozygous *mdr1a*(+/+) animals (V_{brain} of 41.4 ± 2.0 vs. 27.9 ± 6.4 mL · 100 g^{-1}, $P < 0.01$); the weight of the right hemisphere was not significantly different between the two genotypes (142 ± 4 vs. 136 ± 5 mg). No such difference was observed for quinidine (2.5 ± 0.6 vs. 2.5 ± 1.1 mL · 100 g^{-1}).

With the exception of verapamil, no gender-dependent differences in uptake or P-gp effect were observed (Table I). In P-gp-deficient mice, the uptake of verapamil was slightly higher (~24%, $P < 0.05$) in females as compared to males. Conversely, in P-gp-competent mice, uptake was lower (~ 22%; $P < 0.05$) in females. These observations could not be explained by differences in the weight of the right hemisphere or vascular volume (Table II).

Concentration-Dependence of Brain Uptake

In P-gp-competent mice, the brain uptake of verapamil and quinidine at 60 sec was concentration-dependent, with

Table I. Initial Brain Uptake Clearances (Cl_{up}) in Male and Female Mice. P-gp Effect is Defined as the Ratio Between Cl_{up} in P-gp-Competent [*mdr1a*(+/+)] and Deficient Mice [*mdr1a*(–/–)][a]

	Cl_{up} (ml · 100g^{-1} · min^{-1})					
	P-gp competent [*mdr1a*(+/+)]		P-gp deficient [*mdr1a*(–/–)]		P-gp effect [(–/–)/(+/+)]	
	Male	Female	Male	Female	Male	Female
Morphine	1.04 ± 0.03	1.00 ± 0.06	1.29 ± 0.08*	1.30 ± 0.25*	1.24 ± 0.08	1.30 ± 0.26
Verapamil	29.9 ± 2.5	23.4 ± 0.8#	152 ± 17***	188 ± 9***#	5.09 ± 0.70	8.02 ± 0.47
Quinidine	2.99 ± 0.88	2.77 ± 0.68	40.6 ± 3.8***	41.2 ± 4.8***	13.6 ± 4.2	14.9 ± 4.0

[a] Data are presented as mean ± SD of 4 individual experiments at 60 sec. *$P < 0.05$, ***$P < 0.001$ for P-gp genotype within gender; #$P < 0.05$ *vs.* male.

apparent IC_{50} values for efflux of 8.6 ± 2.3 μM (dexverapamil) and 36 ± 2 μM, respectively (Fig. 2). Estimates for $V_{brain,0}$ and $V_{brain,max}$ (mL · 100 g^{-1}), and γ, were: 44 ± 18, 210 ± 10 and 1.25 ± 0.40 for verapamil; and 3.1 ± 1.1, 35.8 ± 1.2 and 2.59 ± 0.43 for quinidine. The inulin space in the presence of 300 μM dexverapamil and 200 μM quinidine was 1.12 ± 0.15 and 1.07 ± 0.08 mL · 100 g^{-1}, respectively; the reference value from previous work is 1.04 ± 0.18 mL · 100 g^{-1} (4). In P-gp-deficient mice, brain uptake of verapamil and quinidine was unaffected by the presence of these concentrations of unlabeled substrate (Fig. 2).

Effect of Vehicles on the Brain Uptake of Verapamil and Quinidine

The presence of 2% organic vehicles (ethanol, DMSO or 4:1 PEG:ethanol) in the perfusate did not change the 60-sec brain uptake of verapamil and quinidine in P-gp-competent mice (Fig. 3).

Effect of Plasma Protein Binding on the Brain Uptake of Verapamil

Human plasma decreased the brain uptake of trace amounts of verapamil in P-gp-competent and deficient mice, and P-gp-competent animals in the presence of 30 μM dexverapamil, to 16 ± 5% ($P < 0.01$), 44 ± 10% ($P < 0.001$) and 36 ± 6% ($P < 0.001$) of corresponding buffer values, respectively (Fig. 4). Dexverapamil (30μM) increased the apparent unbound fraction of [^{3}H]-verapamil in human plasma by 27% (0.138 ± 0.012 *vs.* 0.109 ± 0.006, $P < 0.01$). These values were not corrected for the recovery of [^{3}H]-verapamil following ultrafiltration in phosphate-buffered saline, which was not significantly increased by the presence of dexverapamil (69 ± 6% *vs.* 78 ± 9%).

Table II. Selected Parameters [Body Weight, Weight of Right Hemisphere and Vascular Volume (V_{vasc})] of Animals Used to Investigate the Influence of Gender and *mdrla* P-gp Genotype on the Initial Brain Uptake of Verapamil[a]

	Verapamil group			
	Male		Female	
	mdr1a		*mdr1a*	
	(+/+)	(–/–)	(+/+)	(–/–)
Body weight (g)	33.3 ± 1.7	33.0 ± 2.7	25.0 ± 1.4	29.3 ± 1.7
Right hemisphere (mg)	140 ± 12	149 ± 9	140 ± 5	140 ± 8
V_{vasc} (mL · 100g^{-1})	1.06 ± 0.06	0.99 ± 0.06	0.95 ± 0.06	1.10 ± 0.29

[a] Data are presented as mean ± SD (n = 4).

Influence of P-gp on Brain Distribution Following i.v. Administration

The plasma and brain tissue concentrations of substrates 30 min after i.v. administration to P-gp-competent and deficient mice are reported in Table III. For each compound, the plasma concentrations were identical between the two strains, but the concentration in brain tissue was significantly higher in P-gp-deficient mice. Similar to the *in situ* results, the influence of P-gp varied among substrates, again with quinidine evidencing the largest difference. Blood:plasma concentration ratios were assumed to be equal to unity when correcting for vascular contamination in brain tissue, which was significant only for quinidine in P-gp-competent mice. After correction, the resulting difference was ~20% for quinidine, but was neg-

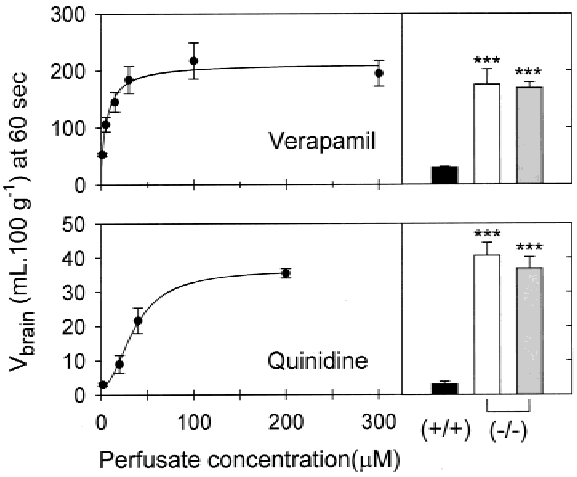

Fig. 2. Concentration-dependent uptake of trace amounts of verapamil (top) and quinidine (bottom) in the right hemisphere (expressed as apparent brain distributional volume, V_{brain}) of P-gp-competent [*mdr1a*(+/+); solid] and deficient [*mdr1a*(–/–); open] mice. The apparent $IC_{50}$for efflux was 8.6 ± 2.3 μmol (dexverapamil) and 36 ± 2 μmol for verapamil and quinidine, respectively. Uptake data in P-gp-competent mice (closed), and P-gp-deficient animals in the absence (open) and presence of an excess concentration of unlabeled substrate (300 μM verapamil or 200 μM quindine; shaded), is shown in the bar graphs for comparison. Data are presented as mean ± SD (n = 5 per point). ***$P < 0.001$ vs. P-gp-competent mice.

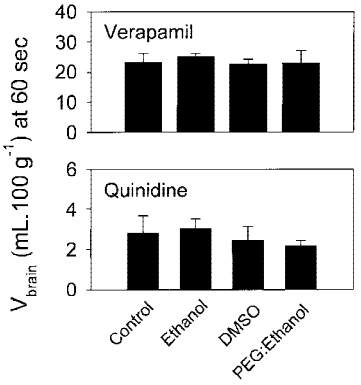

Fig. 3. Effect of vehicles (2% v/v) on the brain uptake of trace amounts of verapamil (top) and quinidine (bottom) in the right hemisphere (expressed as apparent brain distributional volume, V_{brain}) of P-gp-competent [*mdr1a*(+/+)] mice.

ligible for morphine and verapamil (<6%). Use of blood: plasma distribution ratios from (18–20) rats did not change the results significantly.

DISCUSSION

The results of this study demonstrate that *mdr1a* P-gp decreases the initial brain uptake of morphine, quinidine and verapamil *in situ* (Fig. 1; Table I), consistent with the status of these compounds as P-gp substrates. The Cl_{up} of morphine is similar to previously reported values obtained by *in situ* brain perfusion in mice and rats (15,17). For quinidine, there was excellent agreement between the Cl_{up} estimates obtained in P-gp- competent and deficient mice, and the values (11) reported from integration plot analysis after i.v. administration to control and SDZ PSC 833-treated rats (2.55 and 40.0 mL · 100 g^{-1} · min^{-1}, respectively).

Gender differences in brain uptake were observed only for verapamil, but may not be of practical significance (Table I). The results may indicate modest up-regulation of P-gp expression and/or function in female P-gp-competent mice. In the absence of P-gp, higher brain uptake of verapamil in females may reflect a flow-sensitive behavior consistent with a

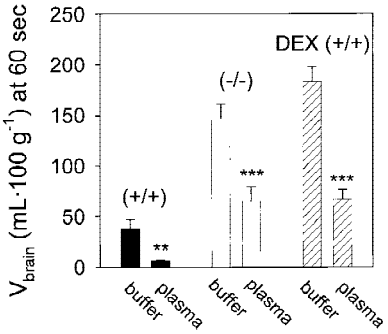

Fig. 4. Effect of human plasma on the brain uptake of trace amounts of verapamil in the right hemisphere (expressed as apparent brain distributional volume, V_{brain}) of P-gp-competent [*mdr1a*(+/+); solid] and deficient [*mdr1a*(–/–); open] mice, and P-gp-competent animals in the presence of 30 μM dexverapamil [DEX; hatched]. Uptake in the presence of plasma was 16 ± 5%, 44 ± 10% and 36 ± 6%, respectively, of the corresponding buffer values. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. buffer.

Table III. Brain-to-plasma Distribution Data for the Substrates 30 Min Following i.v. Administration of 1 μmol · kg^{-1} (Verapamil) or 2 μmol · kg^{-1} (Morphine and Quinidine) to P-gp- Competent [*mdr1a*(+/+)] and Deficient [*mdr1a*(–/–)] Mice[a]

	Concentration (nM) and brain:plasma ratio		
	P-gp competent [*mdr1a*(+/+)]	P-gp deficient [*mdr1a*(–/–)]	(–/–)/(+/+) ratio
Morphine			
plasma	64 ± 25	84 ± 11	1.31 ± 0.54
brain	102 ± 36	222 ± 31**	2.18 ± 0.83
brain:plasma	1.63 ± 0.26	2.67 ± 0.34**	1.63 ± 0.33
Verapamil			
plasma	141 ± 23	150 ± 35	1.07 ± 0.30
brain	75 ± 13	976 ± 149***	13.1 ± 3.0
brain:plasma	0.53 ± 0.06	6.75 ± 1.66***	12.6 ± 3.4
Quinidine			
plasma	229 ± 55	223 ± 52	0.98 ± 0.33
brain	44 ± 7	1897 ± 159***	43.0 ± 8.1
Brain:plasma	0.20 ± 0.07	8.82 ± 1.89***	43.4 ± 16.9

[a] Data are presented as mean ± SD (n = 4–5). $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* P-gp-competent mice.

previous report of higher cerebral blood flow in women as compared to men (21). However, it is unclear whether this observation relates to gender differences outside the brain (e.g., cardiac output), which would be absent under conditions of fixed perfusion rate *in situ,* or if it applies to rodent species.

Although other factors (*e.g.,* hydrogen bonding) also come into play, lipophilicity is an important determinant of passive BBB permeability (3). Interestingly, the brain uptake of morphine, quinidine and verapamil followed the same rank order of reported log $P_{o/w}$ values [log (octanol-water partition coefficients); 0.76, 2.64 and 3.83] (22), with a slight improvement of the relationship in the absence of P-gp (Fig. 5A). It has been hypothesized that P-gp interacts with substrates from within the cell membrane (23). As compared to quinidine, the ~ 4-fold lower apparent IC_{50} for BBB efflux observed with dexverapamil (Fig. 2) may reflect higher lipophilicity, also consistent with the ~ 4-fold higher uptake of verapamil in P-gp-deficient mice (Table I). However, because not all lipid-soluble compounds are transported by P-gp, this property must be dissociated from the intrinsic ability to in-

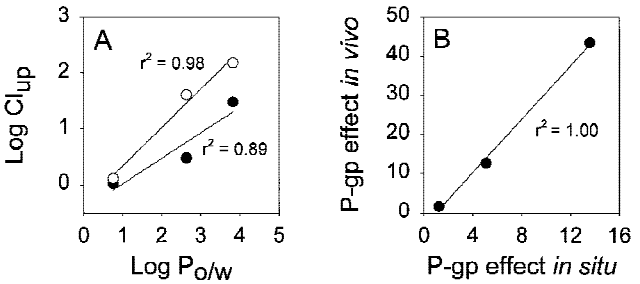

Fig. 5. Panel A. Relationship between the logarithms of initial uptake clearance (log Cl_{up}) of substrates in P-gp-competent [*mdr1a*(+/+); solid] and deficient [*mdr1a*(–/–); open] mice, and octanol-water partition coefficient (log $P_{o/w}$; values from ref. 22). Panel B. Relationship between the P-gp effect *in situ* and the influence of P-gp on brain-to-plasma distribution *in vivo.*

teract with P-gp to some extent. In rat brain capillaries, quinidine inhibits [^{125}I]-iodoarylazidoprazosin (IAAP) photoaffinity labeling of P-gp to a greater extent than verapamil (24), an apparent contradiction with the present results. However, binding to P-gp does not always infer transport, such that these two parameters may be independent (25). The concentration-dependent brain uptake of morphine was not investigated, as the influence of *mdr1a* gene disruption was modest (~30%; Table I). Moreover, it had been reported that the BBB transport of morphine is not saturable (17).

Despite a higher apparent affinity for BBB efflux, verapamil evidenced a lower P-gp effect than quinidine *in situ* (Table I) and *in vivo* (Table III). Rapid diffusion of verapamil across the cell membrane may provide an escape mechanism from P-gp (26), a phenomenon referred to as futile cycling of the transporter (23). Intermediate levels of *mdr1a* P-gp have been observed in the brain of heterozygous *mdr1a*(+/–) mice as compared to the homozygously competent and deficient animals, which translates into intermediate CNS sensitivity to abamectin (2). Consistent with these observations, the Cl_{up} of verapamil was ~ 50% higher in *mdr1a*(+/–) as compared to *mdr1a*(+/+) mice in the present study. For quinidine, relatively slow diffusion may render the residence time in the cell membrane sufficiently long for efficient P-gp-mediated efflux to occur regardless of decreased P-gp expression, such that no difference was observed over a short perfusion time.

No compensatory transport mechanisms (e.g., expression of *mdr1b*) have been identified in the BBB of transgenic *mdr1a*(–/–) mice (1). However, there is evidence supporting the existence of non-P-gp efflux systems in the rat BBB (7). In P-gp-deficient mice, the brain uptake of quinidine and verapamil was unaffected by the presence of excess solute (Fig. 2), indicating that neither compound is transported by other saturable processes, and suggesting that the physical integrity of the BBB was maintained. Thus, it is reasonable to assume that inhibition of P-gp-mediated efflux, as opposed to nonspecific effects, explain the concentration-dependent uptake of quinidine and verapamil in P-gp-competent mice.

The results of this study suggest that the presence of 2% ethanol, DMSO or PEG:ethanol (4:1) in the perfusate did not affect passive diffusion and P-gp-mediated efflux of verapamil or quindine at the BBB over 60 sec (Fig. 3). However, such a high concentration of organic solvent in blood clearly would be neurotoxic *in vivo*. Indeed, brain perfusion with DMSO was associated with mild tremors. Thus, it would be advisable to use the lowest possible concentration of organic vehicle (e.g., $\leq$ 0.25%) to solubilize compounds with low aqueous solubility prior to dilution in physiologic buffers. Maintenance of functional and physical BBB integrity should be demonstrated in control experiments.

Racemic verapamil fully inhibits P-gp at relatively low concentrations *in vitro* (6–10 μM), but its utility as a clinical modulator of multidrug resistance is restricted by dose-limiting cardiovascular effects (e.g., hypotension) at serum concentrations of 1–2 μM (27). Dexverapamil [R-(+)-verapamil] has a similar P-gp inhibitory activity and lower cardiovascular potency (27). Plasma decreased the brain uptake of [^{3}H]-verapamil significantly, but the change in unbound fraction (27%) could not explain the ~10-fold increase in brain uptake in the presence of dexverapamil (30 μM), therefore indicating inhibition of P-gp (Fig. 4). While this concentration of dexverapamil fully inhibits [^{3}H]-verapamil efflux in buffer, plasma significantly decreased brain uptake. Interestingly, the resulting uptake value corresponds to the uptake at a concentration of ~2 μM dexverapamil in buffer (Fig. 2), suggesting that only the unbound fraction was available to inhibit P-gp. While some investigators have measured P-gp modulatory activity in patient serum (28) or reported similar effects of protein binding *in vitro* (29,30), this issue often has been ignored in the design and interpretation of clinical trials. At least in mice, it appears unlikely that systemic administration of dexverapamil would result in significant competitive inhibition of BBB P-gp without toxicity.

Following i.v. administration, plasma concentrations of substrates were unaffected by genetic disruption of *mdr1a* P-gp, suggesting that the increase in brain distribution in P-gp-deficient mice is due to a difference in BBB transport (Table III). The influence of P-gp for morphine, verapamil and quinidine was similar to the 1.7, 7.9 and 27.6-fold differences reported in other studies under different conditions (10–12). Although the data are limited, the results of the present study suggest that the P-gp effect *in situ,* while underestimating the influence of P-gp *in vivo,* follows at least the same rank order (Fig. 5B). It should be noted that the brain: plasma ratios may not represent distributional equilibrium. At this point, the explanation for lower P-gp effect *in situ* remains speculative. Over short perfusion times, the *in situ* brain perfusion technique provides an estimate of unidirectional solute influx from perfusate to brain tissue. We hypothesize that the smaller influence of P-gp *in situ* results in part from the lack of significant P-gp-mediated egress from brain to perfusate, which has been demonstrated for quinidine using the Brain Efflux Index (11). For verapamil, there was a less restrictive effect of protein binding on brain uptake upon genetic P-gp disruption (Fig. 4), which was likely related to the significant increase in brain extraction (from 12 to 60% in male mice). This may translate into a greater influence of P-gp on *in vivo* brain distribution. However, this reasoning is not applicable to drugs with low brain extraction in the absence of P-gp (e.g., quinidine). Alternatively, decreased P-gp function *in situ* appears unlikely with short perfusion times, but an effect of anesthetics cannot be ruled out.

In conclusion, the results of the present study suggest that the *in situ* mouse brain perfusion model may be useful to assess the influence of *mdr1a* P-gp gene disruption on the initial brain uptake of substrates. Differential regulation of P-gp in the BBB by sex hormones may warrant further investigation, but the influence of gender on verapamil brain uptake was modest. The potentially more restrictive effect of plasma protein binding on brain uptake in the presence of P-gp requires confirmation with other substrates. Comparative determination of BBB permeability in P-gp- competent and deficient mice under various conditions may help establish an integrated and predictive model of BBB transport that incorporates the influence of physicochemical properties, plasma protein binding and affinity for P-gp from *in vitro* data.

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