In Situ Transport of Vinblastine and Selected P-glycoprotein Substrates: Implications for Drug-Drug Interactions at the Mouse Blood-Brain Barrier

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Purpose. To study the intrinsic parameters of P-glycoprotein (P-gp) transport and drug-drug interactions at the blood-brain barrier (BBB), as few quantitative *in vivo* data are available. These parameters could be invaluable for comparing models and predicting the *in vivo* implications of *in vitro* studies.

Methods. The brains of P-gp–deficient mice mdr1a(–/–) and wild-type mice were perfused *in situ* using a wide range of colchicine, morphine, and vinblastine concentrations. The difference between the uptake by the wild-type and P-gp–deficient mice gave the P-gp–linked apparent transport at the BBB. Drug-drug interactions were examined using vinblastine and compounds that bind to P-gp sites (verapamil, progesterone, PSC833) other than the vinblastine site to take into account the multispecific drug P-gp recognition.

Results. P-gp limited the brain uptake of morphine and colchicine in a concentration-independent way up to 2 mM. In contrast, vinblastine inhibited its own P-gp transport with an IC_{50} of ~56 μM and a Hill coefficient of ~4. The vinblastine efflux by P-gp was described by a K_m at 16 μM and a maximal efflux velocity, J_{max}, of ~8 pmol s^{-1} g^{-1} of brain. Similarly, vinblastine brain transport was increased by inhibiting P-gp as shown by the IC_{50} ranking, which was PSC833 < verapamil < vinblastine < progesterone.

Conclusions. P-gp is responsible for both capacity-limited and -unlimited transport of P-gp substrates at the mouse BBB. *In situ* perfusion of mdr1a(–/–) and wild-type mouse brains could be used to predict drug-drug interactions for P-gp at the mouse BBB.

KEY WORDS: blood-brain barrier; *in situ* brain perfusion; multidrug resistance; P-glycoprotein; vinblastine.

INTRODUCTION

Much of the specificity of the endothelium forming the blood-brain barrier (BBB) is due to the tight junctions between brain endothelial cells. These mostly restrict the entry of hydrophilic compounds from the blood to the brain via paracellular diffusion. The uptake of some lipophilic compounds by the brain can also be restricted at the BBB by ATP-dependent efflux proteins like P-glycoprotein (P-gp/Abcb1) and Bcrp/Abcg2 (1,2). P-gp was first described as a component of the multidrug resistance (MDR) mechanism of tumor cells, but is now known to be present physiologically at the luminal membrane of the brain capillary endothelial cells (1). P-gp extrudes structurally dissimilar amphiphilic compounds from the endothelial cells into the blood vessel lumen (3,4). The MDR P-gp is encoded by one gene in humans (MDR1) and by two genes in rodents (mdr1a and mdr1b), but mdr1a is the major protein at the rodents BBB.

In vivo experiments with mice lacking the gene encoding the drug-transporting P-gp have contributed greatly to these insights. Measurements of plasma and brain drug concentrations in wild-type and P-gp–deficient mice have demonstrated that P-gp is an important pump that eliminates xenobiotics and protects the brain from them (5). P-gp is now known to play a major role in the pharmacokinetic-pharmacodynamic variability of many drugs and to contribute to drug-drug interactions at the P-gp (6–8). However, very little is known about the capacity of P-gp to transport drugs across physiological barriers, such as the BBB and the velocity of such transport. Most of the *in vivo* studies carried out to date have been designed to measure the blood-brain distribution of drugs after their systemic administration. The objective has been to show how the protein impedes drug accumulation in the brain by comparing drug brain/plasma concentration ratios measured in each strains of mouse at various time(s). However, this technique cannot quantify intrinsic P-gp transport or drug-drug interactions parameters at the BBB *in vivo*. Such data can predict the impact of the P-gp on drug transport across the BBB and thus permit reliable comparisons with *in vivo*/*in vitro* models. The *in situ* brain perfusion technique measures initial transport rates across the BBB and allows the determination of transport parameters (IC_{50}, V_{max}, K_m, K_i). This technique maintains the physiological properties of the BBB and is a very sensitive *in vivo* method for assessing the transport of drugs or physiological compounds across the BBB without the confounding factor of systemic distribution (9). *In situ* brain perfusion in mdr1a(–/–) mice can also be used to more readily identify substrates transported by P-gp at the BBB than can reversal agents injected i.v. into wild-type mice (10). This current study examines the P-gp–assisted transport of three unrelated drugs across the BBB; all three of them are known to be significantly transported across the mouse BBB (10). One, the chemotherapeutic drug vinblastine, has widely been used *in vitro* as a typical P-gp probe, another is the neutral compound colchicine, and the third is the analgesic morphine, one of the lowest molecular weight drugs known to be a P-gp substrate. We first measured the kinetics of their initial brain transport over a wide range of concentrations in P-gp–deficient mdr1a(–/–) and wild-type mice to identify the kinetic components of P-gp pumping at the BBB. The transport of vinblastine by P-gp across the BBB was then measured, together with a wide range of concentrations of other P-gp transported drugs (verapamil, PSC833) or a P-gp modulator (progesterone), as they all interact with sites on P-gp that are distinct from the vinblastine site (11–13). We have identified those drugs transported by P-gp that can inhibit their own P-gp–dependent efflux. We have also assessed the capacity of a compound to modulate the P-gp–mediated transport of vinblastine across the BBB *in vivo*.

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ABBREVIATIONS: ABC, ATP binding cassette; BBB, blood-brain barrier; CNS, central nervous system; MDR, multidrug resistance; P-gp, P-glycoprotein.

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MATERIALS AND METHODS

Chemicals and Reagents

[^{3}H]-Vinblastine (10–12.5 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech (Orsay, France). [^{14}C]-Sucrose (565 mCi mmol^{-1}), [^{3}H]-morphine (83.5 Ci mmol^{-1}), and [^{3}H]-colchicine (61.4 Ci mmol^{-1}) were purchased from Perkin Elmer New England Nuclear Life Sciences (Paris, France). Vinblastine sulfate, morphine hydrochloride, and colchicine were obtained from Lilly (St Cloud, France), Sanofi & Synthelabo (Gentilly, France), and Fluka (St Quentin Fallavier, France), respectively. PSC 833 (Valspodar) was a gift from Novartis (Basel, Switzerland). (±)-Verapamil hydrochloride, dexamethasone sodium phosphate, quinidine hydrochloride, and β-cyclodextrin-progesterone complex were purchased from Sigma (St Quentin Fallavier, France), and (+)-verapamil hydrochloride was purchased from ICN (Orsay, France). Heparin sodium was obtained from Sanofi & Synthelabo. Dimethyl sulfoxide (DMSO) and β-cyclodextrin were purchased from Merck Eurolab (Strasbourg, France) and Fluka, respectively. All other chemicals were of analytical grade.

Animals

The studies were performed on adult male CF-1 mice [mdr1a(+/+) and (–/–), 30–40 g, 7–9 weeks old] bred in-house from progenitors genotyped for mdr1a P-gp that were initially obtained from Charles River Laboratories (Wilmington, MA, USA). The mice were housed in a room with a controlled environment (22 ± 3°C; 55 ± 10% relative humidity) and maintained under a 12-h dark:light cycle (light on from 6:00 a.m. to 6:00 p.m.). They had access to food and tap water *ad libitum*. All experimental procedures complied with the ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (law no. 87-848).

In Situ Brain Perfusion

Surgery and Perfusion

The transport of [^{3}H]-compounds (morphine, colchicine, vinblastine) into the brain were measured by *in situ* brain perfusion (14). Mice were anesthetized by i.p. injection of a mixture of xylazine (Bayer, Puteaux, France) and ketamine (Panpharma, Fougères, France) at 8/140 mg kg^{-1}. Briefly, the common carotid artery was ligated on the heart side. The external carotid was ligated rostral to the occipital artery at the level of the bifurcation of the common carotid artery. Then, the right common carotid was catheterized with polyethylene tubing (0.30-mm i.d. × 0.70-mm o.d.; Biotrol Diagnostic, Chennevières-les–Louvre, France) filled with heparin (25 IU ml^{-1}). Body temperature was maintained at 37 to 38°C throughout surgery, using a rectal thermistor connected to a temperature monitor. The thorax of the mouse was opened and the heart was cut. Perfusion was immediately started (flow rate: 2.5 ml min^{-1}) with the syringe containing the perfusion fluid placed in an infusion pump (Harvard pump PHD 2000, Harvard Apparatus, Holliston, MA, USA) and connected to the catheter. The perfusion fluid consisted of bicarbonate-buffered physiological saline (mM): 128 NaCl, 24 $NaHCO_3$, 4.2 KCl, 2.4 NaH_2PO_4, 1.5 $CaCl_2$, 0.9 $MgCl_2$ and 9 D-glucose. The solution was gassed with 95% O_2 and 5% CO_2 for pH control (7.4) and warmed to 37°C in a water bath. Appropriate concentrations of compounds were added to the perfusate. Each mouse was perfused with [^{3}H]-tracer and [^{14}C]-sucrose (0.2 μCi ml^{-1}) to check the physical integrity of the blood-brain barrier. Perfusion was terminated by decapitation at 90 s for experiments with colchicine and 120 s for transport studies with vinblastine and morphine. The brain was removed from the skull and dissected out on ice. Each right cerebral hemisphere was placed in a tared vial and weighed. Aliquots of the perfusion fluid were also collected and weighed to determine tracer concentrations in the perfusate. Samples were digested in 1 ml of Solvable (Packard, Rungis, France) at 50°C and mixed with 9 ml of Ultima gold XR scintillation cocktail (Packard). The dual labels were counted simultaneously in a Packard Tri-Carb model 1900 TR.

Transport Studies

The initial rates of [^{3}H]-drug (0.2–0.3 μCi ml^{-1}) transport into the brain of mdr1a(–/–) and wild-type mice were measured at different concentrations under trans-influx zero conditions (15). The perfusion fluid contained labeled compound with or without unlabeled drugs to produce an appropriated drug concentration.

The stock solutions of vinblastine, colchicine, morphine, verapamil, dexamethasone, and progesterone were prepared in 0.9% NaCl and in DMSO for PSC833 and quinidine. All these solutions were freshly made on the day of the experiment. The stock solutions were diluted with the bicarbonate-buffered saline used for perfusion. The final DMSO concentration never exceeded 0.5% (v v^{-1}); this concentration did not alter the integrity of the BBB, as measured by the permeability of [^{14}C]-sucrose and transport of the tritiated drugs.

Calculation of BBB Transport Parameters

All calculations were done as previously described (9,14). Brain vascular volume (V_{vasc}; μl g^{-1}) was estimated from the tissue distribution of [^{14}C]-sucrose, which diffuses very slowly across the BBB, using the following equation:

$$V_{vasc} = \frac{X^*}{C^*_{perf}} \quad (1)$$

where X* (dpm g^{-1}) is the amount of sucrose in the right brain hemisphere and C^*_{perf} (dpm μl^{-1}) is the concentration of labeled sucrose in the perfusion fluid.

Transport across the BBB is expressed in terms of three parameters: the apparent volume of distribution (V_{brain}), the transport coefficient (K_{in}) corresponding to the brain uptake clearance, and brain flux (J_{net}). The apparent volume of distribution was calculated from the amount of radioactivity in the right brain hemisphere using the following equation:

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

where X_{brain} (dpm g^{-1}) is the calculated amount of [^{3}H]-compound in the right cerebral hemisphere, and C_{perf} (dpm μl^{-1}) is the labeled tracer concentration in the perfusion fluid. Brain tissue radioactivity was corrected for vascular contamination with the following equation:

$$X_{brain} = X_{tot} - V_{vasc}C_{perf} \quad (3)$$

where X_{tot} (dpm g^{-1}) is the total quantity of tracer measured in the brain tissue sample (vascular + extravascular).

Initial brain transport is expressed as a blood-brain transfer coefficient K_{in} ($\mu l\ s^{-1}\ g^{-1}$) and was calculated from:

$$K_{in} = \frac{V_{brain}}{T} \quad (4)$$

where T is the perfusion time (s).

We performed time-course studies of the brain distributions in deficient and wild-type mice to select an appropriate perfusion time; that is, one that permitted study of both the initial rate of uptake and ensured that at least 40% of the total radioactivity in the tissue resided outside of the vascular space ($X_{brain} \geq 0.4\ X_{tot}$) (9,16). The increase in brain content was linear and unidirectional from zero to 120 s (data not shown; 10,14). The perfusion time selected was 90 s for colchicine and 120 s for morphine and vinblastine.

The flux J_{net} (pmol $s^{-1}\ g^{-1}$) was calculated assuming that K_{in} values are flow-independent for K_{in} lower than ~16 $\mu l\ s^{-1}\ g^{-1}$ (9,14) according to:

$$J_{net} = PS\ C = K_{in}C \quad (5)$$

where PS is the surface permeability, and C is the total drug concentration (labeled and unlabeled) in the perfusate.

BBB Transport and Drug-Drug Interactions

Kinetic and statistical analyses were carried out using Systat 5.01 software (Systat Inc., Point Richmond, CA, USA). Kinetic relationships were calculated by a nonlinear least squares method fitted to a sigmoid curve using a mathematical function based on the Hill equation as previously described (17):

$$K_{in} = K_{in,min} + \frac{(K_{in,max} - K_{in,min})C^n}{IC_{50}^n + C^n}$$

equivalent to

$$K_{in} = K_{in,max} - \frac{(K_{in,max} - K_{in,min})IC_{50}^n}{IC_{50}^n + C^n} \quad (6)$$

where C is drug concentration, $K_{in,min}$ is the minimal and $K_{in,max}$ the maximal brain transport coefficient (K_{in}) value for the labeled substrate, n is the Hill coefficient, and IC_{50} is the concentration giving 50% inhibition of brain transport.

Combining Eqs. 5 and 6 yields the brain flux J_{net} (pmol $s^{-1}\ g^{-1}$):

$$J_{net} = K_{in,max}C - \frac{(K_{in,max} - K_{in,min})IC_{50}^n C}{IC_{50}^n + C^n} \quad (7)$$

The brain flux in wild-type mice could be expressed as the result of two components:

$$J_{net} = J_{in} - J_{efflux}$$

$$\text{where} \quad J_{in} = K_{in,max}C \quad \text{and} \quad J_{efflux} = \frac{(K_{in,max} - K_{in,min})IC_{50}^n C}{IC_{50}^n + C^n} \quad (8)$$

For P-gp–deficient mice, J_{net} can be written as an independent P-gp saturable efflux transport, and Eq. 7 becomes a nonsaturable diffusion equation:

$$J_{net} = J_{in} = K_{in,max}C \quad (9)$$

When the concentration of substrate C is lower than IC_{50}, Eq. 7 becomes:

$$J_{net} = K_{in,max}C - (K_{in,max} - K_{in,min})C = K_{in,max}C \quad (10)$$

Subtracting the brain flux (J_{net}) calculated with Eqs 9 and 7 at different concentrations in mdr1a(–/–) mice and wild-type mice gave the component of P-gp pumping at the BBB (J_{efflux}) that takes place when the drug interacts with P-gp sites at the luminal membrane of the BBB endothelial cells. The P-gp maximal velocity of pumping (J_{max}; pmol $s^{-1}\ g^{-1}$) and the concentration that gives one-half of the maximal increment in transport and maximal decrease in transport velocity, K_{m1} and K_{m2} (μM), were calculated as solutions of

$$J_{efflux} = \frac{J_{max}}{2}$$

When the substrate concentration is lower than IC_{50} and there is no saturation, J_{efflux} is calculated by subtracting Eqs. 9 and 10, which results in:

$$J_{efflux} = (K_{in,max} - K_{in,min})C \quad (11)$$

Equation 6 was used to fit the data of the drug-drug interaction studies.

All values are means ± standard deviation (s.d.) for 3–8 animals, unless otherwise specified, except in the case of the kinetic parameters IC_{50}, Hill coefficient (n), $K_{in,min}$, and $K_{in,max}$. The error values associated with the kinetic parameters are asymptotic standard errors returned by a nonlinear regression routine and are a measure of the certainty of the best fit value. Student's two-tailed unpaired *t* test was used to identify significant differences between groups when appropriate. Statistical significance was set at $p < 0.05$.

RESULTS

Concentration-Independent Efflux of Morphine via P-glycoprotein

The influx (J_{net}) into the brains of wild-type and P-gp deficient mdr1a(–/–) mice of the potent opioid agonist, morphine, a poorly hydrophobic (octanol/water coefficient: log P ~0.76) low-molecular-weight (285 g/mol) P-gp substrate, varied with the perfusate morphine concentration (Fig. 1a). The slope of the J_{net} curve (K_{in}) was 0.32 ± 0.03 $\mu l\ s^{-1}\ g^{-1}$ in the P-gp–deficient mice and 0.21 ± 0.02 $\mu l\ s^{-1}\ g^{-1}$ in the wild-type mice. Morphine uptake at the blood-brain barrier was not saturable over the concentration range 3 nM to 2 mM in either strain of mouse. Nevertheless, the J_{efflux} curve obtained by subtracting the brain flux values for P-gp–deficient mice from those for wild-type mice confirmed that a P-gp–dependent process significantly limited the morphine influx, a constant J_{efflux} slope component of 0.11 $\mu l\ s^{-1}\ g^{-1}$ (Fig. 1a). This is about 34% of the brain uptake in wild-type mice. The tolerance of the BBB to morphine was explored in each animal by including [^{3}H]-sucrose in the perfusate because it does not measurably cross the BBB in the short times used. The

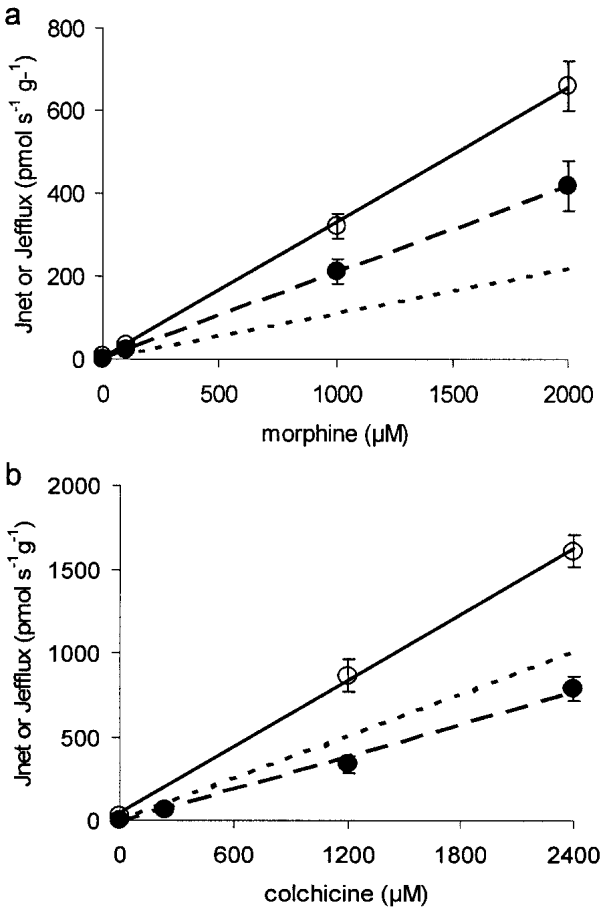

Fig. 1. The brain fluxes (J_{net}) of (a) morphine and (b) colchicine were measured in wild-type (closed circles) and mdr1a(–/–) (open circles) mice by *in situ* brain perfusion. Mouse brains were perfused via the common carotid artery for 90 s for colchicine or 120 s for morphine. Lines are computer-fitted using Eqs. 9 for mdr1a(–/–) and 10 for wild-type mice. Subtracting the brain flux (J_{net}) measured in wild-type and mdr1a(–/–) mice at different concentrations gave the component of mdr1a pumping at the BBB (J_{efflux}, dotted line, Eq.11). The values are means ± SD for 4–6 mice.

BBB of neither wild-type nor P-gp–deficient mice was altered at any of the morphine concentrations used (data not shown).

Concentration-Independent P-glycoprotein–Mediated Colchicine Efflux

The overall pattern of colchicine fluxes, an uncharged P-gp substrate with a molecular weight of 399.4 g/mol and a log P of 2.2, was broadly similar to that of morphine. The colchicine flux was measured over a concentration range of 10 nM to 2.4 mM in wild-type and mdr1a(–/–) mice. The net colchicine flux (J_{net}) was not saturable; the K_{in} slope was 0.28 μl s^{-1} g^{-1} in wild-type mice and 0.72 μl s^{-1} g^{-1} in P-gp–deficient mice (Fig. 1b). P-gp significantly influenced the transport of colchicine out of the brain with a constant J_{efflux} of slope 0.44 μl s^{-1} g^{-1}. Thus, P-gp impeded the uptake of colchicine by the brain by about 58% in wild-type mice. The vascular volumes determined from cerebral sucrose space agreed with reported values over the colchicine concentration range used (14). Colchicine concentrations greater than 3 mM were associated with an increase in the sucrose space in both wild-type and deficient mice, suggesting opening of the BBB (data not shown).

Concentration-Dependent P-glycoprotein–Linked Vinblastine Efflux

The transport of [^{3}H]-vinblastine (811 g/mol), which has a log P value of 3.1 (3), into the brain was measured in wild-type and deficient mdr1a(–/–) mice. The [^{3}H]-vinblastine transport coefficient (K_{in}) was constant in mdr1a(–/–) at all the vinblastine concentrations perfused (25–175 μM; Fig. 2). Conversely, the transport of vinblastine into the brain of wild-type mice was not linear, as for morphine and colchicine. The curve of the transport rate was sigmoid and fitted by a Hill function with a slope (n) of 4.0 ± 1.0. The IC_{50} was 56.2 ± 4.5 μM, $K_{in,min}$, was 0.25 ± 0.01 μl s^{-1} g^{-1}, and $K_{in,max}$ was 0.49 ± 0.02 μl s^{-1} g^{-1}. Though vinblastine did not saturate its own efflux, P-gp decreased the uptake of vinblastine by about 49%. The maximal $K_{in,max}$ values measured in wild-type and P-gp–deficient mice were not significantly different (Fig. 2).The difference calculated from J_{in} measured in wild-type and P-gp–deficient mice gives the apparent pumping J_{efflux} curve of vinblastine by P-gp at the BBB (Fig. 3, inset). The maximal velocity of pumping by P-gp was thus 7.8 ± 1.0 pmol s^{-1} g^{-1}, and the transport constant K_{m1} was 16.1 μM and K_{m2} was 79.2 μM.

The vascular space of [^{14}C]-sucrose agreed with normal values in all experiments. Therefore, the increase in J_{net} was not due to vinblastine leaking across the BBB. Vinblastine concentrations greater than 200 μM had to be perfused to obtain significantly greater sucrose spaces in both wild-type and P-gp–deficient mice, suggesting that vinblastine is toxic for the endothelial cells at the BBB.

Influence of Verapamil and Quinidine on Vinblastine Transport

Several noncytotoxic therapeutic compounds can act as multidrug resistance modulators and restore the chemo-

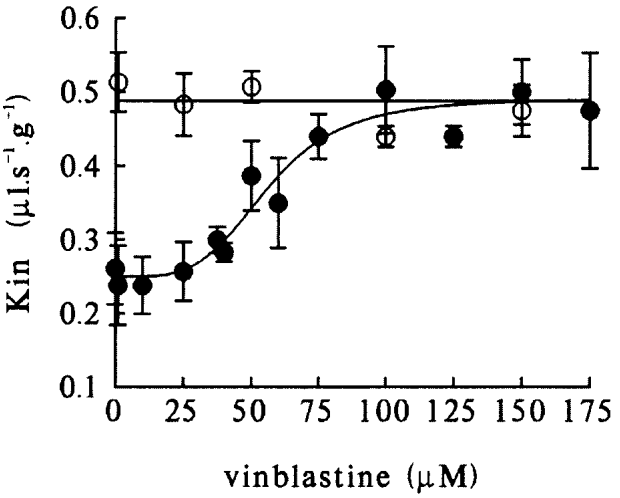

Fig. 2. The brain transport of [^{3}H]-vinblastine (25 nM), expressed as a transport parameter K_{in} (μl s^{-1} g^{-1}), was measured in wild-type (closed circles) and mdr1a(–/–) (open circles) mice over a range of vinblastine concentrations. The resulting sigmoid relationship between [^{3}H]-vinblastine K_{in} and total vinblastine concentration perfused via the common carotid artery for 120 s was fitted by the Hill Eq. 6. All values are means ± SD of 4–8 mice.

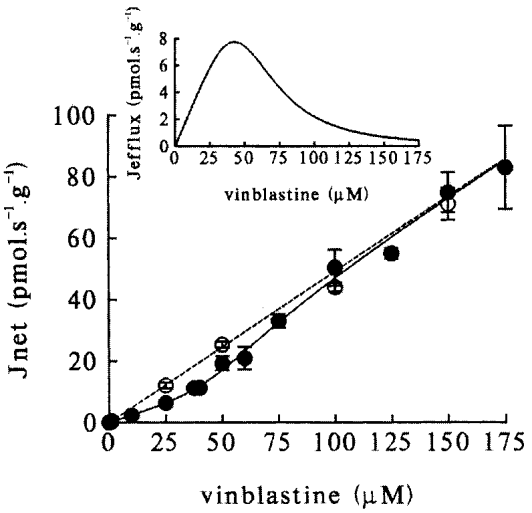

Fig. 3. The brain fluxes (J_{net}) of vinblastine in wild-type (closed circles) and mdr1a(–/–) (open circles) mice were calculated from results obtained by *in situ* brain perfusion. Mice were perfused with vinblastine via the common carotid artery for 120 s. Lines are computer-fitted using Eqs. 8 mdr1a(–/–) and 7 for wild-type mice. Inset: Subtracting the brain fluxes (J_{net}) measured in wild-type and mdr1a(–/–) mice at different concentrations gave the component of mdr1a pumping at the BBB (J_{efflux}). The values are means ± SD for 4–8 mice.

sensitivity of mdr cells by inhibiting the efflux of P-gp substrates. We used 0 to 300 μM racemic verapamil (455 g/mol), a cardiovascular drug with P-gp modulator properties, to modulate the transport of 25 nM [^{3}H]-vinblastine into the brain. The racemic verapamil inhibited vinblastine transport in wild-type mice with a half inhibitory value (IC_{50}) of 23.4 ± 3.0 μM, Hill coefficient of 3.6 ± 1.3, $K_{in,min}$ of 0.28 ± 0.02 μl s^{-1} g^{-1}, and a $K_{in,max}$ of 0.49 ± 0.01 μl s^{-1} g^{-1} (Fig. 4a). Similarly, 20 μM dextrogyre verapamil, a less active cardiovascular agent, inhibited the P-gp–mediated efflux of [^{3}H]-vinblastine (25 nM) to the same extent as 20 μM racemic verapamil, suggesting that there is no stereospecific effect for P-gp inhibition with verapamil. But neither of the two forms of verapamil produced any significant change in the brain transport of [^{3}H]-vinblastine in mdr1a(–/–)–deficient mice, showing that the verapamil inhibition acted via P-gp in wild-type mice (data not shown). Perfusion of another cardiovascular drug, 5 μM quinidine, which modulates P-gp, did not affect the transport of [^{3}H]-vinblastine in wild-type mice (data not shown). This concentration of quinidine inhibits the P-gp–dependent transport of digoxin in mice (7).

Influence of Progesterone and Dexamethasone on Vinblastine Transport

We measured the influence of progesterone, a very lipophilic steroid (log P: 3.3), and dexamethasone, a slightly lipophilic steroid (log P: 1.9) (18) on the brain transport of [^{3}H]-vinblastine (25 nM). Unlike the other compounds used, progesterone is not transported by P-gp and binds to a "regulatory" P-gp site that does not allow an apparent transport of drugs (12,13). Progesterone (perfusate concentration: 0 to 1 mM) inhibited vinblastine transport. The inhibition curve was sigmoid, with a slope (Hill coefficient) of 1.9 ± 0.8, an IC_{50} of 153.8 ± 30.8 μM, a $K_{in,min}$ of 0.20 ± 0.03, and

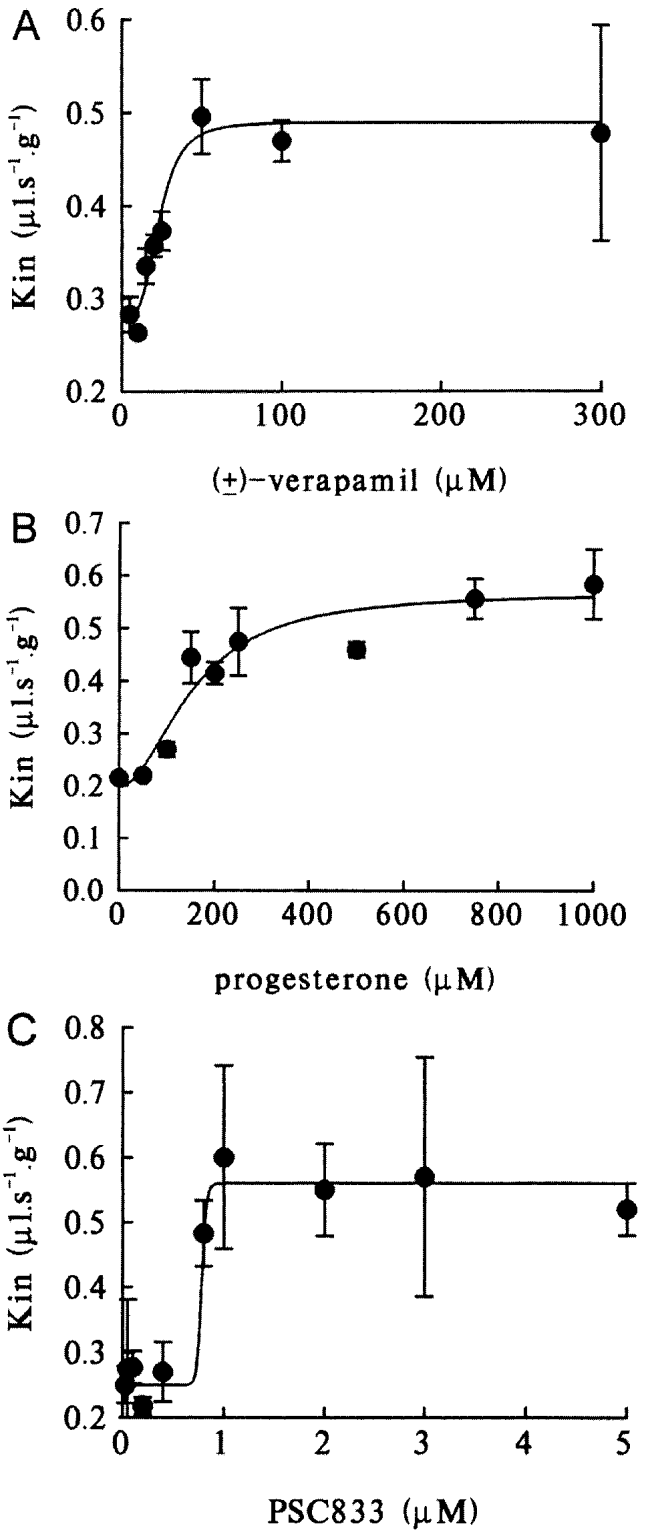

Fig. 4. Effects of the modulators (A) (±)-verapamil, (B) progesterone, and (C) PSC833 on [^{3}H]-vinblastine (25 nM) K_{in} brain transport was measured in wild-type mice by *in situ* brain perfusion. The concentration-dependent effects of each drug on the K_{in} of [^{3}H]-vinblastine were fitted by the Hill Eq. 6. All values are means ± SD for 3–8 mice.

$K_{in,max}$ of 0.57 ± 0.04 μl s^{-1} g^{-1} (Fig. 4b). We checked that this effect of progesterone was not due to water-soluble β cyclodextrin, in which progesterone is formulated. The highest β cyclodextrin concentration (4 mg ml^{-1}) alone (no progesterone) had no significant effect on the transport of [^{3}H]-vinblastine in wild-type mice. Also, the transport of [^{3}H]-vinblastine perfused with or without progesterone (1 mM) in mdr1a(–/–) mice was not significantly different (data not shown), showing that progesterone modulates the P-gp transport of vinblastine into the brain of wild-type mice. In contrast, adding the P-gp substrate dexamethasone (1 mM) to the perfusion fluid did not alter the transport of vinblastine (data not shown). The dose (1 mM) was the same as that at which progesterone inhibits vinblastine transport. This capacity to modulate P-gp transport of some chemicals families seems linked to lipophilicity (19).

Modulation of Vinblastine Transport with PSC833

We also examined the action of a potent P-gp modulator, the cyclosporin analog PSC833 (1214.6 g/mol), which is one of the highest molecular weight P-gp substrates. The effect of 0–5 μM PSC833 on the transport of [^{3}H]-vinblastine in wild-type mice is shown in Fig. 4c. The brain transport fitted a sigmoid plot giving a PSC833 IC_{50} of 0.78 ± 0.02 μM, an (n) value of 35, $K_{in,min}$ of 0.25 ± 0.02 μl s^{-1} g^{-1}, and a $K_{in,max}$ of 0.56 ± 0.03 μl s^{-1} g^{-1}. A control assay showed that the brain transport of [^{3}H]-vinblastine in mdr1a(–/–) mice with 5 μM PSC833 was not statistically different from the transport of [^{3}H]-vinblastine alone in these mice.

DISCUSSION

The drugs transported by P-gp follow two patterns, one concentration-dependent (vinblastine) and the other concentration-independent (colchicine and morphine) as was shown *in situ* at the rat BBB (17). The K_{in} values for colchicine and vinblastine are identical to those measured in the rat (17). Both morphine and colchicine cross the BBB poorly, and their uptakes by the brain are not saturated up to a concentration of 2 mM. The concentrations of colchicine and morphine in the blood cannot be greater than micromolar even in acute poisoning. This unsaturated nature of P-gp–mediated colchicine and morphine transport at the BBB can thus limit the potential pharmacotoxicologic effects on the brain, whatever the systemic concentration of these drugs. As was shown in wild-type mice, the transport of colchicine and morphine is not saturated over the concentration ranges tested, although P-gp was shown to transport these compounds. In the same way, the lack of saturation measured in our study with P-gp–deficient mice does not demonstrate the involvement or not of other transporters at the luminal membrane of the BBB. But, this is unlikely for colchicine as no transporters other than P-gp are presently known to transport this drug (20). However, a probenecid-sensitive transporter different from P-gp has been reported to efflux morphine from the brain at the BBB (21).

In contrast, vinblastine is transported across the BBB by a concentration-dependent P-gp–mediated efflux. Unlike TXD258, a chemotherapeutic taxoid drug that inhibits its own P-gp–mediated efflux at the BBB *in vivo* and *in situ* (6), there is little risk of vinblastine saturating its own P-gp transport at the BBB, as the current plasma concentrations of vinblastine never exceeded nanomolar values in mice (22). The failure of some P-gp–transported substrates to show significant inhibitory potential is well illustrated in a study in which 167 diverse compounds were classified according to whether they were P-gp substrates and/or P-gp inhibitors *in vitro* (23). Vinblastine has also been shown to bind to P-gp at a site different from those of colchicine and morphine and more tightly than either morphine or colchicine (24–27). These differences are in agreement with the multiple transport sites of P-gp and indicate that vinblastine transport is of the high affinity, low capacity type, whereas morphine and colchicine transport is low affinity, high capacity. However, the way a compound binds to P-gp is not always an indication that it is transported and is not correlated with its ability to inhibit P-gp transport (23,28). Published studies have highlighted the importance of hydrogen bonding, lipophilicity, and the shape of the compounds in their ability to recognize/inhibit P-gp that could then give us some indications of how vinblastine saturates P-gp and why colchicine and morphine do not (4,19). Recent three-dimensional computational approaches and models of P-gp have yielded useful molecular information showing that the three-dimensional hydrophobic and polar surfaces of the molecules constitute elements that identify pharmacophores and can predict P-gp recognition and inhibition (24,28,29).

The saturation of P-gp by vinblastine was modeled by an Hill equation. It gave an IC_{50} close to the value measured at the rat ileum (~48 μM) and at the rat BBB (~71 μM) (17,30). The efflux of vinblastine at the mouse BBB is described by apparent K_m and V_{max}. These values are similar to those measured at the rat BBB (22 μM and 9 pmol s^{-1} g^{-1}) (17). A similar trans-influx zero study with vinblastine performed on murine fibroblasts expressing the human P-gp gave a K_m at 37 ± 10 μM (31). We extended the analysis of drug interaction by investigating the effects of a pair of substrates. Drug-drug interactions were examined using vinblastine and compounds that bind to P-gp sites distinct from the vinblastine site (verapamil, progesterone, PSC833) to take into account the multispecific drug recognition of P-gp (11–13,24,32). A wide range of concentrations of these compounds was then perfused together with 25 nM of [^{3}H]-vinblastine. The vinblastine-drug interaction series gave an IC_{50} from 0.78 (PSC833) to 154 μM (progesterone) and generated the rank order: PSC833 < (+) = (±)-verapamil < vinblastine < progesterone, similar to that observed *in vitro* (33). Verapamil and cyclosporin A, a compound very similar to PSC833, interact at the same P-gp site, which is different from the vinblastine binding site (24). PSC833 inhibits the P-gp–linked transport of vinblastine in an "all or nothing" fashion, explaining why the Hill coefficient is greatest. Although vinblastine and PSC833 bind to different P-gp sites, the high molecular weight and shape of these compounds mean that they overlap, producing steric exclusion as shown by Garrigues *et al.* (24). This situation could explain in part the modulation of the P-gp transport when vinblastine and PSC833 are both present. In contrast, verapamil, a lower molecular weight compound, could bind together with vinblastine but to a different P-gp site, to produce negative allosteric modulation (24). Nevertheless, the maximal brain transport coefficient $K_{in,max}$ is always attained in the same way as with vinblastine, whatever the interacting compound. Beside the clinical relevance of drug-drug interactions studies, the mutual effects of pairs of substrates on

P-gp transport, binding, and ATPase activity have widely been used to characterize further their interactions with P-gp. These interactions have then been used to investigate the topology of the "binding" domain of this protein (11–13,24). However, transport studies with various concentrations of the second modulator vinblastine were not done because too many mice were required. This prevented us from determining a substrate-related transport site topology and measuring the K_i values, which are more reliable than IC_{50} for comparing models (28). The progesterone-vinblastine interaction shows that *in situ* brain perfusion can also be used to evaluate the capacity of a molecule not transported by P-gp to modulate the P-gp–linked transport of one that is.

In situ brain perfusion allows sufficient exposure of the BBB to verapamil and progesterone for the P-gp–dependent transport of vinblastine to be inhibited. However, these blood concentrations of verapamil or progesterone cannot be reached by the systemic administration of verapamil or progesterone (34). Thus, in agreement with our results, intravenous or intraperitoneal administration of high doses of verapamil or quinidine do not modulate vinblastine transport into the mouse brain *in vivo* (10,35,36). In contrast, a bolus injection of 10 mg/kg of PSC833 gave a plasma concentration of 16 μM after 5 min (37) and improved the delivery of vinblastine into the brain (10). The *in situ* brain perfusion exposes endothelial brain cells to a controlled concentration of free drug. Knowledge of the free fraction in plasma is thus essential to scale the *in situ* perfusion data to the *in vivo* situation. As determined by ultrafiltration, about 97% of the PSC833 in the human plasma is bound to proteins and lipoproteins (38) and about 50% in rat plasma (37). Both in rat and humans, the free plasma fraction of PSC833 after its systemic administration can overlap the IC_{50} of 0.78 μM and lead to drug-drug interaction that could improve the delivery of vinblastine to the mouse brain as previously shown *in vivo* (10). Nevertheless, such prediction for the BBB must be regarded with some caution as the unbound fraction of some drugs can be underestimated due to a facilitated protein-bound drug dissociation in the capillary cerebral bed (39,40).

Communication between the various P-gp pharmacophores could lead to substantial drug recognition and drug-drug interactions that could be associated with undesirable brain pharmacological activity and toxicity (7,8). Although several studies on Caco-2 cells and transfected cells have suggested that mdr1a-mediated substrate transport at the BBB is correlated with MDR1 (41), more studies are needed before we can determine the relevance and predictive capacity of our mouse findings for humans. *In situ* brain perfusion of mdr1a(–/–) and wild-type mice offers significant advantages over existing conventional *in vivo* pharmacokinetic studies for determining potential drug-drug interactions and gives P-gp–mediated drug efflux parameters in a native environment without recourse to inhibitors. These results could also be readily used to compare and validate *in vitro* BBB models designed to determine P-gp transport properties and drug-drug interactions at the BBB. In conclusion, we have shown that P-gp may carry out a capacity-limited or capacity-unlimited transport at the BBB depending on the P-gp substrate. Although *in vivo* studies cannot be used for high-throughput screening but only in the development phase, *in situ* brain perfusion in mdr1a(–/–) and wild-type mice could be valuable for determining the effect of P-gp on drug transport (10) and quantifying the capacity of a compound to modulate the P-gp transport at the mice BBB.

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