In Vivo Saturation of the Transport of Vinblastine and Colchicine by P-Glycoprotein at the Rat Blood–Brain Barrier

Salvatore Cisternino,[1,3] Christophe Rousselle,[1] Marcel Debray,[2] and Jean-Michel Scherrmann[1]

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Purpose. To determine concentration-dependent P-gp–mediated efflux across the luminal membrane of endothelial cells at the blood–brain barrier (BBB) in rats.

Methods. The transport of radiolabeled colchicine and vinblastine across the rat BBB was measured with or without PSC833, a well known P-gp inhibitor, and within a wide range of colchicine and vinblastine concentration by an *in situ* brain perfusion. Thus, the difference of brain transport achieved with or without PSC833 gives the P-gp–mediated efflux component of the compound transported through the rat BBB. Cerebral vascular volume was determined by coperfusion with labeled sucrose in all experiments.

Results. Sucrose perfusion indicated that the vascular space was close to normal in all the studies, indicating that the BBB remained intact. P-gp limited the uptake of both colchicine and vinblastine, but the compounds differ in that vinblastine inhibited its own transport. Vinblastine transport was well fitted by a Hill equation giving IC_{50} at ~71 μM, a Hill coefficient (n) ~2, and a maximal efflux velocity J_{max} of ~9 pmol s^{-1} g^{-1} of brain.

Conclusions. P-gp at the rat BBB may carry out both capacity-limited and capacity-unlimited transport, depending on the substrate, with pharmacotoxicologic significance for drug brain disposition and risk of drug–drug interactions.

KEY WORDS: blood–brain barrier; *in situ* brain perfusion; P-glycoprotein; brain transport; saturation.

INTRODUCTION

The ATP-dependent P-glycoprotein (P-gp) lies on the luminal membrane of the brain capillary endothelial cells (1) that form the blood–brain barrier (BBB) and is a key element in the extrusion of structurally dissimilar compounds from the brain endothelial cells into the blood vessel lumen. But little is known about the capacity of P-gp to transport drugs across physiologic barriers, such as the BBB, *in vivo*. The P-gp–mediated transport of some substrates can be capacity limited, as can other drug transporters. Knowledge of the properties of this transport can be critical for anticipating the risks of nonlinear drug brain disposition or drug-drug interactions, depending on the systemic concentration of the P-gp substrates.

We have used the *in situ* brain perfusion method of Takasato *et al.* (2) to determine the kinetic properties of P-gp at the BBB and so obtain answers to this question. This technique, in which the physiologic properties of the BBB are maintained, can be used to measure initial transport rates and determine the mechanisms of transport and their intrinsic kinetic parameters. Two specific substrates of P-gp were studied: the high-affinity chemotherapeutic drug vinblastine and the low-affinity antigout drug colchicine. We measured the kinetics of their initial brain transport in the rat with or without a P-gp modulator, the cyclosporin analogue PSC833. We thus quantified P-gp–mediated transport across the BBB in rat and demonstrated that it can be capacity limited for vinblastine.

MATERIALS AND METHODS

Chemicals and Reagents

[^{3}H]Vinblastine (12.5 Ci/mmol) was purchased from Amersham (Orsay, France). [^{14}C]Sucrose (565 mCi/mmol) and [^{3}H]colchicine (61.4 Ci/mmol) were purchased from Perkin Elmer (Paris, France). Vinblastine sulfate was from Lilly (St Cloud, France), and colchicine from Fluka (St. Quentin, France). PSC833 was a gift from Novartis (Basel, Switzerland). All other chemicals were of analytic grade.

Animals

The studies were performed on adult male Sprague-Dawley OF1 rats, 6–8 weeks old, obtained from Iffa-Credo (L'Arbresle, France). The rats were housed in a controlled environment with a 12-h dark:light cycle. They had access to food and tap water *ad libitum*. All experimental procedures complied with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

In Situ Brain Perfusion

Surgery and Perfusion

The transport of [^{3}H]colchicine and [^{3}H]vinblastine into the brain was measured by *in situ* brain perfusion (2,3). Briefly, rats were anesthetized by intraperitoneal injection of ketamine-xylazine (70/6 mg/kg ip). The right external carotid branch and occipital artery were ligated, and the cerebral hemisphere was perfused through the right common carotid artery (polyethylene tubing 0.76 mm i.d. × 1.22 mm o.d.). The syringe containing the perfusion liquid (buffered Krebs-bicarbonate) was placed in an infusion pump and connected to the catheter. Before perfusion, the thorax of the animal was opened, the heart was cut, and perfusion immediately started with a flow rate of 10 ml/min. The perfusion buffer was gassed to pH 7.4 with 95% O_2/ 5% CO_2 and warmed to 37°C. Each rat was perfused with [^{3}H]tracer and [^{14}C]sucrose (0.2 μCi/ml) to check the physical integrity of the BBB. Perfusion was carried out for 90 s in experiments with colchicine and for 120 s in transport studies with vinblastine. It was terminated by decapitating the rat. The brain was removed from the skull and dissected out on ice. The right cerebral

[1] INSERM U26, Hôpital Fernand Widal, 200 Rue du Faubourg Saint-Denis, 75475 Paris Cedex 10, France.

[2] Faculté de Pharmacie, Département de Biomathématiques, 4 avenue de l'Observatoire, 75006 Paris, France.

[3] To whom correspondence should be addressed. (email: Salvatore.Cisternino@fwidal.inserm.fr)

ABBREVIATIONS: BBB, blood–brain barrier; CNS, central nervous system; K_{in}, transport coefficient; MDR, multidrug resistance; P-gp, P-glycoprotein.

hemisphere and aliquots of the perfusion fluid were placed in tared vials and weighed. Samples were digested in 2 ml of Solvable (Packard, Rungis, France) at 50°C and mixed with 9 ml of Ultima gold XR (Packard). Dual-label counting was performed in a Packard Tri-Carb 1900TR.

Transport Studies

The initial rates of [^{3}H]drug (0.2–0.3 μCi/ml) transport into the rat brain were measured at different drug concentrations. The perfusion fluid contained labeled compound with or without unlabeled drugs to produce an appropriate drug concentration. The stock solutions of vinblastine and colchicine were prepared in 0.9% NaCl, and PSC833 (3 μM) was dissolved in DMSO. All these solutions were freshly prepared 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); this concentration did not alter the integrity of the BBB, as measured by the permeability of [^{14}C]sucrose.

Calculation of BBB Transport Parameters

All calculations have been described previously (2,3). The brain vascular volume (V_{vasc}; μl/g) was estimated from the tissue distribution of [^{14}C]sucrose, which diffuses very slowly across the BBB, using the following equation:

$$V_{vasc} = \frac{X^*}{C^*_{perf}} \tag{1}$$

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

Transport across the BBB was expressed in terms of three parameters: the apparent volume of distribution (V_{brain}; μl/g), 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:

$$V_{brain} = \frac{X_{brain}}{C_{perf}} \tag{2}$$

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

$$X_{brain} = X_{tot} - V_{vasc}C_{perf} \tag{3}$$

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

Initial brain transport was expressed as a K_{in} (μl s^{-1} g^{-1}) and was calculated from:

$$K_{in} = \frac{V_{brain}}{T} \tag{4}$$

where T is the perfusion time (s).

We measured the time course of the brain distributions in rats to select an appropriate perfusion time, one that permitted us to measure the initial rate of uptake and that ensured that at least 40% total radioactivity in the tissue was outside the vascular space (3).

The flux J_{net} (pmol s^{-1} g^{-1}) was calculated assuming that K_{in} values were flow-independent for K_{in} lower than ~16 μl s^{-1} g^{-1} (3) according to:

$$J_{net} = K_{in}C \tag{5}$$

where C is the total drug concentration (labeled and unlabeled) in the perfusate.

BBB Transport Modeling

Kinetic and statistical analyses were carried out using Systat 5.01 software (Systat Inc., IL, 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:

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

which is equivalent to:

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

where C is drug concentration, $K_{in,min}$ is the minimal and $K_{in,max}$ the maximal brain K_{in} value for the labeled substrate, n is the Hill coefficient, and IC_{50} is the concentration at which half-inhibitory effect on brain transport was achieved.

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

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

The brain flux could be expressed as the result of two components:

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

where:

$$J_{in} = K_{in,\max}C$$

$$J_{efflux} = \frac{(K_{in,\max} - K_{in,\min})IC_{50}^nC}{IC_{50}^n + C^n} \tag{8}$$

When P-gp is saturated with PSC833 or vinblastine, J_{net} can be written as an independent P-gp–saturable efflux transport, and Eq. (7) becomes a nonsaturable diffusion equation:

$$J_{net} = J_{in} = K_{in,\max}C \tag{9}$$

If the concentration C of substrate is low in comparison with IC_{50}, Eq. (7) becomes:

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

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 was obtained by subtracting the brain flux (J_{net}) calculated with Eqs. (9) and (7) at different concentrations in rats also perfused with the P-gp modulator PSC833. The P-gp maximal pumping velocity (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 $J_{efflux} = J_{max}/2$

J_{efflux} was calculated by subtracting Eqs. (10) and (7) when the substrate concentration was low compared to IC_{50} and there was no saturation, which results in:

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

All values are presented as means ± standard deviations (SD) for four to six rats except those for 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 unpaired *t* test was used to identify significant differences between groups when appropriate. All the tests were two-tailed, and statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Colchicine and vinblastine, two P-gp substrates, had the same intrinsic transport through the rat BBB, with a $K_{in,min}$ of 0.24 ± 0.01 μl s^{-1} g^{-1} for both drugs. These values are identical to those measured by *in situ* brain perfusion in wild-type mice (4). The studies with the P-gp modulator PSC833 showed that K_{in} increased significantly to 0.43 ± 0.03 μl s^{-1} g^{-1} for [^{3}H]vinblastine and 0.57 ± 0.05 μl s^{-1} g^{-1} for [^{3}H]colchicine, so that the brain transport of vinblastine increased about twofold and that of colchicine about 2.5-fold (Figs. 1 and 2). PSC833, a potent P-gp modulator, was previously shown to completely and specifically inhibit the P-gp–mediated efflux component of vinblastine and colchicine, as shown in wild-type and P-gp-deficient mice (4). Thus, their poor brain penetration could be partly related to P-gp–mediated efflux at the BBB. Several authors have used mice with and without P-gp to show that the brain distribution of a large number of drugs can be altered by a defective P-gp (5). Most of these conventional pharmacokinetics studies measured the P-gp effect by calculating the ratio of the brain concentrations in P-gp–deficient and wild-type mice at a given time. But no studies have carefully determined whether this P-gp effect can be capacity-limited *in vivo.* Exceeding the P-gp transport capacity at the BBB might have pharmacotoxicologic significance for drug brain disposition linearity and risk of drug-drug interactions. *In situ* brain perfusion can be used to assay a wide range of P-gp substrates at known concentrations, as was done for these two P-gp substrates.

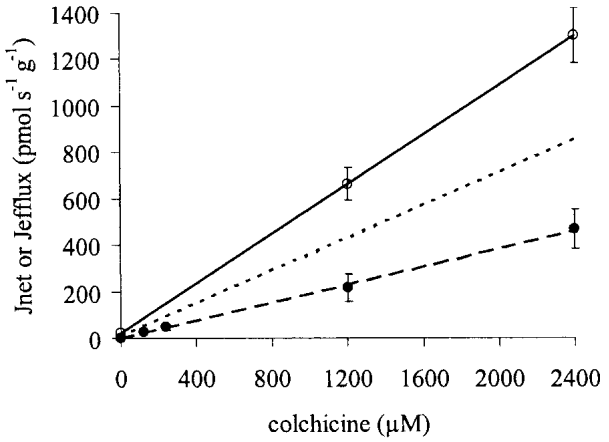

Fig. 1. The brain fluxes of colchicine were measured in rats perfused with colchicine alone (dotted line) and rats perfused with both colchicine and PSC833 (3 μM; solid line) by *in situ* brain perfusion. Rats were perfused via the common carotid artery for 90 s with colchicine (with or without PSC833). Lines were computer-fitted using Eq. (9) for rats perfused with colchicine and PSC833 and Eq. (10) for rats perfused with colchicine alone. Subtracting the brain flux (J_{net}) measured in rats with or without PSC833 gave the component of mdr1a pumping at the BBB (J_{efflux}, stippled line) that fitted Eq. (11). The values are means ± SD for four to six rats.

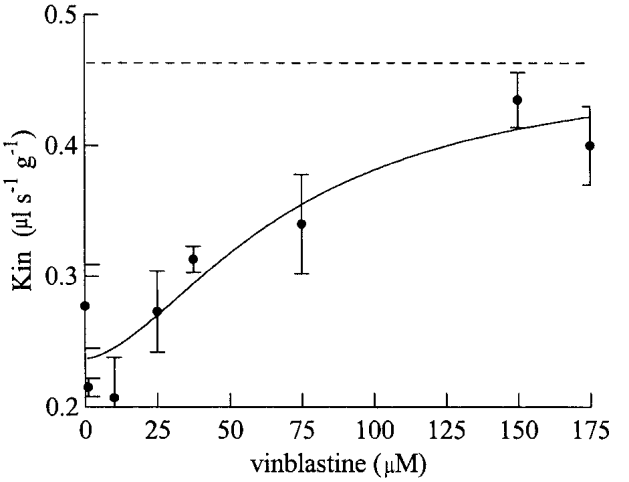

Fig. 2. The brain transport of [^{3}H]vinblastine (25 nM), expressed as a transport parameter K_{in} (μl s^{-1} g^{-1}), was measured in rats (solid line) over a range of vinblastine concentrations and in rats perfused with vinblastine plus PSC833 at 3 μM (dotted line). The sigmoid relationship between the [^{3}H]vinblastine K_{in} and total vinblastine concentration perfused via the common carotid artery for 120 s was fitted by the Hill equation [Eq. (6)]. All values are means ± SD of four to six rats.

We find two patterns of P-gp–linked drug transport, a concentration-dependent one for vinblastine and a concentration-independent one for colchicine. In all experiments, the distribution volume of [^{14}C]sucrose (15 ± 2 μl/g) agreed with normal values (6), indicating that BBB integrity was maintained during the transport experiments. It was not possible to measure colchicine and vinblastine transport up to 3 mM and 200 μM, respectively, because of opening of the BBB, as suggested by the increase in the vascular sucrose space (data not shown). Nevertheless, because therapeutic or even toxic systemic concentrations of colchicine are never more than nanomolar, it appears impossible to exceed the P-gp capacity. In contrast, vinblastine is transported across the BBB by a concentration-dependent P-gp–mediated system whose transport kinetics were fitted by a Hill equation: IC_{50} of 71.3 ± 39 μM and Hill slope (n) of 1.7 ± 0.7 (Fig. 2). This was not a result of disruption of the BBB because the vascular space of [^{14}C]sucrose remained normal. Also, the $K_{in,max}$ obtained with high vinblastine concentrations was 0.46 ± 0.08 μl s^{-1} g^{-1}, a value not different from that obtained with PSC833 inhibition. However, there is little risk of saturating the P-gp transport of vinblastine at the BBB *in vivo* because the current therapeutic plasma concentration never exceeds

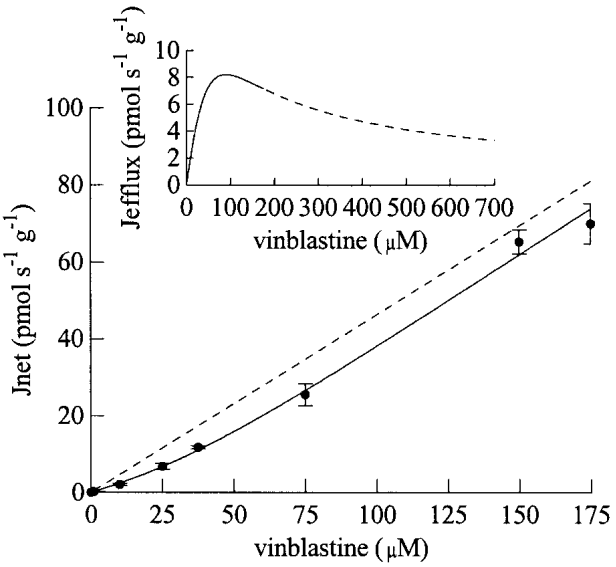

Fig. 3. The brain fluxes (J_{net}) for vinblastine (solid line) in rats perfused with vinblastine alone and in rats perfused with vinblastine plus PSC833 (3μM) (dotted line) were calculated from results obtained by *in situ* brain perfusion. Rats were perfused via the common carotid artery for 120 s. Lines were computer-fitted using Eq. (8) for rats treated with vinblastine plus PSC833 and Eq. (7) for rats treated with vinblastine alone. Inset: Subtracting the brain fluxes (J_{net}) measured in rats with or without PSC833 gave the component of mdr1a pumping at the BBB (J_{efflux}) that fitted Eq. (8). The values are means ± SD for four to six rats.

nanomolar range in rodents. Like colchicine, P-gp can protect the brain whatever vinblastine concentration is given.

Nevertheless, this saturable P-gp efflux has provided new insights into the P-gp efflux mechanisms acting at the BBB *in vivo*. The differences in transport capacity and inhibition properties of these two drugs may be related to the presence of several distinct drug-binding sites on P-gp, possibly overlapping or allosterically coupled (7). However, the binding of compound to P-gp is not always an indication that it is transported, as transport and binding properties can be independent (8,9). Hence, like mdr cells (10), the *in vivo* resistance to colchicine is similar to that of vinblastine, although their P-gp binding K_d for colchicine is very low (158 μM), and they bind vinblastine tightly (0.77 μM) (10). This suggests that P-gp expressed at the rat brain endothelial cells could be similarly resistant to drugs with different binding affinities for P-gp. *In vitro* studies also suggest that the resistance level of P-gp could be correlated to the density of P-gp expressed at the membrane as for vinblastine but not for some P-gp substrates such as colchicine, where the resistance level was not found correlated to the density of P-gp (11). Hence, Druley *et al.* (11) suggested that other factors, such as the cell environment, could alter the ability of P-gp to confer drug resistance.

This attempt to explain the mechanisms underlying the colchicine and vinbalstine transport and saturation of P-gp does not impede the modeling of its transport. Our *in vivo* kinetic data are very similar to those for the *in vitro* transport of vinblastine and daunorubicin, where the Hill coefficient was around 2–5, probably reflecting in part the cooperative nature of the substrate interaction with P-gp (12). The IC_{50} for vinblastine measured in our study is also very close to the IC_{50} at the rat ileum, ~48 μM (13). The efflux rate J_{efflux} of vinblastine (Fig 3, inset) was described by a bell-shaped curve giving a V_{max} of 8.8 ± 0.7 pmol s^{-1} g^{-1}, a half-maximal increment K_{m1} of 21.7 and extrapolated decrease K_{m2} of 598 μM. This indicates that the maximal velocity of the transport process decreased at high substrate concentrations, as sometimes occurs for enzyme kinetics in drug metabolism. This type of bell-shaped curve has been found for some drugs, including vinblastine, to describe the kinetics of the ATPase activity for P-gp (14,15). The rate of ATP hydrolysis suggested that two drug-binding sites, one stimulatory and one inhibitory, could be responsible for this biphasic and bell-shaped response of ATPase (16,17), thus giving this transport velocity pattern (18). Moreover, the release of ADP from P-gp is also a rate-limiting step in the catalytic ATPase cycle. This could limit the recovery of the substrate binding to P-gp (19), so explaining why the rate of ADP dissociation limits P-gp–dependent transport at elevated vinblastine concentrations.

This capacity-limited transport at the BBB implies that drug–drug interactions at P-gp must be carefully investigated because of the communication between the various drug-binding sites on P-gp, which could lead to substantial drug recognition and drug–drug interactions. These can be associated with undesirable brain pharmacologic activity and toxicity (20). Beside this negative effect, inhibiting P-gp could also be a way of overcoming the P-gp efflux at the BBB, resulting in improved delivery of drugs to the CNS. Several studies on Caco-2 cells and transfected cells have suggested that mdr1a-mediated substrate transport at the BBB is correlated with MDR1 (21), suggesting that rodents could be a predictive model for humans. The similar values for IC_{50} at the rat BBB and the rat intestine for vinblastine P-gp transport require more studies with more compounds to determine any correlation between the P-gp–mediated effluxes of drugs in these two tissues. It has also been shown that the IC_{50} of digoxin P-gp–mediated efflux at the rat ileum and human colon are similar, suggesting that "the rat might well be a quantitatively predictive model of human intestinal efflux" mediated by P-gp (13). However, the presence of several distinct drug-binding sites on P-gp (7) may confuse any attempt at defining a single rule for extrapolation between species.

Thus, we find that P-gp at the BBB may carry out both capacity-limited and capacity-unlimited transport, depending on the substrate. This, together with the risk of drug–drug interaction, could make P-gp an important influence on CNS drug distribution and variations in their action.

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