Apparent Lack of Mrp1-Mediated Efflux at the Luminal Side of Mouse Blood–Brain Barrier Endothelial Cells

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Purpose. The purpose of this work was to determine mrp1-mediated efflux across the luminal membrane of endothelial cells at the blood–brain barrier (BBB) in mice.

Methods. The transport of radiolabeled etoposide, 17β-estradiol-D-17β-glucuronide ($E_2$17βG), vincristine, and doxorubicin across the BBB of mrp1(–/–) and wild-type mice was evaluated by *in situ* brain perfusion. Etoposide transport was also determined in P-glycoprotein-deficient mdr1a(–/–) mice perfused with both etoposide and mrp1 inhibitors like probenecid or MK571. Cerebral vascular volume was determined by co-perfusion with labeled sucrose.

Results. Sucrose perfusion indicated that the vascular space was close to normal in all the studies, indicating that the BBB remained intact. The transport of etoposide, $E_2$17βG, vincristine, and doxorubicin into the brain was not affected by the lack of mrp1. Trans-efflux studies in mrp1-deficient mice with etoposide and $E_2$17βG confirmed that mrp1 was not involved in the efflux of these substrates across the BBB. There was also a significant P-gp-mediated efflux of etoposide in studies with P-glycoprotein-deficient mdr1a(–/–) mice. Perfusion of mdr1a(–/–) mice etoposide plus probenecid or MK571 did not affect the brain transport of etoposide.

Conclusion. Efflux mediated by mrp1 does not seem to occur across the luminal membrane of the endothelial cells forming the mouse BBB.

KEY WORDS: mrp1; blood–brain barrier; etoposide; P-glycoprotein; *in situ* brain perfusion.

INTRODUCTION

The blood–brain barrier (BBB), which is formed by tight junctions between brain capillary endothelial cells, restricts the movement of compounds from the circulating blood into the brain. Efflux transport systems, such as the P-glycoprotein (P-gp), also act as a barrier at the BBB. Three drug extrusion pumps belonging to the ATP-binding cassette (ABC) transporter have been found in human and rodent brains, the P-gps, the multidrug resistance-associated proteins (MRPs; Ref. 1), and the breast cancer resistance protein (BCRP; Ref. 2). P-gp was first found on the luminal side of endothelial cells (3) and is believed to limit the entry of a wide spectrum of compounds into the brain. The precise functions and locations of the other ABC transporters, the MRPs, and BCRP at the BBB and on brain parenchyma cells have yet to be determined.

The mRNAs encoding MRP1 and MRP5 have been detected in the normal human brain (4) whereas mRNAs from MRP3 and MRP4 have been found in human glioma cells (5). MRP1 is present on the endothelial cells of microvessels in the brains of cattle and rats (6,7) and on the epithelial cells of the human and rat choroid plexus (8,9). Zhang *et al.* (10) recently used reverse transcription polymerase chain reaction analysis to detect the expression of MRP1, MRP4, MRP5, and MRP6 in both primary cultures of bovine brain microvessel endothelial cells (BMECs) and in capillary-enriched fractions of homogenates. Nevertheless, the functions of several MRP homologues in the brain microvessel endothelial cells remain to be investigated. They could either limit the uptake of various MRP substrates by the brain or remove similar compounds from it.

P-gp transports amphipathic, cationic, and neutral compounds across membranes in their native forms whereas mrp1 transports amphipathic anions like glutathione conjugates (LTC_4), glucuronates, and sulfates, such as 17β-estradiol-D-17β-glucuronide ($E_2$17βG) and dianionic bile salts, respectively (11). However, mrp1 can also actively transport certain unmodified and unconjugated cationic (vincristine, doxorubicin) and neutral (etoposide) compounds using a glutathione co-transport mechanism (12–16).

Mice lacking the genes encoding mdr1, such as mdr1a(–/–) or mdr1a/1b(–/–) strains, are good experimental models for examining the role of P-gp in the uptake of xenobiotics by the brain (for review, see Ref. 17). We have also used *in situ* brain perfusion in P-gp-deficient mdr1a(–/–) mice (18) to screen substrates that are transported across the BBB by P-gp and/or modulate its activity (19). The present study was done on mrp1(–/–) and mdr1a(–/–) mice; we used anionic and non-anionic substrates and modulators of mrp1 and *in situ* brain perfusion to investigate the function of mrp1 at the BBB. The results indicate that the mrp1 at the luminal side of the mouse brain endothelial cells does not contribute to drug transport.

MATERIALS AND METHODS

Drugs and Chemicals

[^{3}H]-vincristine (6.6 Ci/mmol) and [^{14}C]-doxorubicin (55 mCi/mmol) were purchased from Amersham Pharmacia Biotech (Orsay, France). [^{14}C]-sucrose (401 mCi/mmol), [^{3}H]-sucrose (12.3 Ci/mmol) and [^{3}H]-estradiol 17β-D-glucuronide ([^{3}H]-$E_2$17βG; 40.5 Ci/mmol) were from Perkin–Elmer NEN Life Sciences (Brussels, Belgium). [^{3}H]-etoposide (411 mCi/mmol) was from Moravek Biochemicals (Brea, CA, USA). (±)-Verapamil hydrochloride, etoposide and probenecid were from Sigma (St Quentin Fallavier, France). MK571 was kindly provided by Merck Frosst (Kirkland, Canada). Dimethyl sulfoxide was from Merck Eurolab (Strasbourg, France). All other chemicals were of analytical grade.

Animals

Adult male CF-1 mice [mdr1a(+/+) and mdr1a(–/–) mice, 30–40 g, 6–8 weeks old] were bred in-house from progenitors genotyped for mdr1a P-gp that were initially ob-

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tained from Charles River Laboratories (Wilmington, MA, USA). The targeted disruption of the mrp1 locus and generation of mrp1(–/–) mice (129 × c57/BL6 genotype) has been reported (20). The study was performed on sex and age-matched 6- to 8-week-old mice of the F5 generation. No expression of the MRP1 protein was observed in all examined tissues and organs of mrp1(–/–) mice (20). Animals were housed in a room with a controlled environment (22 ± 3°C; 55 ± 10% relative humidity) with a 12-h dark:light cycle (light 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 "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

In Situ Brain Perfusion

Surgical Procedure and Transport Studies

We measured blood–brain transport using the *in situ* brain perfusion technique (18). Mice were anesthetized by intraperitoneal injection of xylazine (Bayer, Puteaux, France; 8 mg/kg) and ketamine (Parke Davis, Courbevoie, France; 140 mg/kg). The common carotid artery was ligated caudally. The external carotid was ligated rostral to the occipital artery at the level of the bifurcation of the common carotid artery. The right common carotid was catheterized with polyethylene tubing (0.30 mm inner diameter × 0.70 mm outer diameter, Biotrol Diagnostic, Chennevières-les–Louvre, France) filled with heparin (25 U/mL), and mounted on a 26-gauge needle. The thorax of the animal was opened and the heart was cut just before starting perfusion. The syringe containing the perfusion fluid was placed in an infusion pump (Harvard pump PHD 2000, Harvard Apparatus, Holliston, MA, USA) and connected to the catheter. The perfusion rate was 2.5 mL/min. The perfusion fluid consisted of bicarbonate-buffered physiologic 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 to pH 7.4 with 95% O_2/5% CO_2 and warmed to 37°C in a water bath. Tracers (0.2–0.5 μCi/mL) were added to the perfusate. Perfusion was terminated by decapitation at the selected time. The brain was removed from the skull and dissected out on ice. The 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 (Packard). The dual labels were counted in a Packard Tri-Carb model 1900 TR (Packard).

Trans Influx and Efflux Zero Transport Experiments

We first measured the trans-influx zero brain transport of [^{3}H]-vincristine (50 nM), [^{3}H]-etoposide (0.7 μM), [^{14}C]-doxorubicin (3 μM), and [^{3}H]-$E_2$17βG (7 nM) in wild-type mrp1(+/+) and mrp1(–/–) mice using a perfusion time of 120 s.

To study the trans-efflux zero transport, the mice were first loaded with [^{3}H]-etoposide or [^{3}H]-$E_2$17βG, which was followed by a "washing procedure" in mrp1(–/–) and wild-type mice. Syringe "A" of a dual-syringe pump contained the bicarbonate-buffered physiologic saline plus the radiotracer, whereas syringe "B" contained tracer-free bicarbonate buffered physiologic saline. The carotid and syringe catheters were connected to a four-way valve (Hamilton, Bonnaduz, Switzerland). The carotid was cannulated and the appropriate connections were made. Syringe A was then discharged at 2.5 mL/min for 120 s. Syringe A was switched off and syringe B was switched on for 60 s to wash-out the capillary space. The mouse was decapitated and its brain removed for radioactivity count.

We also measured the trans-influx and trans-efflux zero brain transports of etoposide in P-gp-deficient mdr1a(–/–) and wild-type mice with and without co-perfusion of probenecid (1 mM) or MK571 (50 μM) in the perfusion fluid.

Calculation of BBB Transport Parameters

Brain vascular volume (V_{vasc}; μL/g) was estimated from the tissue distribution of [^{14}C]- or [^{3}H]-sucrose, which diffuses very slowly across the BBB, using the following equation:

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

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

Transport across the BBB is expressed in terms of two parameters: the apparent volume of distribution (V_{brain}) and the transport coefficient (K_{in}).

The apparent volume of distribution was calculated from the amount of radioactivity in the right brain hemisphere using the equation:

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

where X_{brain} (dpm/g) is the calculated amount of [^{14}C]- or [^{3}H]-tracer in the right cerebral hemisphere and C_{perf} (dpm/μL) is the tracer concentration in the perfusion fluid. Brain tissue radioactivity was corrected for vascular contamination with the equation:

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

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

Brain uptake also was expressed as a blood–brain transfer coefficient K_{in} (μL/s/g) calculated from the following:

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

where T is the total perfusion time (s).

The perfusion time used in single time in trans-influx zero studies was long enough to ensure that at least 40% of the total radioactivity in the tissue was outside of vascular space ($X_{brain} \geq 0.4\ X_{tot}$; Ref. 21).

Statistical Analysis

Data are presented as means ± standard deviation (SD) for three to six animals unless otherwise specified. Student's unpaired t test was used to identify significant differences between groups when appropriate. All tests were two-tailed and statistical significance was set at $p < 0.05$.

RESULTS

Assessment of BBB Integrity

The physical integrity of the BBB and the brain vascular space were checked with radiolabeled sucrose. This radioactive marker was perfused with mrp1 substrate to detect any opening of the BBB by these drugs or any other changes in BBB integrity after the disruption of the ABC transporter in the genetically deficient mice. The vascular sucrose volumes of the mrp1-deficient mice (15.8 ± 1.2 μL/g) and the wild-type mice (16.3 ± 1.3 μL/g) did not differ significantly, and were similar to published values (18,19).

Blood-Brain Transport Coefficients for 4 mrp1 Substrates

The blood–brain transfer coefficients (K_{in}) of the four compounds tested after 120 s of brain perfusion in deficient mrp1(–/–) and wild-type mice are shown in Fig. 1. The K_{in} values ranged from 0.06 μL/s/g (vincristine) to 0.22 μL/s/g (doxorubicin). The initial brain uptake of each compound was not affected by the lack of mrp1.

Blood-Brain Transport of [^{3}H]-E_2 17βG and [^{3}H]-Etoposide in Wild-Type and Mrp1(–/–) Mice

The efflux transport of [^{3}H]-$E_2$17βG and [^{3}H]-etoposide was measured in both deficient and proficient mrp1 mice with and without the wash-out procedure. The wash-out procedure did not significantly influence the brain Kin of [^{3}H]-$E_2$17βG in wild-type (0.15 ± 0.01 μL/s/g) or mrp1-deficient mice (0.14 ± 0.03 μL/s/g) as compared, respectively, to the wild-type (0.16 ± 0.02 μL/s/g) or mrp1-deficient mice (0.14 ± 0.02 μL/s/g) that did not receive the wash-out procedure. Moreover, the [^{3}H]-$E_2$17βG Kin coefficients for the mrp1(–/–) and wild-type mice determined by the wash-out procedure were not significantly different, despite the difference in mrp1 status. The transport of [^{3}H]-etoposide was measured under similar conditions. Unlike [^{3}H]-$E_2$17βG, the [^{3}H]-etoposide K_{in} was affected by the wash-out procedure in both deficient or proficient mrp1 mice (Fig. 2). Moreover, the [^{3}H]-etoposide K_{in} values obtained with wash-out were similar for the mrp1-deficient mice and the wild-type mice. This suggests that the etoposide efflux could be caused by another efflux transporter and/or to passive retrodiffusion into the capillary space.

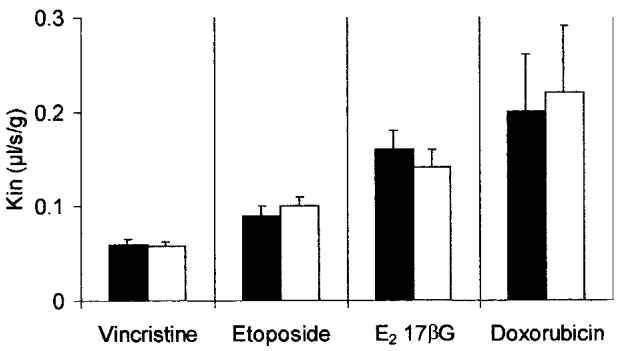

Fig. 1. Brain transport coefficients (K_{in}, μL/s/g) of 4 mrp1 substrates in wild-type (solid columns) and mrp1(–/–) mice (empty columns) measured by *in situ* brain perfusion. The mice were perfused via the common carotid artery for 120 s. Data are means ± SD of four to six animals. We found no statistically significant differences between wild-type and mrp1(–/–) for any of the compounds.

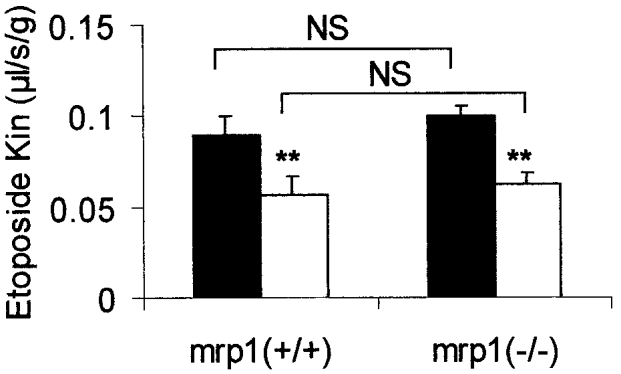

Fig. 2. The brain transport coefficient (K_{in}, μL/s/g) of [^{3}H]-etoposide was measured by *in situ* brain perfusion in wild-type and mrp1(–/–) mice. [^{3}H]-etoposide was perfused for 120 s (solid columns). The empty columns indicate the mice given a 60 s tracer free wash-out after the 120 s of brain perfusion with [^{3}H]-etoposide. Data are means ± SD for 4 – 6 mice. ** $p < 0.01$ comparing wash-out and nonwash-out groups of each strain.

Influence of mdr1a P-Glycoprotein on the Blood–Brain Transport of [^{3}H]-Etoposide

We examined the capacity of P-gp to modulate the blood-brain transport of etoposide by perfusing wild-type and P-gp-deficient mdr1a(–/–) mice, with [^{3}H]-etoposide for 120 s. The K_{in} for [^{3}H]-etoposide was significantly ($p < 0.001$) 1.5-fold greater in mdr1a(–/–) mice (K_{in} = 0.12 ± 0.01 μL/s/g) than in the wild-type mice (K_{in} = 0.08 ± 0.01 μL/s/g). We then assessed the effect of P-gp on efflux by measuring the trans-efflux of [^{3}H]-etoposide in mdr1a(–/–) and wild-type mice. Wash-out significantly decreased the transport of [^{3}H]-etoposide in wild-type mice, but had no significant effect on the [^{3}H]-etoposide Kin of mice lacking P-gp mdr1a (Fig. 3).

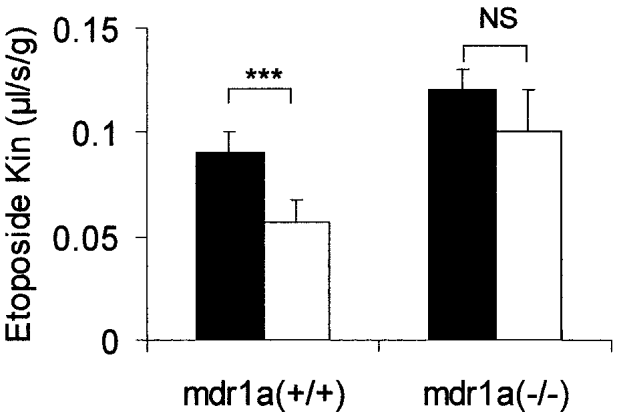

Fig. 3. The brain transport coefficient (K_{in}, μL/s/g) of [^{3}H]-etoposide was measured by *in situ* brain perfusion. Wild-type and mdr1a(–/–) mice were perfused with [^{3}H]-etoposide for 120 s (solid columns). The empty columns indicate mice that were given a 60-s tracer-free wash-out after 120 s of brain perfusion with [^{3}H]-etoposide. Data are means ± SD for four to six mice. ***$p < 0.001$ comparing washout and nonwash out group of each mouse strain.

Effect of Probenecid and MK571 on the Blood–Brain Transport of [^{3}H]-Etoposide in P-gp mdr1a(–/–) Deficient Mice

We used mdr1a(–/–) mice to minimize the contribution of P-gp to the transport of [^{3}H]-etoposide. The K_{in} for [^{3}H]-etoposide were measured in trans-influx or -efflux zero conditions with or without co-perfusion of modulators known to inhibit mrp1 transport. The K_{in} for [^{3}H]-etoposide were not significantly different with or without probenecid (1 mM) in the perfusion fluid (Fig. 4). Adding 50 μM MK571 to the perfusion fluid also had no effect on the transport of [^{3}H]-etoposide measured with wash-out.

DISCUSSION

There has been no clear indication to date of how mrp1 may influence the transport of xenobiotics across the BBB. Although mrp1 seems to be present in primary cultures of rodent BMEC (7), the *in vivo* function of mrp1 needs to be assessed because cells in culture frequently overproduce certain proteins. The luminal or abluminal location of mrp1 in polarized endothelial cells like those of the BBB must be also determined. If the BMEC produce mrp1, its substrates might be transported in two ways. The first assumes that mrp1 is anchored, like P-gp, in the luminal membrane of the BMECs. The substrates would then be transported from the brain to the blood in a same way as those of P-gp. Hence mrp1 could be another restrictive determinant of BBB permeability. If however, mrp1 is present at the abluminal membrane of the BMEC, its substrates could be transported from the cytosol of the BMECs into the brain extracellular fluid. These two possible mrp1 location on the endothelial cells are exclusive because ABC proteins more often transport their substrates from the cell cytosol or plasma membrane to the outside using the energy source provided by the cytosolic ATP catalytic domains. The mrp1 protein has been found on the basolateral side of the epithelial cells of the choroid plexus. It has also been detected in many other tissues, including the basal layer of the oropharyngeal mucosa, the bronchial epithelium and the basal membrane of the testicular Sertoli cells (Ref. 22, for a review, see Ref. 23). *In situ* brain perfusion can only investigate the transport of compounds at the luminal membrane of endothelial cells. Because mrp1, unlike P-gp, is generally found on the basolateral membrane of polarized cells it is more likely to be on the abluminal side of BMECs. Several known mrp1 substrates were assayed, some of them, like vincristine, doxorubicin and etoposide, are also substrates of P-gp. This is why we also used P-gp-deficient mice to isolate the action of P-gp from that of the hypothetical mrp1 in the blood–brain transport of several substrates.

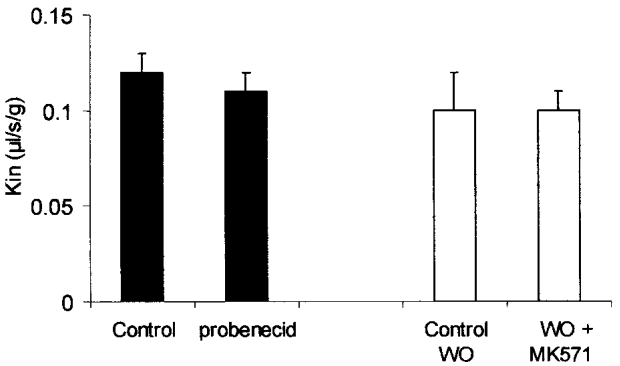

Fig. 4. Brain transport coefficient (K_{in}, μL/s/g) for [^{3}H]-etoposide. P-gp-deficient mdr1a(–/–) mice were perfused with [^{3}H]-etoposide, with or without 1 mM probenecid, for 120 s (solid column). The empty columns represent mice that were given a 60 s tracer free wash-out (WO) with or without MK571 (50 μM) after a brain perfusion of [^{3}H]-etoposide with or without coperfusion of MK571 (50 μM). Data are means ± SD for four to six mice.

The *in situ* perfusion of the brain with vincristine, doxorubicin, and etoposide, three known unmodified and overlapping substrates of both P-gp and mrp1, indicates that the entry of these three substrates into the brain is not controlled by mrp1. The transport of the glucuronate conjugate $E_2$17βG, which is transported by mrp1 and by at least two organic anion transporters (like OATP2 and OAT; Ref. 24) but not by P-gp, was not affected by the mrp1 status. We looked more specifically at the efflux mediated by these ABC pumps using a trans-efflux zero study with etoposide and $E_2$17βG. This experimental procedure showed no mrp1-mediated efflux of $E_2$17βG. The brain transport of etoposide was affected by the wash-out procedure in both wild-type and mrp1-deficient mice, suggesting that an other efflux transporter is involved, or that [^{3}H]-etoposide binds non-specific to the luminal membrane of the endothelial cells and was removed by the wash-out. Etoposide is a substrate of P-gp (25) unlike $E_2$17βG. Our brain trans-efflux zero study of etoposide transport in P-gp-deficient mdr1a(–/–) mice confirmed that the efflux of etoposide from the brain is due mainly to P-gp-mediated transport at the luminal membrane of the endothelial cells. We also measured mrp1-linked etoposide efflux with known inhibitors like the broad-specific organic anion transporter inhibitor probenecid, and a more specific mrp1 inhibitor, MK571, co-perfused with [^{3}H]-etoposide in P-gp-deficient mdr1a(–/–) mice to circumvent the significant contribution of P-gp. The transport of etoposide was not modulate in trans-influx or trans-efflux zero studies, despite the inclusion of mrp1 inhibitors. Hence, the P-gp at the BBB may well be critical for the brain disposition of etoposide in the brain, and mrp1 may make no significant contribution. Previous studies support our findings. Wijnholds *et al.* (26) used mrp1(–/–) single knockout and mrp1(–/–), mdr1a/1b(–/–) triple knockout mice in a conventional pharmacokinetic study and demonstrated that a lack of the mrp1 transporter did not influence the amount of etoposide in the whole brain. They concluded that mrp1 does not limit the entry of etoposide into the brain. However, the concentration of etoposide in the cerebrospinal fluid of the triple knockout mice was higher than in the cerebrospinal fluid of the P-gp-deficient mice. This may indicate that mrp1 acts as an efflux pump at the choroid plexus (26), as indicated by *in vitro* and *in vivo* studies with other compounds like ^{99m}Tc-sestamibi (8). Studies using intraperitoneal arsenite, another efficiently transported mrp1 substrate, showed no significant difference in the whole brains of mrp1(–/–) and wild-type mice (27). However, one study on rodents suggested that mrp1 at the BBB was involved in efflux (28). Tamai *et al.* showed that, grepafloxacin, a new quinolone antibacterial agent, was effluxed from the brains of mice and rats *in vivo* and a study on immortalized rat brain capillary endothelial cells showed that they had both P-gp and mrp1 and another anion-efflux transporter sensitive to 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and bicarbonate

(28). The authors concluded that several efflux transporters are involved in the efflux of quinolones from the brain. It is possible that another MRP isoform, such as mrp2 that transports several quinolones like grepafloxacin, and their conjugated metabolites (29–31), may also be involved since confocal transport experiments have shown that mrp2 is an efflux transporter in the luminal membrane of isolated porcine brain microvessels (32,33). Nevertheless, *in situ* brain perfusion of grepafloxacin in the rat, showed no influence of mrp1 efflux (28). We therefore conclude that the possibility of mrp1 at the luminal side of the BMEC should be discarded.

In vivo experiment to characterize transport-mediated efflux across the BBB can be complicated by factors such as the presence of other transporters like the multiple members of organic anion and cation families, which have overlapping interactions with many drugs. Although animals lacking specific transporters are ideal for evaluating the contribution of each transporter to drug resistance, the basal production or up-regulation of other transporters could mask or reveal other transport pathways, so preventing arrival at a clear conclusion. The particular cellular architecture of the BBB may add another confusing element. The concentration of mrp1 is higher in rat primary cultured astrocytes than in endothelial cells, suggesting that its preferential presence in astrocyte endfeet that surround the brain microvessels (34) may help to concentrate mrp1 substrates in the brain extracellular fluid. This would create a steep concentration gradient in this fluid, facilitating clearance of the mrp1 substrate across the BBB. In conclusion, our findings tend to support that the mrp1 is not present at the luminal side of the mouse endothelial cells forming the BBB.

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REFERENCES

1. P. Borst, R. Evers, M. Kool, and J. Wijnholds. A family of drug transporters: the multidrug resistance-associated proteins. *J. Nat. Cancer Inst.* **92**:1295–1302 (2000).
2. T. Eisenblätter and H. J. Galla. A new multidrug resistance protein at the blood-brain barrier. *Biochem. Biophys. Res. Commun.* **293**:1273–1278 (2002).
3. C. Cordon-Cardo, J. P. O'Brien, D. Casals, L. Rittman-Grauer, J. L. Biedler, M. R. Melamed, and J. R. Bertino. Multidrug resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* **86**:695–698 (1989).
4. M. Kool, M. de Haas, G. L. Scheffer, R. J. Scheper, M. J. van Eijk, J. A. Juijn, F. Baas, and P. Borst. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res.* **57**:3537–3547 (1997).
5. X. Decleves, A. Fajac, J. Lehmann-Che, M. Tardy, C. Mercier, I. Hurbain, J. L. Laplanche, J. F. Bernaudin, and J. M. Scherrmann. Molecular and functional MDR1-Pgp and MRPs expression in human glioblastoma multiforme cell lines. *Int. J. Cancer* **98**:173–180 (2002).
6. H. Huai-Yun, D. T. Secrest, K. S. Mark, D. Carney, C. Brandquist, W. F. Elmquist, and D. W. Miller. Expression of multidrug resistance-associated protein (MRP) in brain microvessel endothelial cells. *Biochem. Biophys. Res. Commun.* **243**:816–820 (1998).
7. A. Regina, A. Koman, M. Piciotti, B. El Hafny, M. S. Center, R. Bergmann, P. O. Couraud, and F. Roux. Mrp1 multidrug resistance-associated protein and P-glycoprotein expression in rat brain microvessel endothelial cells. *J. Neurochem.* **71**:705–715 (1998).
8. V. V. Rao, J. L. Dahlheimer, M. E. Bardgett, A. Z. Snyder, R. A. Finch, A. C. Sartorelli, and D. Piwnica-Worms. Choroid plexus epithelial expression of MDR1 P glycoprotein and multidrug resistance-associated protein contribute to the blood-cerebrospinal-fluid drug-permeability barrier. *Proc. Natl. Acad. Sci. USA* **96**:3900–3905 (1999).
9. N. Strazielle and J. F. Ghersi-Egea. Demonstration of a coupled metabolism-efflux process at the choroid plexus as a mechanism of brain protection toward xenobiotics. *J. Neurosci.* **19**:6275–6289 (1999).
10. Y. Zhang, H. Han, W. F. Elmquist, and D. W. Miller. Expression of various multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain Res.* **876**:148–153 (2000).
11. G. Jedlitschky, I. Leier, U. Buchholz, K. Barnouin, G. Kurz, and D. Keppler. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res.* **56**:988–994 (1996).
12. D. W. Loe, R. G. Deeley, and S. P. Cole. Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. *Cancer Res.* **58**:5130–5136 (1998).
13. Q. Mao, R. G. Deeley, and S. P. Cole. Functional reconstitution of substrate transport by purified multidrug resistance protein MRP1 (ABCC1) in phospholipid vesicles. *J. Biol. Chem.* **275**: 34166–34172 (2000).
14. K. Nagata, M. Nishitani, M. Matsuo, N. Kioka, T. Amachi, and K. Ueda. Nonequivalent nucleotide trapping in the two nucleotide binding folds of the human multidrug resistance protein MRP1. *J. Biol. Chem.* **275**:17626–17630 (2000).
15. G. Rappa, A. Lorico, R. A. Flavell, and A. C. Sartorelli. Evidence that the multidrug resistance protein (MRP) functions as a cotransporter of glutathione and natural product toxins. *Cancer Res.* **57**:5232–5237 (1997).
16. G. J. Zaman, J. Lankelma, O. van Tellingen, J. Beijnen, H. Dekker, C. Paulusma, R. P. Oude Elferink, F. Baas, and P. Borst. Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. *Proc. Natl. Acad. Sci. USA* **92**:7690–7694 (1995).
17. A. H. Schinkel. P-glycoprotein, a gatekeeper in the blood-brain barrier. *Adv. Drug Deliv. Rev.* **36**:179–194 (1999).
18. C. Dagenais, C. Rousselle, G. M. Pollack, and J. M. Scherrmann. Development of an *in situ* mouse brain perfusion model and its application to mdr1a P-glycoprotein deficient mice. *J. Cereb. Blood Flow Metab.* **20**:381–386 (2000).
19. S. Cisternino, C. Rousselle, C. Dagenais, and J. M. Scherrmann. Screening of multidrug-resistance sensitive drugs by in situ brain perfusion in P-glycoprotein-deficient mice. *Pharm. Res.* **18**:183–190 (2001).
20. A. Lorico, G. Rappa, R. A. Finch, D. Yang, R. A. Flavell, and A. C. Sartorelli. Disruption of the murine MRP (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione. *Cancer Res.* **57**:5238–5242 (1997).
21. Y. Takasato, S. I. Rapoport, and Q. R. Smith. An *in situ* brain perfusion technique to study cerebrovascular transport in the rat. *Am. J. Physiol.* **247**:H484–H493 (1984).
22. K. O. Hamilton, E. Topp, I. Makagiansar, T. Siahaan, M. Yazdanian, and K. L. Audus. Multidrug resistance-associated protein-1 functional activity in Calu-3 cells. *J. Pharmacol. Exp. Ther.* **298**: 1199–1205 (2001).
23. A. H. Schinkel. The roles of P-glycoprotein and MRP1 in the blood-brain and blood-cerebrospinal fluid barriers. *Adv. Exp. Med. Biol.* **500**:365–372 (2001).
24. D. Sugiyama, H. Kusuhara, Y. Shitara, T. Abe, and Y. Sugiyama.

Effect of 17 beta-estradiol-D-17 beta-glucuronide on the rat organic anion transporting polypeptide 2-mediated transport differs depending on substrates. *Drug Metab. Dispos.* **30**:220–223 (2002).
25. D. E. Burgio, M. P. Gosland, and P. J. McNamara. Effects of P-glycoprotein modulators on etoposide elimination and central nervous system distribution. *J. Pharmacol. Exp. Ther.* **287**:911–917 (1998).
26. J. Wijnholds, E. C. DeLange, G. L. Scheffer, D. J. Van den Berg, C. A. Mol, M. van der Valk, A. H. Schinkel, R. J. Scheper, D. D. Breimer, and P. Borst. Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood-cerebrospinal fluid barrier. *J. Clin. Invest.* **105**:279–285 (2000).
27. A. Lorico, A. Bertola, C. Baum, O. Fodstad, and G. Rappa. Role of the Multidrug Resistance Protein 1 in protection from heavy metal oxyanions: investigations in vitro and in MRP1-deficient mice. *Biochem. Biophys. Res. Commun.* **291**:617–622 (2002).
28. I. Tamai, J. Yamashita, Y. Kido, A. Ohnari, Y. Sai, Y. Shima, K. Naruhashi, S. Koizumi, and A. Tsuji. Limited distribution of new quinolone antibacterial agents into brain caused by multiple efflux transporters at the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **295**:146–152 (2000).
29. M. Murata, I. Tamai, H. Kato, O. Nagata, and A. Tsuji. Efflux transport of a new quinolone antibacterial agent, HSR-903, across the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **290**:51–57 (1999).
30. K. Naruhashi, I. Tamai, N. Inoue, H. Muraoka, Y Sai, N. Suzuki, and A. Tsuji. Involvement of multidrug resistance-associated protein 2 in intestinal secretion of grepafloxacin in rats. *Antimicrob. Agents Chemother.* **46**:344–349 (2002).
31. H. Sasabe, A. Tsuji, and Y. Sugiyama. Carrier-mediated mechanism for the biliary excretion of the quinolone antibiotic grepafloxacin and its glucuronide in rats. *J. Pharmacol. Exp. Ther.* **284**:1033–1039 (1998).
32. G. Fricker, S. Nobmann, and D. S. Miller. Permeability of porcine blood brain barrier to somatostatin analogues. *Br. J. Pharmacol.* **135**:1308–1314 (2002).
33. D. S. Miller, S. N. Nobmann, H. Gutmann, M. Toeroek, J. Drewe, and G. Fricker. Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol. Pharmacol.* **58**:1357–1367 (2000).
34. X. Decleves, A. Regina, J. L. Laplanche, F. Roux, B. Boval, J. M. Launay, and J. M. Scherrmann. Functional expression of P-glycoprotein and multidrug resistance-associated protein (Mrp1) in primary cultures of rat astrocytes. *J. Neurosci. Res.* **60**:594–601 (2000).