BBB Transport and P-glycoprotein Functionality Using MDR1A (-/-) and Wild-Type Mice. Total Brain Versus Microdialysis Concentration Profiles of Rhodamine-123

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Purpose. The effect of P-glycoprotein (Pgp) on brain distribution using mdr1a (-/-) mice was investigated.

Methods. Fluorescein (Flu) and FD-4 were used to check whether blood-brain barrier (BBB) integrity was maintained in mdr1a (-/-) mice. The Pgp substrate rhodamine-123 (R123) was infused and total brain, blood and brain microdialysate concentrations in mdr1a (-/-) mice and wild-type mice were compared.

Results. Maintenance of BBB integrity was indicated by equal total brain/blood ratios of Flu and FD-4 in both mice types. R123 concentrations in brain after i.v. infusion were about 4-fold higher in mdr1a (-/-) than in wild-type mice ($P < 0.05$), without changes in blood levels. After microdialysis experiments the same results were found, excluding artifacts in the interpretation of Pgp functionality by the use of this technique. However the 4-fold ratio in brain was not reflected in corresponding microdialysates. No local differences of R123 in the brain were found. By the no-net-flux method *in vivo* recovery appeared to 4.6-fold lower in mdr1a (-/-) mice compared with wild-type mice.

Conclusions. Pgp plays an important role in R123 distribution into the brain. Using intracerebral microdialysis, changes in *in vivo* recovery by the absence or inhibition of Pgp (or active efflux in general) need to be considered carefully.

KEY WORDS: intracerebral microdialysis; blood-brain barrier; mdr1a (-/-) mice; *in vivo* recovery; rhodamine-123; P-glycoprotein.

INTRODUCTION

Multidrug resistance (MDR) is defined as the ability of cells to develop resistance to a broad range of structurally and functionally unrelated drugs after being exposed to these drugs. MDR can be mediated by the (increased) activity of P-glycoproteins (Pgp's) (1,2). These 170 kDa proteins act as ATP-dependent drug efflux pumps, leading to lower intracellular concentrations of a variety of natural, toxic products like anthracyclines, taxanes, epipodophyllotoxins, and vinca-alkaloids (Pgp substrates). In humans the gene encoding the drug transporting Pgp is called MDR1. In rodents two genes have been identified that encode drug transporting Pgp's: mdr1a and mdr1b (3,4,5).

As quite a number of tumors express Pgp, MDR may be very important with regard to the development of resistance to chemotherapy. However, Pgp must also play a role in normal tissues, as it is expressed in a variety of different tissues such as epithelial cells of the liver, kidney, and intestine (6). In capillary endothelial cells of the brain, which basically constitute the blood-brain barrier (BBB), the drug transporting Pgp has been detected at the luminal side (7,8,9). The observation that brain penetration of certain drugs is much lower than predicted on the basis of their lipophilicity (10,11) may well be explained by the fact that a number of these drugs are Pgp substrates (12). Strong evidence for this is provided by large differences in drug transport into the brain observed between mdr1a (-/-) and wild-type mice (13,14).

Since distribution into the brain is a major determinant of the action of drugs that potentially affect brain functions, it is of importance to study the role of Pgp at the level of the BBB. While *in vitro* studies may provide basic knowledge on BBB Pgp functioning (15,16,17), *in vivo* studies are needed to assess the effect in quantitative pharmacokinetic terms. In addition *in vivo* studies take into account the influence of endogenous compounds that potentially interact with this transport system, such as the Pgp substrate corticosterone or the inhibitor progesterone (18,19).

Intracerebral microdialysis is an *in vivo* technique that enables local and selective monitoring of drug concentrations in brain and is therefore well suited for pharmacokinetic investigations. It has already been evaluated for passive BBB transport studies in rats (20,21,22,23,24). In the present study this technique has been applied in mdr1a (-/-) and wild-type mice. Comparing results obtained in these mice, the role of mdr1a encoded Pgp at the BBB can be assessed because the mdr1a gene appears to be the predominant (if not the only) Pgp gene expressed at the BBB (13).

A first question to address was whether the disruption of the mdr1a gene would lead to changes in the integrity of the BBB. Therefore BBB permeability to two non-Pgp substrates fluorescein (Flu) and fluorescein-dextran-4000 (FD-4) in mdr1a (-/-) and wild-type mice was compared. Then, the mdr1a (-/-) mouse model was used to study BBB transport of the Pgp substrate rhodamine-123, i.e. R123 (25,26). It was investigated whether the microdialysis procedure (surgery and experiment) would affect BBB transport of R123 by comparison of mdr1a (-/-) and wild-type mice ratios of brain/blood of R123 following a 4h infusion with the ones obtained after other procedures. In separate microdialysis experiments, *in vivo* recovery values of R123 in mdr1a (-/-) and wild-type mice were determined by the no net flux method (27), and the occurrence of local differences in distribution of R123 in the brain was investigated.

METHODS (Project UDEC-9510 of State University Leiden)

Animals

Male mdr1a (-/-) FVB mice were obtained from the Netherlands Cancer Institute and bred under SPF conditions at TNO (Leiden, The Netherlands). Wild-type SPF FVB mice were also

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ABBREVIATIONS: BBB, blood-brain barrier; Pgp, P-glycoprotein; Flu, fluorescein; FD-4, fluorescein-dextran-4000.

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Table Ia. Schematic Presentation of the Surgical Procedures Used for the Experimental Groups I, II, III and IV

	Day 0				Day 1		
Group	Anesthesia	Cannulas	Microdialysis probe	Recovery on 39 C	Anesthesia	Cannulas	R123 administration
I	HDW	i.v. cannula	yes	yes	—	—	i.v. infusion
II	HDW	i.v. cannula	—	yes	—	—	i.v. infusion
III	HDW	—	—	yes	diethyl ether	i.v. cannula	i.v. infusion
IV	—	—	—	—	diethyl ether	i.v. cannula	i.v. infusion

Note: HDW = Hypnorm, dormicum, and water.

obtained from TNO. Male mice at the age of 10–13 weeks were used for this study. After transport, the mice were housed individually in the experimental room (Sylvius Laboratories, Leiden, The Netherlands) until the end of the experiment, at ambient temperature with free access to food (Standard Laboratory diet, RMTH-TH, Hope Farms, Woerden, The Netherlands) and water. Lights were on from 7.00–19.00 h. Individual cages used for housing were also used in the experimental setup, provided with food and water *ad libitum*.

Surgical Procedures

Implantation of a Blood Cannula

Animals were anesthetized by diethyl ether or by i.p. injection of 200 μl mixture of 1:1:2 (v/v) Hypnorm® and Dormicum® in water (HDW)—see Table Ia. Then, a polyethylene cannula (i.d. 0.28 mm, o.d. 0.61 mm), filled with 20E of heparin, was inserted into a tail vein, to be used for infusion of R123.

Microdialysis Surgery

Following HDW anesthesia and insertion of the blood cannula, the head of the mouse was shaved and fixed in a stereotaxic frame. Incisions were made to expose the periosteum that was locally anesthetized with a 1% solution of lidocaine (Bufa, Castricum, The Netherlands). The periosteum was removed to expose the skull. Then, two 1.5 mm holes were drilled at both sides of the curved skull. The use of a tungsten wire (TW5-3 Clark Electro Medical Instr., UK) allowed the horizontal (transversal) introduction of the microdialysis probe through the cortex, 1.1 mm below the bregma point. The microdialysis membranes used in this study (o.d. 290 μm, C-DAK artificial kidney 201–800 D 135 SCE, CD Medical B.V., Rotterdam, The Netherlands) had been previously covered with silicon glue except for a 5 mm zone to be positioned centrally in the cortex. Thereafter the tungsten wire was gently pulled out to leave the dialysis membrane in the cortical brain. Stainless steel needles, glued to both ends of the dialysis membrane, were secured with cement (Poly F-Plus, Zinc Polycarboxylate Cement, De Trey Dentsply, Dental House Nijmegen, The Netherlands) on top of the skull. A polyethylene cannula (i.d. 0.28 mm, o.d. 0.61 mm, length about 6 cm) was placed subcutaneously in order to equilibrate the perfusion medium to mouse body temperature before dialysis exchange (de Lange *et al.* 1994).

Table Ib. Concentrations of R123 in Brain (ml = g) and Blood Following a 4 h i.v. Infusion of R123 at a Rate of 9 nmol.min^{-1}

	C_{brain} (μM)		C_{blood} (μM)	
Group	(-/-)	wild-type	(-/-)	wild-type
I (n = 11)	2.88 ± 0.60*	0.72 ± 0.09	2.95 ± 0.53	2.53 ± 0.27
II (n = 5)	3.26 ± 1.01*	0.39 ± 0.07	3.53 ± 0.90	2.15 ± 0.29
III (n = 5)	2.07 ± 0.23*	0.43 ± 0.09	2.16 ± 0.97	2.73 ± 0.59
IV (n = 11)	3.03 ± 0.45*	0.63 ± 0.08	2.70 ± 0.44	2.79 ± 0.42

Note: Total blood concentrations appeared to be equal to total plasma concentrations, and the free fraction of R123 in plasma was about 25%. * = significantly different from corresponding values in wild-type mice ($P < 0.05$).

Recovery from Surgery

Following surgery using HDW anesthesia, the mice were placed on a warm paraffin pad (37–39°C; Deltaphase™ isothermal pad, Braintree Scient. Inc., MA, USA). After first signs of movement the animals were placed back into their own cage to allow further recovery from surgery for in total 24–27 h before the start of the experiment. Following diethyl ether inhalation anesthesia the mice were directly placed back into their cages for and had a 2 hour recovery period before the start of the experiment.

Experimental Procedure Flu and FD-4

To study BBB transport of fluorescein (Flu) and Fluorescein-dextran 4000 (FD-4), 3 mdr1a (-/-) and 3 wild-type mice were used. An i.v. cannula was placed under hypnorm anesthesia and the animals were allowed to recover for 24 hours before the start of the experiment. Flu and FD-4 were infused at a rate of 50 nmol.min^{-1} (10 mM, 5 μl/min). After 2 hours the mice were decapitated and brains were removed and blood collected in heparinized tubes and centrifuged to obtain plasma.

Experimental Procedures R123

Anesthesia and surgery affect physiological conditions (Claassen, 1995). Therefore, it was investigated whether different surgical procedures would affect Pgp functionality at the level of the BBB. Mdr1a (-/-) (26.5 ± 0.4 g, n = 32) and wild-type mice (28.4 ± 0.6 g, n = 32) were randomly allocated to four experimental groups receiving different surgical procedures. Surgical procedures used ranged from implantation of a blood cannula at the day of the experiment under diethyl ether inhalation anesthesia, to implantation of a microdialysis probe and the blood cannula one day before the experiment under Hypnorm anesthesia (Table Ia):

Infusion Experiments

Infusion of R123 at a rate of 9 nmol.min^{-1} was started between 10.00 and 12.00 h, using a 3 mM aqueous solution with a flow rate of 3 μl.min^{-1}. To investigate possible effects of surgical procedures (Table Ia) on Pgp functionality, a 4 h infusion period was used. At the end of the experiment, mice were anesthetized with a 200 μl HDW solution (HDW: see surgical procedure). Blood was collected from the eye plexus, diluted 10-fold by a 1:1 (v/v) mixture of methanol and water. The blood mixture samples were stored at −20°C until analysis. Brains were removed and directly frozen by liquid nitrogen and stored at −80°C until analysis.

In vivo recovery was determined during a 10–11 h period of infusion of R123 (3 mM, 3 μl.min^{-1}). At the end of the experiment mice were anesthetized. Blood was collected from the eye plexus and brains were removed and in this case first rapidly dissected into right and left hemisphere. The left hemisphere was further divided into cortex, cerebellum and rest. Brain parts were frozen by liquid nitrogen and stored at −80°C until analysis. Part of the blood was diluted ten-fold by a 1:1 (v/v) mixture of methanol and water, and another part was heparinized and centrifuged to obtain plasma. The blood mixture and plasma samples were stored at −20°C until analysis.

Microdialysis Procedure

Animals were weighed and put back in their cage that was placed in the experimental setup. Fused silica tubing (i.d. 100 μm, o.d. 280 μm) was used to connect the perfusion pump (Pump 22, Harvard Apparatus Inc, USA) with the microdialysis probe via the subcutaneous cannula. The outlet of the probe was connected with the sample loop of the h.p.l.c. ("on-line") by fused silica as well (i.d. 200 μm, o.d. 280 μm, ±60 cm). The microdialysis probe was perfused with medium at a rate of 2 μl min^{-1}. The medium consisted of 145 mM NaCl, 0.6 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.2 mM ascorbic acid, in a 2 mM potassium phosphate buffer, pH = 7.4 (28). Blank chromatographic data were obtained for 30–60 min before starting the infusion of R123. R123 levels in the dialysate samples were determined during the experiment in 10 min interval samples. The blank perfusion medium was used until a rather stable concentration of R123 in the dialysate was measured. Then the probe was connected to tubing containing perfusion medium with 5 nM of R123. Perfusion of the probe with the 5 nM R123 medium was performed until rather stable dialysate R123 concentrations were measured. Then, a perfusion medium containing 15 nM of R123 was applied in the same way.

Sample Preparation Flu and FD-4

Plasma

To 10 μl of plasma an icecold aliquot of 990 μl perfusion buffer was added, and vortexed for 10 sec. A 50 μl aliquot of this mixture was injected directly into the h.p.l.c. system.

Brain Tissue

Whole brains were weighed and a volume of icecold perfusion buffer 5 times the weight of the tissue was added. The brain tissues were thoroughly homogenized on ice (Ultra Turrax, IKA Werk, Germany). Brain homogenates were centrifuged (4°C, 5000 rpm, 15 min) and 50 μl of the supernatant was directly injected into the h.p.l.c. system.

Sample Preparation R123

Plasma

To 20 μl of plasma an icecold aliquot of 180 μl methanol/water (1:1, v/v) and 150 μl tris.HCl buffer (0.05 M, pH = 7.4) was added, and vortexed for 10 sec. For extraction of R123, 5 ml of ethylacetate/n-butanol (freshly made, 9:1, v/v) was added and the mixture was vortexed for 1 min, and centrifuged (4°C, 4000 rpm, 20 min). Of the upper layer 4 ml was removed and evaporated to dryness under reduced pressure at 37°C (Vortex evaporator, Buchler, Germany) and stored at −20°C pending analysis.

Blood

To a 150 μl sample of blood mixture an aliquot of 150 μl of Tris.HCl buffer (0.05 M, pH = 7.4) was added. The extraction procedure was identical to that described for plasma. Residues were stored at −20°C pending analysis.

Brain Tissue

Whole brains were weighed and a volume of icecold Tris.HCl buffer (0.05 M, pH = 7.4) 5 times the weight of the tissue was added, for brain parts 19 times the weight. The brain tissues were thoroughly homogenized on ice (Ultra Turrax, IKA Werk, Germany). Of brain homogenates a 100–500 μl aliquot was extracted by 5 ml of the ethylacetate/n-butanol mixture, and vortexed for 1 min. After centrifugation (4°C, 4000 rpm, 20 min) the supernatant was decanted and evaporated to dryness with reduced pressure at 37°C. The residue was stored at −20°C pending analysis.

Analysis of Flu and FD-4

The h.p.l.c. system for analysis of Flu and FD-4 consisted of a HEMA SEC BIO 300 column (10 μM, 25 cm * 4.6 mm i.d.) and a fluorescence detector (Perkin Elmer, LC 240) set at an excitation and emission wavelength of 498 and 525 nm, respectively. The mobile phase was a mixture of 75 mM of Na_2HPO_4, pH = 9, and 10% methanol (v/v). The flow rate was 0.75 ml.min^{-1}, and the temperature was set at 30°C. Chromatographic data were recorded and processed with a SP4100 computing integrator (Spectra Physics B.V., Eindhoven, The Netherlands). The retention time of Flu and FD-4 were 3 and 5 min respectively.

Analysis of R123

The R123 h.p.l.c. system consisted of a reversed phase column (Altima DB C8, 5 μ, 150 × 4.6 mm, Altech, Breda, The Netherlands) and a fluorescence detector (Perkin Elmer, LC 240) set at an excitation and emission wavelength of 498 and 525 nm, respectively. The mobile phase consisted of a mixture of 0.025 mM phosphate solution and methanol (1:1, v/v). The flow rate was 0.5 ml.min^{-1}, and the temperature was

set at 40°C. Chromatographic data were recorded and processed with a SP4100 computing integrator (Spectra Physics B.V., Eindhoven, The Netherlands). The retention time of R123 was 8 min.

Microdialysate

10 min interval-samples of microdialysate were injected on-line through a Valco injection valve equipped with a 10 µl sample loop. The coefficient of variation (C.V.) of microdialysate standards was concentration dependent, being 39% (k = 10), 13% (k = 9) and 6% (k = 8) for 0.5, 5, and 15 nM respectively. The detection limit of the assay was 0.2 nM (2 fmol absolute).

Plasma, Blood and Brain Tissue Extracts

The residues of plasma, blood and brain tissue extracts were dissolved in 500 µl ice cold methanol and vortexed for 30 sec. 5 µl was injected by an autosampler (WISP 710B, Waters Associate, The Netherlands). The C.V. values for analysis of blood, plasma and brain were 15% (k = 9), and 13% (k = 7), and 12% (k = 9) respectively.

Data Analysis

Estimation of Brain ECF Concentrations and In Vivo *Recovery*

The concentration versus time profiles of R123 in brain dialysate were corrected for the interval mean of the lagtime between probe (mouse) and injection into the h.p.l.c. system, so that t represents the mid sample interval point. The dialysate concentration-time profiles for each perfusion or inlet concentration (C_{in}) were fitted to the equation $C_{dial,t} = C_{dial,ss}(1 - e^{-kss.t})$ with a computerized minimization POWELL algorithm (Siphar Modelling Package, SIMED, Creteil, France). For the no-net-flux calculations of brain ECF concentrations and *in vivo* recovery, the dialysate values corresponding to 3*ln2/kss ($= 3*t^1/_2$) were used. These values are referred to as C_{out} in this study. The difference between C_{in} and C_{out} was determined and depicted as a function of C_{in}. Linear regression of the individual data points was performed and the intercept of the x-axis (point of no-net-flux were C_{in} equals brain ECF) was calculated together with the standard error of intercept according to McCormick and Roach (29). The values in mdr1a (-/-) and wild-type mice were compared with the students t-test at a significance level of $P < 0.05$.

Statistical Procedure

Comparison of the brain homogenate, blood data was performed by analysis of variance by ranks non-parametric tests (Kruskal-Wallis test), at a significance level of $P < 0.05$. Data are presented as mean values ± SEM.

Distribution of R123 in Blood

Ratio Between Blood and Plasma

The ratio between concentrations of R123 in blood over plasma was determined for individual mice and appeared to be 1.0.

Plasma Protein Binding

The extent of protein binding of R123 in plasma of mdr1a (-/-) and wild-type mice was determined *in vitro* by ultrafiltration at 37°C using the Amicon Micropunction System (Amicon, Div., Danvers, MA, USA). Spiked plasma samples with concentrations covering the plasma concentrations found in this study were used. Total plasma concentrations were determined after extraction (see plasma sample preparation). Separation of free drug from protein bound drug was accomplished by ultrafiltration of 200 µl plasma through a YMT ultrafiltration membrane (Amicon) at 1090 g for 20 min at 37°C, yielding about 75 µl ultrafiltrate that was analyzed by direct injection. The free fraction in plasma of mdr1a (-/-) and wild-type mice was 28 ± 1.9% (mean ± SEM, n = 3) and 23 ± 3.8% respectively (n.s.).

RESULTS

BBB Integrity; Flu and FD-4 Levels in Brain Homogenate and Plasma

Flu and FD-4 were infused i.v. at a rate of 50 nmol.min^{-1} for a 2 h period. In Fig. 1, plasma, brain, and brain/plasma ratio values of Flu and FD-4 in mdr1a (-/-) and wild-type mice are depicted. No differences existed in the brain/plasma ratios of Flu and FD-4 between mdr1a (-/-) and wild-type mice, indicating that there were no differences in BBB transport of these hydrophilic molecules known as good probes for BBB integrity.

R123 Levels in Brain Homogenate and Blood After Different Surgical Procedures, and in Microdialysate

R123 i.v. infusion experiments were performed in groups consisting of mdr1a (-/-) and wild-type mice with different surgical procedures (Table Ia). Total brain and blood concentrations were determined after a 4 h period of R123 infusion at a rate of 9 nmol.min^{-1}. The absolute concentrations found in brain and blood appeared to be independent of the surgical procedure used (Table Ib). Brain concentrations were consistently about 4-fold higher in mdr1a (-/-) mice than in wild-type mice ($P < 0.05$), while blood concentrations were equal. Either no difference in free concentrations of R123 in blood of both

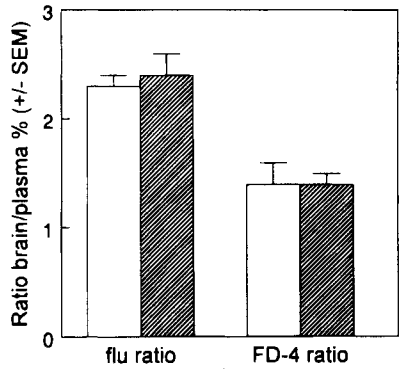

Fig. 1. Brain/plasma ratios (mean of n = 8, and SEM) of fluorescein and FD-4 following a 2 hr infusion at a rate of 50 nmol.min^{-1} for mdr1a (-/-) (striped bars) and wild-type mice (filled bars). No significant differences were observed between mdr1a (-/-) and wild-type values.

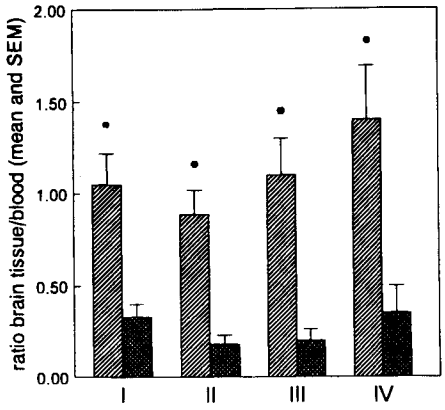

Fig. 2. Ratio of brain homogenate over blood concentrations in mdr1a (-/-) (striped bars) and wild-type mice (double striped bars), following a 4 h period of R123 i.v. infusion at a rate of 9 nmol.min^{-1}. Group I (n = 11), II (n = 5), III (n = 5) and IV (n = 11) represent groups in which different surgical procedures have been applied (see Table I). Data are presented as mean ± SEM. * = significantly different from values obtained in wild-type mice of the corresponding group ($P < 0.05$).

types of mice were found. The brain/blood ratio for the different groups is depicted in Fig. 2. It shows that this ratio is profoundly higher in mdr1a (-/-) than in wild-type mice ($P < 0.05$) and not significantly affected by the various surgical procedures applied here.

R123 microdialysate concentrations were obtained during a 4 h infusion period (group I; Table Ia). The difference found between brain homogenate R123 concentrations of mdr1a (-/-) and wild-type mice, however, was not observed to the same degree in the associated end-of-experiment dialysate levels (Fig. 3). It was hypothesized that this could be due to differences in *in vivo* recovery values in mdr1a (-/-) compared to wild-type mice, or to differences between cortical and total brain concentrations. Both these possibilities were tested in the next series of experiments.

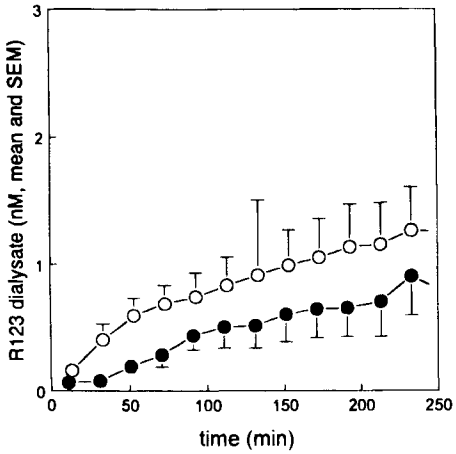

Fig. 3. Microdialysate concentration versus time profiles obtained in mdr1a (-/-) (○, n = 6) and wild-type mice (●, n = 5), during a 4 h period of i.v. infusion of R123 at a rate of 9 nmol.min^{-1}. Data are presented as mean ± SEM.

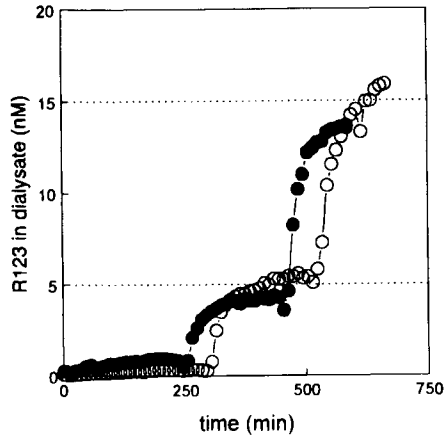

Fig. 4. Individual examples of concentration versus time profiles of R123 in the dialysate of a mdr1a (-/-) (○) and a wild-type mouse (●) obtained with the consecutive perfusion of the dialysis probe with 0, 5 and 15 nM during i.v. infusion of R123 at a rate of 9 nmol.min^{-1}.

In Vivo *Recovery of R123 in Microdialysate of mdr1a (-/-) and Wild-Type Mice, and R123 Tissue Concentrations in Different Parts of the Brain*

In vivo recovery was determined using the no net flux method (Lonnroth *et al.*, 1987). Microdialysate profiles of R123 were obtained in mdr1a (-/-) and wild-type mice during a 10–11 h infusion of R123 (9 nmol.min^{-1}). The probe was consecutively perfused with different perfusion concentrations (or inlet concentrations: 0, 5 and 15 nM; Fig. 4). The difference between inlet-concentration (C_{in}) and dialysate concentration at approximately steady-state conditions (C_{out}) was determined and depicted as a function of C_{in} (Fig. 5). Linear regression of the individual data points resulted in lines with the point of no net flux [$C_{in} = C_{out} = C_{brainECF}$] at significant different C_{in} values

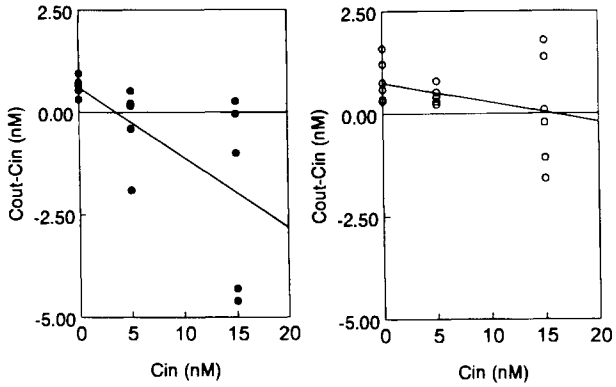

Fig. 5. Differences between steady-state dialysate concentrations (C_{out}) and perfusion concentrations (C_{in}) depicted as a function of C_{in}. Data are presented as individual data points for mdr1a (-/-) (○) and wild-type (●) mice. The section of the x-axis represents the estimated concentration in $brain_{ECF}$ (value and standard error), which was 15.7 ± 7.8 and 3.6 ± 2.1 for mdr1a (-/-) and wild-type mice ($P < 0.05$). The slope of the lines is determined by linear regression and is an estimate of *in vivo* recovery of R123 out of the $brain_{ECF}$ into the dialysate. *In vivo* recovery values (value and standard error) were 4.8 ± 6.2% and 17 ± 5.8% respectively for mdr1a (-/-) and wild-type mice ($P < 0.05$).

of 15.7 ± 7.77 nM for mdr1a (-/-) and 3.6 ± 2.1 nM for wild-type mice (intercept of x-axis and standard error, $P < 0.05$). The slope of the line reflects *in vivo* recovery, which was 4.8 ± 6.2% for mdr1a (-/-) and 17 ± 5.8% for wild-type mice (significantly different, $P < 0.05$).

At the end of the *in vivo* recovery experiments, blood was collected and brains were dissected into right and left hemisphere. The latter was divided into cortex, cerebellum and a rest group. The brain/blood ratio was significantly higher in the mdr1a (-/-) compared with wild-type mice ($P < 0.05$), but no significant differences were found between the different parts of the brain (Fig. 6).

Comparison of Microdialysate, Brain ECF, and Brain Homogenate Concentrations

Figure 7 shows the ratios of mdr1a (-/-) over wild-type values for microdialysate, brain ECF (microdialysate corrected for *in vivo* recovery), and brain homogenate concentrations. It indicates that Brain ECF and total brain R123 concentration differences between mdr1a (-/-) and wild-type mice are similar.

DISCUSSION

An existing classical paradigm for drug transport across the BBB is that it depends on lipid solubility and molecular weight of the unbound drug in plasma (10,30). However, major discrepancies have been observed for some rather lipophilic drugs with much lower brain entrance than expected (10,11). It has been suggested that the drug transporting Pgp at the luminal site of the brain capillary endothelial cells (7,9) is the cause of this extra barrier function, as most of the "outlier compounds" appeared to be Pgp substrates (12). An animal model that allows more detailed studies on this subject has become available with the mdr1a (-/-) mice (13). This mdr1a gene appears to be the dominant (if not the only mdr1 gene) that encodes for the Pgp at the BBB in mice. It has been shown that the absence of the mdr1a encoded Pgp leads to substantially higher brain/blood ratios of, e.g., ivermectin, vinblastine, digoxin, and cyclosporin A (13,14). Interestingly, levels in other tissues were only slightly altered. This demonstrates the important role of Pgp in BBB translocation of certain well-known drugs.

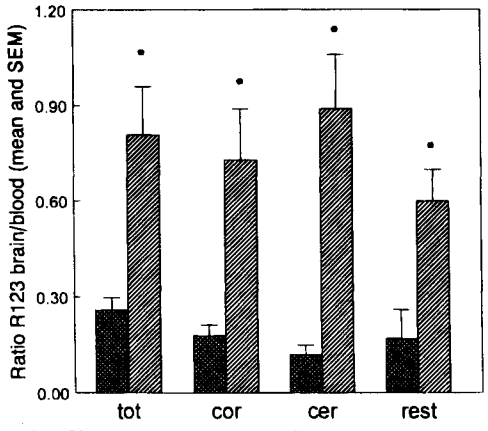

Fig. 6. Ratio of brain homogenate over blood concentrations in mdr1a (-/-) (striped bars) and wild-type (double striped bars) mice following a 10–11 hours of R123 i.v. infusion at a rate of 9 nmol.min^{-1}. Abbreviations: tot = right hemisphere; cor = left cortical brain; cer = left cerebellar brain; rest = rest of the left hemisphere. Data are presented as mean ± SEM. * = significantly different from corresponding values obtained in wild-type mice ($P < 0.05$).

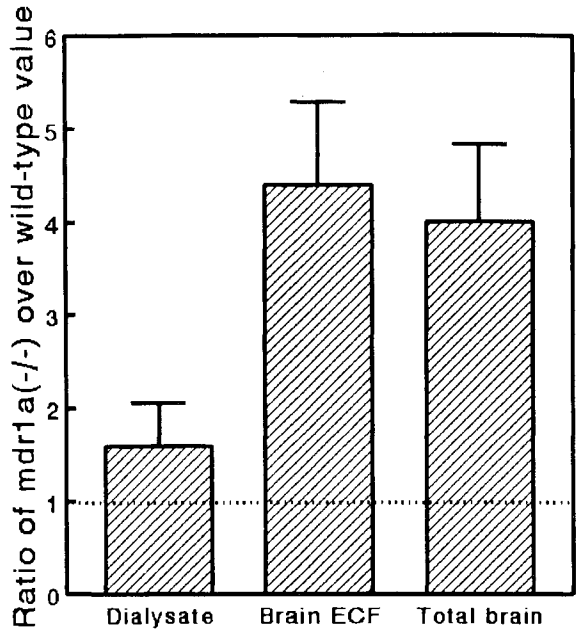

Fig. 7. Ratios (mean ± SEM) of dialysate, brain ECF, and total brain concentrations of mdr1a (-/-) over wild-type mice.

In using the mdr1a (-/-) mice to elucidate the effects of the absence of the Pgp on the transport of drugs across the BBB, it is of importance to know whether the absence of Pgp has any effects on BBB integrity. Experiments performed here using fluorescein and FD-4 as integrity markers (31) showed that there was no difference in brain/plasma ratio of these compounds between mdr1a (-/-) and wild-type mice (being respectively 2.3 and 1.4%), indicating maintenance of BBB integrity in the absence of Pgp.

After 4 h of i.v. infusion of the Pgp substrate R123, the total brain concentrations were about 4-fold higher in the mdr1a (-/-) mice compared with wild-type mice, while blood and plasma concentrations were equal in both types of mice. However, because free drug concentrations in plasma are assumed to be the driving force for passive diffusion of drugs into the brain, the percentage of unbound R123 was determined in plasma of mdr1a (-/-) and wild-type mice *in vitro*. Also blood and plasma levels of individual mice *in vivo* were compared. These were all in the same range, and cannot account for the differences found in brain concentrations between mdr1a (-/-) and wild-type mice. The higher brain/blood ratio of R123 in mdr1a (-/-) mice should therefore be caused by the absence of the mdr1a Pgp at the level of the BBB.

Brain tissue homogenization and CSF sampling may be used to obtain information on brain distribution of Pgp drugs in a general sense. However, under local pathological conditions such as brain tumors (32) or even under physiological circumstances, ultimate activity of this transport protein may vary locally within the brain because biosynthesis, processing and turn-over rate of Pgp may be different (33,34,35,36). Hence,

an *in vivo* technique is needed that enables the study of local drug distribution quantitatively. A potentially powerful technique is intracerebral microdialysis that allows the selective measurement of drug concentrations in brain at multiple time points. In previous studies we have already shown in rats that this technique allows a quantitative assessment of BBB drug transport *in vivo*. Moreover, it has been substantiated that dialysate concentrations reflect local brain concentrations. Therefore it can be used to investigate drug transport into selected areas of the brain (20,21,22,23,24).

In mice, far less experience exists with intracerebral microdialysis. It requires miniaturization of a number of technical aspects. In the present investigation, the use of intracerebral microdialysis in mdr1a (-/-) and wild-type mice was evaluated as a tool to study effects of Pgp on the entry of R123 into the brain. Experiments were performed to find optimal microdialysis conditions in mice as was done in previous studies in rats (data not shown). Subsequently, the potential influence of microdialysis surgical and experimental procedures on Pgp functionality was investigated. In rats, anesthesia as well as microdialysis surgery are known to increase corticosterone levels (unpublished investigations). As this steroid is a Pgp substrate (18) it could interfere with the amount of other Pgp substrates transported. Also, the anesthetic drugs used could not be excluded to have a direct effect on Pgp activity. In the experimental groups undergoing different surgical procedures (Table I), total brain and blood levels of R123 were determined after an i.v. infusion of 4 hours. These included the microdialysis surgery and three different surgical procedures to insert a cannula into the tail vein (for R123 administration). No significant changes in total brain and blood concentrations were observed in mdr1a (-/-) or wild-type mice, excluding artifacts in the interpretation of Pgp functionality by the use of the intracerebral microdialysis methodology.

The difference in brain levels between mdr1a (-/-) and wild-type mice after the 4 h infusion of R123, however, was not observed to the same degree in dialysate concentrations of these mice. It was investigated whether this could be due to differences in local brain distribution of R123 (as microdialysis measures at specific sites in the brain), or by differences between *in vivo* recovery of R123 in mdr1a (-/-) and wild-type mice. In order to evaluate this, R123 levels in cortex, cerebellum and rest of the brain were determined in a subsequent series of experiments. No significant differences were found in R123 levels of different parts of the brain (Fig. 6) Within this experiment also *in vivo* recovery was determined by the no net flux method (27; Fig. 4). Cortical $brain_{ECF}$ values could be estimated (Fig. 5) and an on average 5-fold difference evolved in R123 levels between mdr1a (-/-) and wild-type mice, as were found between the brain homogenate levels.

Different values for *in vivo* recovery of R123 of the two types of mice seems a bit surprising at first sight, but, can be explained on the basis of *in vivo* recovery theory (37). *In vivo* recovery is defined as the ratio of the concentration in the dialysate over that in the "bulk" (undisturbed ECF). It depends on mass transfer (or diffusion) of the compound from the ECF to the probe and mass transfer across the membrane into the dialysate. The probe characteristics and the flow rate of perfusion determine the latter, and can be considered as constants in this study. It leaves the mass transfer from the bulk to the probe to cause the difference found between the *in vivo* recovery values of the mdr1a (-/-) and wild-type mice. Bungay *et al.* (37) have developed a mathematical framework to explain *in vivo* recovery differences at steady-state conditions on the basis of tissue dependent parameters like metabolism, intracellular-extracellular exchange, extracellular-microvascular exchange, and diffusion. Influx of the compound (or its generation in case of neurotransmitters) does not affect the *in vivo* recovery, but determines the concentration $brain_{ECF}$. Loss of the compound, by active efflux across the BBB or by metabolism, does affect *in vivo* recovery. In the appendix the equations of Bungay *et al.* (37) have been used to compare mdr1a (-/-) with wild-type mice *in vivo* recoveries. It is assumed that the only difference between these mice is the absence of Pgp-dependent extracellular-microvascular exchange in the (-/-) mice. Thus, it is predicted that Pgp absence results in a higher resistance to tissue mass transfer of a Pgp substrate from the bulk to the probe, and therefore leads to a lower *in vivo* recovery in mdr1a (-/-) mice compared with the wild-types. This is in line with the findings of the present study. In analogy, effective inhibition of Pgp functionality should also decrease *in vivo* recovery. Thusfar one paper on Pgp and R123 using microdialysis (in rats) did not report such observation (38) but correction for *in vivo* recovery was not made on the basis of R123 itself, but on calibration by retrodialysis of rhodamine-116 (R116).

In short, Pgp plays an important role in the distribution of R123 into the brain. This was demonstrated by differences in total brain concentrations between mdr1a (-/-) and wild-type mice, as blood concentrations were equal. Also the intracerebral microdialysis technique can be applied to study brain distribution in mdr1a (-/-) and wild-type mice as a function of time, because the technique did not interfere with Pgp activity. The 4-fold difference found between total brain concentrations in the mdr1a (-/-) and wild-type mice was reflected by the end-of-experiment $brain_{ECF}$ concentrations, determined by correction of dialysate concentrations *in vivo* recovery values on the basis of the no-net-flux method.

APPENDIX

Bungay *et al.* (1990) have developed a mathematical framework to provide a quantitative basis for *in vivo* recovery in microdialysis of tissue. *In vivo* recovery is dependent on mass transport. They approached mass transport (or diffusional transport) as a series of transports through tissue, across the probe membrane and into the dialysate. A mass transport resistance expresses the proportionality between a concentration gradient and the resultant mass flow. They have shown that for *in vivo* recovery the following equation can be used:

$$E_d = (C_{out} - C_{in})/(brain_{ECF} - C_{in}) \quad (1)$$
$$= 1 - \exp\{-1/[F(R_d + R_m + R_e)]\}$$

in which E_d = *in vivo* recovery (or dialysate extraction fraction); C_{out} = dialysate concentration at steady-state; C_{in} = perfusate concentration; F = flow rate of the perfusate; R = mass transfer resistance with subscripts d = dialysate, m = membrane and e = external medium.

For a probe in tissue $R_e >> R_m >> R_d$, leaving only the R_e to be further considered:

$$R_e = \{(K_0/K_1)/(2 \cdot r_o \cdot L \cdot D_e \cdot \varphi_e)\} \cdot \Gamma \quad (2)$$

in which K_0/K_1 are modified Bessel functions, r_0 is the radius of the probe, L is the length of the semipermeable part of the membrane, D_e is the diffusion coefficient for the extracellular phase, φ_e is the accessible volume fraction of the extracellular phase, and Γ is the profile depth parameter that can be described by:

$$\Gamma = \{D_e/(k_1 + k_2 + k_3)\} \quad (3)$$

with k_1, k_2 and k_3 representing the first-order rate constants for efflux to the microvasculature, irreversible extracellular metabolism, and the composite of irreversible intracellular metabolism and extracellular-intracellular exchange respectively.

For our specific situation subdivide k_1 into k_{1a} and k_{1b}, representing the rate constants for elimination to microvasculature that is not Pgp dependent and the Pgp dependent one, respectively. Assuming that all parameters in mdr1a (-/-) and wild-type mice are equal except for the absence of the Pgp related efflux to the microvasculature in (-/-) mice, one will obtain (after some rearrangements):

$$R_{e(-/-)}/R_{e\ \text{wild-type}} = \{(k_{1a} + k_{1b} + k_2 + k_3)/(k_{1a} + k_2 + k_3)\} \quad (4)$$

It shows that $R_{e(-/-)}/R_{e\ \text{wild-type}} > 1$, and therefore $R_{e(-/-)}$ is higher than $R_{e\ \text{wild-type}}$. This means that the mass transfer resistance in the (-/-) mice is larger than the mass transfer resistance in the wild-type mice. A larger value for R_e will lead to a lower value of E_d. In other words a lower *in vivo* recovery is expected for the mdr1a (-/-) mice.

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