Effect of Probenecid on Fluorescein Transport in the Central Nervous System Using *In Vitro* and *In Vivo* Models

Haiying Sun,[1] Donald W. Miller,[1] and William F. Elmquist[1,2]

Received July 24, 2001; accepted August 7, 2001

Purpose. The purpose of this study was to characterize the function of multidrug resistance-associated proteins (MRPs) (or MRP-like organic anion transport systems) in the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) using both an *in vitro* BBB model and an *in vivo* microdialysis model.

Methods. *In vitro* functional studies were performed using bovine brain microvessel endothelial cells (BBMEC). The accumulation of fluorescein, an anionic fluorescent dye, in BBMEC was determined with and without the presence of inhibitors of various efflux transport proteins. *In vivo* microdialysis simultaneously monitored fluorescein concentrations in cortical extracellular fluid and cerebrospinal fluid. The effect of probenecid on the *in vivo* distribution of fluorescein was studied using a balanced crossover design in the rat.

Results. *In vitro* experiments showed that probenecid, indomethacin, LY-329146, and all MRP inhibitors significantly increased (two- to threefold) the accumulation of fluorescein in BBMEC, whereas LY-335979, a P-gp inhibitor, had no effect on the accumulation of fluorescein. Probenecid significantly increased fluorescein plasma concentration and the plasma free fraction *in vivo*. The distribution of fluorescein across the BBB and BCSFB was enhanced by 2.2- and 1.9-fold, respectively, when probenecid was coadministered, even after correction for increased fluorescein plasma concentrations and free fraction.

Conclusions. These results demonstrate that MRPs or MRP-like transport system(s) may play an important role in fluorescein distribution across both BBB and BCSFB. This study showed that microdialysis proved to be a powerful *in vivo* technique for the study of transport systems in the central nervous system, and *in vitro*/*in vivo* correlations are possible using these model systems.

KEY WORDS: microdialysis; fluorescein; probenecid; multidrug resistance-associated protein; blood-brain barrier; blood-CSF barrier.

INTRODUCTION

Transport systems at the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) are important in regulating the concentration of drugs in the central nervous system (CNS). It has long been recognized that acidic compounds can be removed from the CNS by organic anion transport systems located at the choroid plexus epithelium (BCSFB) and brain capillary endothelium (BBB). The putative physiological function of these efflux transport systems includes, but is not limited to, the transport of glucuronide and glutathione conjugates, inflammatory mediators (e.g., leucotriene C4) (1), acid metabolites of neurotransmitters (2), and xenogenous neurotoxins (3). Previous studies also suggest that these organic anion transporters are involved in the efflux clearance of therapeutic compounds from the CNS, such as zidovudine (4).

Recently, the molecular biology of organic anion transport proteins (Oatps) at the BBB and BCSFB has been undertaken, and several transport protein families have been recognized, such as the multidrug resistance-associated protein family (MRPs) (5–7), the Oatps (8–10), and the monocarboxylic acid transporter (11). Several isoforms of MRP have been cloned (12), and it has been established that particular isoforms of the MRP family (MRP1-5) are responsible for the cellular extrusion of various organic anions (13,14). The expression of MRP1 at the BCSFB has been demonstrated in freshly isolated choroid plexus membrane (7,9). Recent studies have also shown that different isoforms of MRP such as MRP1, MRP5, possibly MRP4, and possibly MRP6 are expressed in the isolated brain capillary endothelial cell (15,16) the cells that form the blood-brain-barrier. These studies suggest the possibility that one or more specific MRP isoform may contribute to the transport of organic anions across BBB and BCSFB.

The characterization of MRP1 transport of organic anions at the BBB has been performed in several *in vitro* models (7,17). However, the expression of transport proteins, including MRP1 and p-glycoprotein in brain capillary endothelial cells may depend on different cell culture conditions, including time in culture and co-culture with astrocytes or astrocyte-conditioned media (5,17,18). Therefore, the *in vivo* environment surrounding the brain endothelial cells may play a role in the induction or inhibition of the expression of the transport proteins, which in turn would regulate their functional role as part of the BBB.

There has been a growing interest in inhibiting MRP to modulate multidrug-resistant cancers. The present study was performed to obtain further insight into the magnitude of the function of MRP or MRP-like drug efflux proteins in the transcellular transport of organic anions across the BBB and BCSFB, using both *in vitro* and *in vivo* models. The current study examines organic anion transport in the CNS in the *in vivo* situation by the use of the brain microdialysis sampling, which is a technique that has been widely applied to drug distribution studies particularly in the CNS (19). *In vivo* microdialysis in the cortex and the lateral ventricle was used to study the transport properties of fluorescein across BBB and BCSFB, respectively. This transport was examined in a crossover design with or without the coadministration of probenecid, an established inhibitor of MRP1 (20). Fluorescein, a fluorescent dye, is a compound that exists as a negatively charged species under physiological conditions and has been used as a substrate of MRP (6).

[1] Department of Pharmaceutical Sciences, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, Nebraska 68198-6025.

[2] To whom correspondence should be addressed. (e-mail: wfelmqui@unmc.edu)

ABBREVIATIONS: BBB, blood-brain barrier; BCSFB, blood cerebrospinal fluid barrier; MRP, multidrug resistance-associated protein; Oatp, organic anion transport protein; ECF, extracellular fluid; CSF, cerebrospinal fluid; BBMEC, bovine brain microvessel endothelial cells; PBS, phosphate buffered saline; 2FF, difluorofluorescein.

0724-8741/01/1100-1542$19.50/0

MATERIALS AND METHODS

Chemicals

Fluorescein and difluorofluorescein were purchased from Molecular Probes (Eugene, OR). Probenecid and indomethacin were purchased from Sigma (St. Louis, MO). LY-335979 and LY-329146 were gifts from Eli Lilly (Indianapolis, IN). Solvents were of high-performance liquid chromatography (HPLC) grade, and all other chemicals were reagent grade or better.

In Vitro Experiments

Isolation and Culture of Brain Endothelial Cells

The procedure for the isolation and culture of bovine brain microvessel endothelial cells (BBMEC) was described in an earlier study (6). Briefly, the BBMEC were isolated from fresh cow brains through enzyme digestion and centrifugation (21). The cells were plated on collagen-coated, fibronectin-treated 6-well plates at a density of 50,000 cells/cm^2. The accumulation and permeability studies were performed when the cell monolayers reached confluency (10–12 days).

Fluorescein Accumulation in Brain Capillary Endothelial Cells

The effect of various drug efflux transport inhibitors on the accumulation of fluorescein was examined in confluent monolayers of BBMEC. Cells were incubated in media containing fluorescein (100 μM) for 60 min at 37°C, either alone or in the presence of LY-335979 (1 μM), probenecid (100 μM), LY-329146 (5 μM), or indomethacin (10 μM). BBMECs were preincubated with phosphate-buffered saline (PBS) or PBS containing the inhibitors for 60 min prior to the fluorescein. The 60-min time point was chosen because in previous studies we have shown that steady-state flux of fluorescein is reached in the BBMECs by this time point (6). After the incubation, cells were rapidly washed three times with ice-cold PBS. The cell monolayers ($n = 3$) were solubilized with Triton X-100, and aliquots (250 μl) were removed for determination of fluorescein using a fluorescence spectrophotometer (RF5000, Shimadzu, Columbia, MD) with excitation and emission wavelengths of 488 and 510 nm, respectively. Percent accumulation of fluorescein was normalized to protein content using the method of Lowry *et al.* (22).

Fluorescein Flux across Brain Capillary Endothelial Cells

BBMECs were grown to confluency on polycarbonate membranes coated with collagen and fibronectin. Confluent monolayers were mounted on side-by-side diffusion chambers and preincubated in PBS for 30 min at 37°C. PBS (3 ml) containing 1 μM fluorescein was then added to the apical (donor) side with either no probenecid (control, $n = 3$) or 100 μM probenecid ($n = 3$). Samples (150 μl with replacement) were removed from the donor side at time zero and from the receiver side at 5, 10, 15, 30, and 60 min. Fluorescein concentration in the receiver (basolateral) compartment was not measurable until 30 min. Fluorescein concentrations in donor and receiver PBS were measured using spectrofluorometry as above.

In Vivo Experiments

Microdialysis Probe Placement

Male Wistar rats weighing between 260 to 340 g were used in this study. The surgical procedures for implantation of the microdialysis probe guide cannula, probe placement, and the cannulation of the femoral artery and vein were similar to Yang *et al.* (23) with slight modification.

Microdialysis Sampling Procedure

For both *in vitro* and *in vivo* microdialysis sampling, CMA-12 probes were perfused with artificial CSF (119.5 mM NaCl, 4.75 mM KCl, 1.27 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 1.6 mM Na_2HPO_4 (pH 7.2)) using a microprocessor controlled syringe pump (Harvard 22, Harvard Apparatus, Natick, MA). The perfusate flow through both the ventrical probe (1 mm) and the cortical probe (3 mm) was 0.5 μl/min. Microdialysates were collected over 20-min intervals directly into the injection loops of a multiport valve (E-36, Valco, Houston, TX) controlled by a digital valve sequence programmer (DVSP2, Valco). Microdialysates were then directly injected into the HPLC for analysis.

Microdialysis Probe Calibration

To quantify the fluorescein concentration in the extracelluar fluid of the cortical and the ventricular CSF using microdialysis sampling, the *in vivo* recovery of the probes was determined using the method of retrodialysis (24). This method uses the loss of a structural analog from the perfusate to determine the fractional recovery of the analyte of interest from the sampled extracellular fluid (ECF), i.e., cortical ECF or ventricular CSF. Difluorofluorescein (2FF), a fluorinated analog of fluorescein, was added to the perfusion fluid; its relative loss was used to determine the recovery of fluorescein through the following relationship:

$$\text{Recovery}_{in\ vivo} = 1 - \frac{C_{\text{out}}}{C_{\text{in}}} = 1 - \frac{PA_{2\text{FF(out)}}}{PA_{2\text{FF(in)}}} \qquad (1)$$

in which PA_{2FF}(out) was the peak area of 2FF in the dialysate leaving the probe, and PA_{2FF} (in) was the peak area of difluorofluorescein in the perfusate entering the probe. The concentration of fluorescein in the dialysates from the cortical ECF and ventricular CSF was then corrected using the *in vivo* recovery to obtain the fluorescein concentration-time profiles in the respective brain tissues.

Determination of Fluorescein Free Fraction in Rat Plasma

The extent of protein binding of fluorescein in rat plasma and the influence of probenecid on that binding were determined *in vitro*. Aliquots of rat plasma were spiked with fluorescein concentrations covering the range found in the *in vivo* microdialysis studies. Total plasma concentrations were determined by HPLC. Plasma was placed in an ultrafiltration device (Centrifree Micropartition Device, Millipore, Bedford,

MA), incubated at 37°C for 20 min, and free drug was separated from protein-bound drug by centrifugation at 1,600*g* for 20 min at 37°C. Free fluorescein concentration in the ultrafiltrates was determined by HPLC. In some fluorescein-spiked plasma aliquots, probenecid was also added in concentrations of 50 and 300 μg/ml to examine the effect of probenecid on fluorescein free fraction.

Sample Analysis

The determination of fluorescein concentration in plasma, ultrafiltrate, and microdialysate was done using HPLC with fluorescent detection. The HPLC system consisted of a LC-10AD pump, RF-10A fluorometric detector, and CR501 integrator (Shimadzu). Separations were carried out on a BDS-Hypersil C-18 column (2.0 × 150 mm, 5 μm) (Keystone Scientific, Inc., Bellefonte, PA). The mobile phase was an acetonitrile:buffer mix (13.6:86.4, w/w) with a buffer composition of 10 mM ammonium phosphate and 10 mM sodium citrate (pH 6.4). The flow rate was 0.25 ml/min, and the column eluate was monitored at excitation and emission wavelengths of 488 and 510 nm, respectively. Plasma samples were deproteinized with acetonitrile prior to HPLC injection. The fluorescein plasma assay was linear from 0.01 to 2 μg/ml in a low-range standard curve, and it was linear from 0.5 to 50 μg/ml for a high-range standard curve. The specimen preparation was different in these two ranges to avoid saturation of the fluorescent detector. The on-line analysis of the microdialysates had a linear range of 0.001 to 0.35 μg/ml, adequate to cover all concentrations measured from the cortical ECF and the ventricular CSF. The interday variability was always less than 10% coefficient of variations.

The concentration of probenecid in plasma was assayed according to the method reported by Galinsky *et al.* (25) with modification. The HPLC system was the same as described above except for ultraviolet detection (SPD-10AV, Shimadzu). The column was a μBondapak™ (Water, Millford, MA) C-18 column (3.9 × 300 mm, 10 μm). The mobile phase was acetonitrile:10 mM KH_2PO_4 buffer (22:78 w/w), and the flow rate was 1.0 ml/min with the column eluate monitored at a 254-nm ultraviolet wavelength. The probenecid plasma assay was linear from 25 to 500 μg/ml (our standard curve range), however, the sensitivity was 2 μg/ml. The interday variability was less than 10% coefficient of variations.

Study Design and Drug Administration

The distribution of fluorescein to the CNS was examined *in vivo* using an intravenous infusion with balanced-crossover design. A dose of 6 mg/kg/h fluorescein was administered by intravenous infusion to 6 rats with or without probenecid. In the probenecid-treatment phase, rats received a 100-mg/kg loading dose of probenecid followed by a constant probenecid infusion of 30 mg/kg/h. Fluorescein was administered immediately following the probenecid loading dose. In the study, rats were equally divided into two groups. Group A received fluorescein with probenecid in phase one and received fluorescein without probenecid in phase two. Group B received the opposite treatment order. There was a minimum of 24 h washout between treatment arms. Blood sampling began at 12 h after the initiation of the infusion (steady state). Microdialysate samples from frontal cortex and lateral ventricle were continuously taken for approximately 24 h. The concentration of fluorescein in the microdialysate was corrected using the *in vivo* probe recovery (see above) to obtain the fluorescein brain concentration-time profile.

Data Analysis

The rate of change of the amount of fluorescein in the CSF or in the ECF of a region of brain tissue can be expressed by the following differential equation:

$$dA_{brain}/dt = CL_{in} \times C_{plasma} - CL_{out} \times C_{brain} \quad (2)$$

in which A_{brain} is the amount of fluorescein in the respective CNS compartments, C_{plasma} is the total fluorescein concentration measured in plasma, and C_{brain} is the fluorescein concentration in the brain space (i.e., cortical ECF or ventricular CSF) measured by microdialysis after correction for recovery. Cl_{in} and Cl_{out} represent the clearance into, and out of, each brain compartment, respectively.

In the case of intravenous infusion, when fluorescein concentrations reach steady state, we obtain:

$$\frac{CL_{in}}{CL_{out}} = \frac{C_{ss,brain}}{C_{ss,plasma}} \quad (3)$$

in which C_{ss} is the concentration at steady state in the respective tissues.

The ratios of $C_{ss\ brain}$ to $C_{ss\ plasma}$ (Eq. 3) were used to estimate the ratio of distributional clearances and the equilibrium distribution coefficient of fluorescein across the BBB and BCSFB in the *in vivo* intravenous infusion study. A parameter designated the distributional enhancement index, *DE,* was calculated from the ratio of probenecid treated-to-control equilibrium distribution coefficients, as shown in Eq. 4.

$$DE = \left(\frac{C_{ss,brain}}{C_{ss,blood}}\right)_{treated} \Big/ \left(\frac{C_{ss,brain}}{C_{ss,blood}}\right)_{control} \quad (4)$$

Statistical Analysis

The statistical tests used for comparison between groups was a Student's *t* test with $P < 0.05$ taken as a significant difference.

RESULTS

Effects of Various Inhibitors on Fluorescein Accumulation in BBMEC

The effect of efflux transport inhibitors on the accumulation of fluorescein in the BBMEC monolayers is shown in Fig. 1. The specific and potent p-glycoprotein inhibitor LY-335979 (26) shows no significant increase in accumulation of fluorescein over control. However, probenecid, LY-329146, and indomethacin, which are organic anion transport inhibitors and have been shown to inhibit MRP-mediated transport (20,27,28) show significant increases in accumulation over control, as high as 300% of the control value. Figure 2 shows a dose response curve for the effect of probenecid on fluorescein accumulation in the BBMEC monolayers. Maximal effects occurred at concentrations at or above 100 μM probenecid.

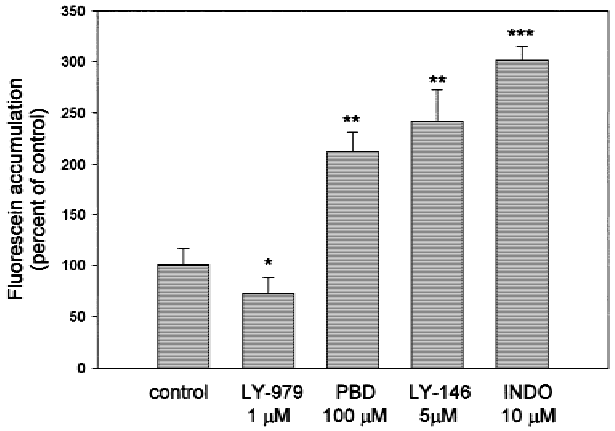

Fig. 1. Effect of various drug efflux protein inhibitors on the accumulation of fluorescein in the bovine brain microvessel endothelial cell monolayers. Mean ± SE. *$P > 0.1$, not significantly different from control value; **$P < 0.05$; ***$P < 0.001$, significantly different from control value.

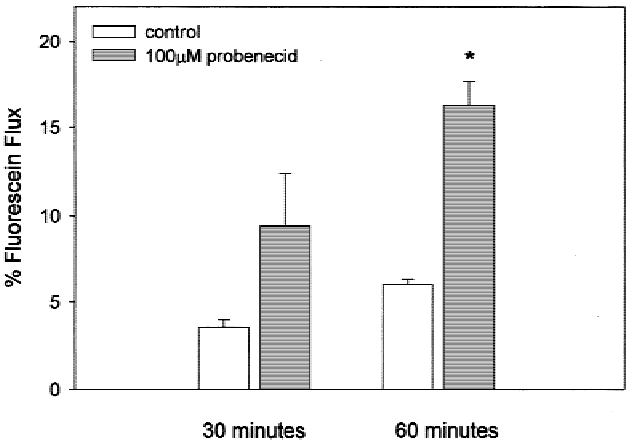

Fig. 3. Effect of probenecid on the apical to basolateral permeability of fluorescein across bovine brain microvessel endothelial cell monolayers. Mean ± SE.

Fluorescein Permeability Study

The effect of probenecid on the flux of fluorescein across the BBMEC monolayer is shown in Fig. 3. Probenecid significantly increased the apical to basolateral flux of fluorescein.

Microdialysis Probe Recovery

The *in vitro* recovery by loss of the probe calibrator, 2FF, determined by measuring the loss of 2FF from the perfusate to buffer medium surrounding the probe at 20°C was 32%. This was equivalent to the fluorescein recovery by gain (33%), which was determined by measuring the gain of fluorescein from the buffer medium into the perfusate. However, the equivalence of 2FF and fluorescein probe recovery *in vivo* must be demonstrated, because it has been shown that the determinants of probe recovery *in vitro* and *in vivo* are different (24). The *in vivo* recoveries by loss of fluorescein and 2FF were 17% and 16% (3-mm probe length), respectively, in the cortex, and 13% and 11% (1-mm probe length), respectively, in the ventricle. With the coadministration of probenecid, the *in vivo* recovery of fluorescein was decreased. However, the magnitude of this effect was the same on the *in vivo* recovery of retrodialysis calibrator, 2FF (29). Additionally, the use of 2FF as the retrodialysis calibrator could conceivably affect the transport of fluorescein via active transport by an efflux transport system in the capillary, given that 2FF is a close structural analog of fluorescein. We have published evidence to the contrary, indicating that the fluorescein efflux transport system must have a relatively high capacity and is still in the linear range even with the local delivery of the structural analog, 2FF, via the microdialysis probe. In this recently published study, the local delivery of 2FF, given at the same concentration as the current study, did not affect the recovery of the FLR or the effect of probenecid on that recovery (29). Therefore, 2FF has been validated as an appropriate *in vivo* recovery calibrator in the absence and presence of the drug efflux inhibitor.

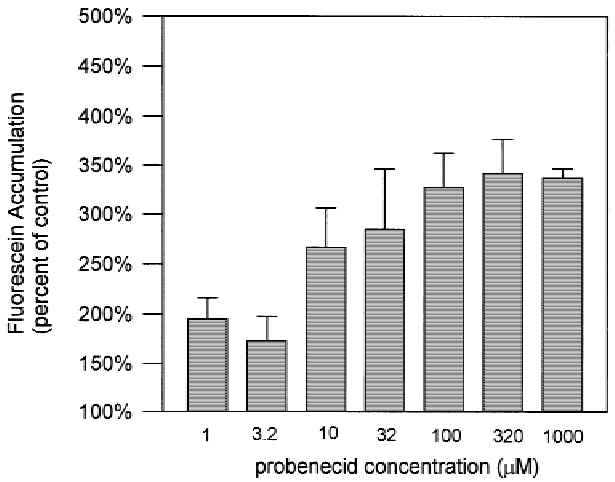

Fig. 2. Effect of various concentrations of probenecid on fluorescein accumulation in bovine brain microvessel endothelial cell monolayers. Mean ± SE.

In Vivo Brain Distribution of Fluorescein

Rats were given a 6-mg/kg/h constant infusion of fluorescein for 16 h. In the control phase, fluorescein concentrations in both the cortex and CSF reached a steady state after 4 to 5 h. While in the probenecid-treated phase, because of the increasing probenecid plasma concentrations, the time for fluorescein concentrations to reach steady state in both the cortex and CSF was prolonged. At 12 h after the start of the experiment, probenecid plasma concentration reached steady state and was 335 ± 59 μg/ml. At this time, fluorescein concentrations in cortical ECF and ventricular CSF in probenecid-treated phase were relatively constant and were considered at steady state (Fig. 4). Probenecid treatment significantly increased fluorescein concentrations at steady state in cortical ECF from 118 ± 26 to 2,045 ± 440 ng/ml, and in ventricular CSF from 101 ± 72 to 1,421 ± 734 ng/ml, respectively. The total fluorescein plasma concentration in both phases reached a steady state after 4 to 5 h (Fig. 4). Probenecid increased fluorescein plasma concentration but to a much less extent, i.e., from 6.8 ± 0.33 to 28 ± 2.03 μg/ml. Table I lists the tissue (i.e., cortical ECF and ventricular CSF) to plasma concentration ratio at the steady state with or without the coadministration of probenecid. Probenecid significantly

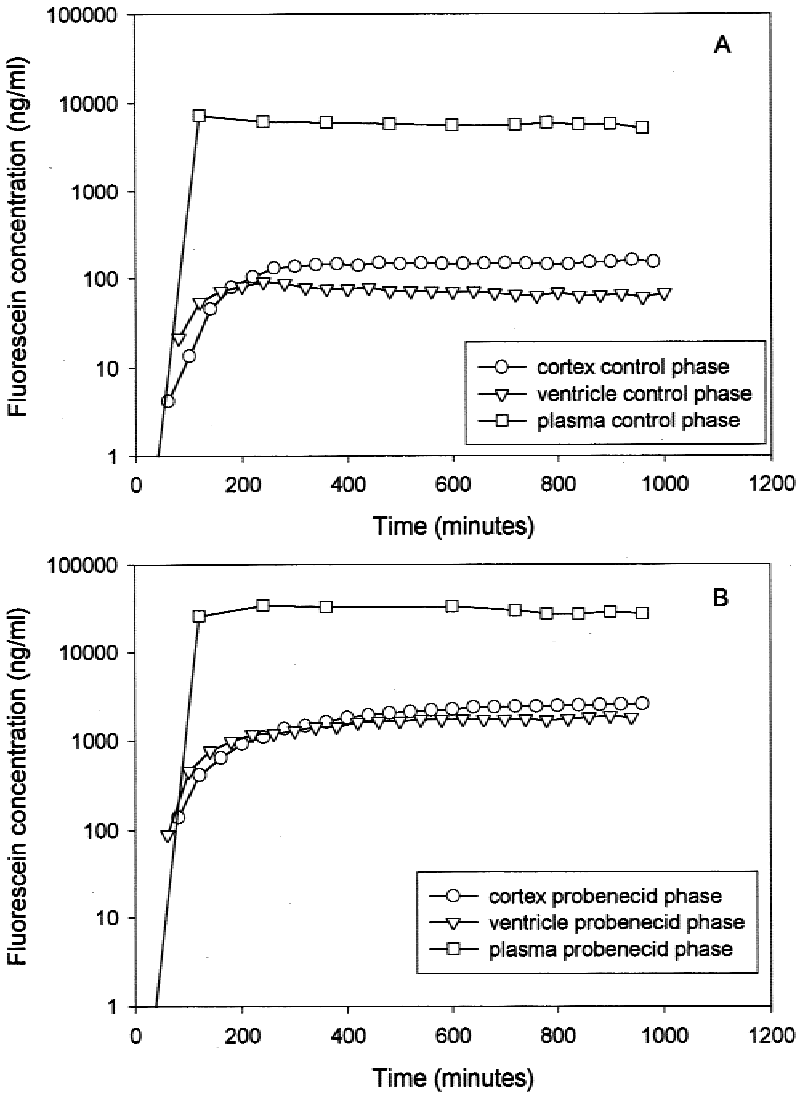

Fig. 4. Fluorescein concentration-time profile in cortical ECF (○), ventricular CSF (▽), and plasma (□) in control phase (A) and probenecid treated phase (B) during a 6-mg/kg/h intravenous infusion of fluorescein in a representative rat.

increased the $C_{ss,\ tissue}$ to $C_{ss,\ plasma}$ ratio, and the distribution enhancement index calculated from Eq. 4 was 4.44 ± 0.95 and 3.90 ± 1.63 in cortex and ventricle, respectively.

Fluorescein free fraction was studied to examine the effect of probenecid on the free fraction of fluorescein in rat plasma. The total concentrations of fluorescein used in the protein-binding experiments to establish linearity were 10 to 50 μg/ml plasma, which encompasses the range observed of

Table I. The Effect of Probenecid Treatment on the Distribution of Fluorescein in Cortical ECF and Ventricular CSF

	Cortex			Ventricle		
Rat	($C_{SStissue}/C_{SSplasma}$) control	($C_{SStissue}/C_{SSplasma}$) treated	Distribution enhancement treated/control	($C_{SStissue}/C_{SSplasma}$) control	($C_{SStissue}/C_{SSplasma}$) treated	Distribution enhancement treated/control
1	0.023	0.110	4.85	0.016	0.042	2.69
2	0.011	0.040	3.64	0.003	0.008	3.28
3	0.015	0.085	5.61	0.013	0.081	6.26
4	0.013	0.067	5.03	0.029	0.063	2.17
5	0.019	0.067	3.54	0.016	0.054	3.47
6	0.026	0.087	3.31	0.011	0.062	5.53
Mean	0.018	0.076	4.33*	0.015	0.052	3.90*
SD	0.006	0.024	0.95	0.009	0.025	1.63

* Significantly greater than unity, $P < 0.01$.

steady-state concentrations in the control and probenecid-treated phases of the crossover. Fluorescein free fraction in control groups was ~32% and not concentration dependent. However, when 50 μg/ml probenecid was added to the plasma, the free fraction of fluorescein was slightly but significantly increased to 37 ± 0.3%. Three-hundred micrograms/milliliter probenecid, which is around the average steady-state probenecid concentration reached in infusion study, increased fluorescein free fraction by ~1.74-fold to a value of 58 ± 1.8%.

The distribution of fluorescein to brain at steady state was estimated after correction for changes in protein binding and was calculated using the free fraction of fluorescein from both phases. The brain-to-plasma unbound fluorescein concentration ratios in both cortical ECF and ventricular CSF were significantly increased by probenecid (Fig. 5). The brain distribution enhancement index after correction for free fraction was 2.23 ± 0.50 and 1.94 ± 0.88 for cortical ECF and ventricular CSF, respectively, and is significantly different from unity ($P < 0.01$).

DISCUSSION

In the present study, we used both *in vitro* and *in vivo* approaches to study organic anion transport in the CNS. Both *in vitro* and *in vivo* data demonstrate that fluorescein, an organic anion, was removed from the CNS by a probenecid-sensitive efflux system, which could be an MRP isoform or MRP-like (organic anion) efflux protein that exists at the BBB and BCSFB.

Fluorescein has excellent physicochemical properties for microdialysis (i.e., hydrophilicity and detectability) and was used in the present study as an organic anion probe to examine organic anion transport in the CNS. Fluorescein, as well as its derivatives 2′,7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein, carboxy-2′,7′-dichlorofluorescein and calcein, have been used as a substrate of MRP in studies examining MRP-mediated transport (6,27). In the present *in vitro* study, the accumulation of fluorescein was examined in BBMEC, an *in vitro* BBB model that has been shown to express various MRP isoforms, i.e., MRP1, MRP4, MRP5, and MRP6 (6,15). The accumulation of fluorescein in the BBB cells was enhanced by LY-329146, indomethacin, and probenecid. Probenecid is a well-known organic anion transport inhibitor and has also been shown to be a modulator of MRP1-mediated transport (20). LY-329146 is a raloxifene analog that has shown excellent activity against MRP1 with a IC_{50} (0.8 μM) for the inhibition of leucotriene C_4 transport (a high affinity MRP substrate) that is similar to the potent MRP1 inhibitor MK-571 (0.6 μM) (26,30). Indomethacin has been shown to sensitize MRP-overexpressing cells to vincristine and doxorubicin and has also been reported to be an MRP1 inhibitor (28). The enhancement of fluorescein accumulation in the BBMEC by three different MRP1 inhibitors further suggests that fluorescein is a substrate of MRP1. Furthermore, we have recently reported that in MDCKII MRP1-transfected cells, the accumulation of fluorescein was significantly lower (~40% lower) than that in wild-type MDCKII cells (31). Therefore, the use of fluorescein in the present study will help characterize organic anion transport in the CNS and may be indicative of MRP-mediated transport *in vivo*.

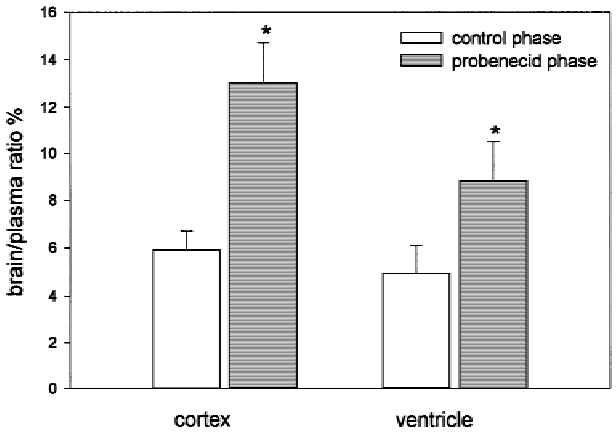

Fig. 5. The brain-to-plasma unbound fluorescein concentration ratios in cortical ECF and ventricular CSF. *$P < 0.01$, significantly different from control in the corresponding tissue ($n = 6$, mean ± SE).

Besides the expression of the MRP isoforms, Oatp1 has been shown expressed in choroid plexus (8) and has been suggested to transport compounds from CSF to choroid plexus epithelium and therefore work in concert with MRP1 in the efflux transport of substrates across BCSFB (9). Moreover, fluorescein may be a substrate for other MRP isoforms or other organic anion transporters such as Oatp 1. Therefore, although this specificity has not been reported, it is possible that other MRP isoform(s) and Oatp1 (BCSFB) is also involved in this process.

The *in vivo* study showed that probenecid increased the distribution enhancement of fluorescein to the ECF and CSF by approximately four- to fivefold (Table I), which is in agreement with the inhibition effect of probenecid on the efflux transport of fluorescein in the *in vitro* BBB model (increased accumulation, see Figs. 1–3). Given that high concentrations of probenecid decreased the protein binding of fluorescein, the distribution enhancement was corrected using the free plasma concentration and was ~1.94 ± 0.88 (range, 1.1–3.2) for the ventricle CSF and 2.23 ± 0.50 (range, 1.72–2.91) for the ECF. It is interesting to note that the distribution enhancement in the cortical ECF (BBB transport) in the *in vivo* study is similar to the increase in the accumulation in the *in vitro* fluorescein study in the BBMEC model (Fig. 1), indicating a reasonable in *vitro*/*in vivo* correlation for the effects of organic anion transport inhibition in the BBB.

The free fraction of probenecid has been shown to increase non-linearly with increasing total concentration, indicating a possible drug interaction at the plasma protein binding level when using high concentration of probenecid (32). Our protein binding study showed that probenecid displaced fluorescein from plasma protein binding sites, increasing the free fraction of fluorescein in plasma. The extent of the displacement was dependent on the total probenecid plasma concentration. The fact that probenecid significantly increased the plasma concentration of fluorescein at steady state (Fig. 4) indicates that probenecid decreased the systemic clearance of fluorescein in rat. Therefore, part of the significant increase in fluorescein brain distribution is due to the effect of probenecid on the systemic clearance of fluorescein. However, it is clear that there are effects on the BBB and BCSFB efflux transport. This is a good example of the complexity of drug interactions that are a result of transport in-

hibition. The interaction must be examined at the systemic level as well as at the specific tissue or organ barrier of interest, such as barriers in the CNS.

Given that there is a significant research effort to develop and use modulators of multidrug resistance proteins (such as MRPs), it is important to recognize that coadministration of those modulators may influence the disposition of other therapeutic agents and even endogenous compounds. If MRP inhibitors affect the CNS transport of MRP substrates, then modulators of MRP-mediated multidrug resistance may affect the CNS exposure of a variety of substrates, including neurotoxic oncolytic agents without regard to whether the transport is actually mediated by MRP or not. The significance of this study will remain important even if the relevant brain efflux transporter is an MRP-related organic anion transport system. In a recent study of the brain distribution of etoposide performed by Wijnholds *et al.* (33), the accumulation of etoposide in various tissues of mrp1/mdr1a/1b triple-knockout mice was compared with those of mdr1a/1b double-knockout mice. It is important to note that an increased accumulation of etoposide was observed in the ventricular CSF in the triple knockout mice compared with the double knockout animals. It is known that mrp1 acts as an efflux transport system in the choroid plexus (7), presumably explaining the increased levels of etoposide in the CSF in the mrp1-deficient mice. However, in the total brain homogenates (to which CSF levels may add insignificant amounts) there was no increase in the levels of etoposide. This result would be expected if the mrp1 genotype did not affect the BBB transport of etoposide. It is therefore possible that probenecid is affecting organic anion transport systems in the BBB other than MRP1.

The finding that Oatp 2 (10) is expressed in the BBB and BCSFB and that this transport protein may serve as an influx transport system would suggest that the effect of probenecid on an efflux transport system (such as an MRP-like transporter) might be attenuated by the concurrent inhibition of an organic anion influx system. However, in our study we see an increase in the brain ECF and CSF concentrations of fluorescein when probenecid is administered. Therefore, one can draw the conclusion that the probenecid-sensitive efflux transport is more active than a probenecid-sensitive influx system in both the BBB and the BCSFB.

Our microdialysis studies show that the blood-brain and blood-CSF barriers formed significant barriers for fluorescein distribution into the cortical ECF and the ventricular cerebrospinal fluid (CSF). The distribution of fluorescein into the brain and CSF was significantly enhanced by the coadministration of probenecid. The *in vivo* results correlated well with the probenecid-enhanced cellular accumulation and directional flux of fluorescein in an *in vitro* model of the BBB, the BBMEC. These results indicate that the CNS distribution of various drugs may be affected when there is the coadministration of inhibitors of drug efflux systems related to the MRP family of transport proteins. Moreover, the *in vivo* microdialysis model has been shown to be useful in the examination of drug interactions that affect drug efflux at the BBB and the BCSFB.

The results of the present study further characterize the processes that influence organic anion transport in the CNS. A more complete appreciation of the kinetics of the active transport systems in the CNS will help describe the distribution of organic anions in brain and allow predications of the relative significance of drug-drug interactions. This information will aid in avoiding drug interactions that may result in CNS toxicity and also it may allow the design of new drug delivery strategies to specifically enhance the brain distribution of therapeutically desirable agents to the CNS.

ACKNOWLEDGMENTS

This work was supported by grants NIH-NCI CA-75466 (to W.F.E.) and R03AG-17294 (to D.W.M.) from the National Institutes of Health and by Graduate Fellowships from the University of Nebraska Medical Center and a Presidential Fellowship awarded by the University of Nebraska (to H.S.).

REFERENCES

1. R. Spector and E. J. Goetzl. Leukotriene C4 transport and metabolism in the central nervous system. *J. Neurochem.* **46**:1308–1312 (1986).
2. B. M. Emanuelsson, L. Paalzow, and M. Sunzel. Probenecid-induced accumulation of 5-hydroxyindoleacetic acid and homovanillic acid in rat brain. *J. Pharm Pharmacol.* **39**:705–710 (1987).
3. C. S. Kim and J. B. Pritchard. Transport of 2,4,5-trichlorophenoxyacetic acid across the blood-cerebrospinal fluid barrier of the rabbit. *J. Pharmcol. Exp. Ther.* **267**:751–757 (1993).
4. S. L. Wong, K. van Belle, and R. J. Sawchuk. Distributional transport kinetics of zidovudine between plasma and brain extracellular fluid/cerebrospinal fluid in the rabbit: investigation of the inhibitory effect of probenecid utilizing microdialysis. *J. Pharmacol. Exp. Ther.* **264**:899–909 (1993).
5. A. Regina, A. Koman, M. Piciotti, B. E. 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).
6. H. Huai-Yun, D. T. Secrest, S. M. Karen, 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. V. V. Rao, J. L. Dahlheimer, M. E. Bardgett, A. Z. Snyder, R. A. Finch, A. 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).
8. R. H. Angeletti, P. M. Novikoff, S. R. Juvvadi, J. M. Fritschy, P. J. Meier, and A.W. Wolkoff. The choroid plexus epithelium is the site of the organic anion transport protein in the brain. *Proc. Natl. Acad. Sci. USA* **94**:283–286 (1997).
9. J. I. Nishino, H. Suzuki, D. Sugiyama, T. Kitazawa, K. Ito, M. Hanano, and Y. Sugiyama. Transepithelial transport of organic anions across the choroid plexus: possible involvement of organic anion transporter and multidrug resistance-associated protein. *J. Pharmcol. Exp. Ther.* **290**:289–294 (1999).
10. B. Gao, B. Stieger, B. Noe, J. M. Fritschy, and P. J. Meier. Localization of the organic anion transporting polypeptide 2 (Oatp2) in capillary endothelium and choroid plexus epithelium of rat brain. *J. Histochem. Cytochem.* **47**:1255–1264 (1999).
11. R. L. Leino, D. Z. Gerhart, and L. R. Drewes. Monocarboxylate transporter (MCT1) abundance in brains of suckling and adult rats: a quantitative electron microscopic immunogold study. *Brain. Res. Dev. Brain. Res.* **113**:47–54 (1999).
12. M. Kool, M. de Haas, G. L. Scheffer, R. J. Sheper, M. J. T. 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).
13. P. Borst, R. Evers, M. Kool, and J. Wijnholds. The multidrug resistance protein family. *Biochim. Biophys. Acta.* **1461**:347–357 (1999).
14. M. A. McAleer, M. A. Breen, N. L. White, and N. Mattews. pABC11 (also known as MOAT-C and MRP5), a member of the

ABC family of proteins, has anion transporter activity but dose not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. *J. Biol. Chem.* **274**:23541–23548 (1999).
15. Y. Zhang, H. Han, W. F. Elmquist, and D. W. Miller. Expression of multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain Res.* 876:148–153 (2000).
16. H. Suzuki. Analysis of xenobiotic detoxification system mediated by efflux transporters. *Yakugaku Zasshi* **119**:822–834 (1999).
17. H. Gutmann, M. Torok, G. Fricker, J. Huwyler, C. Beglinger, and J. Drewe. Modulation of multidrug resistance protein expression in porcine brain capillary endothelial cells *in vitro*. *Drug Metab. Dispos.* **27**:937–941 (1999).
18. B. El Hafny, O. Chappey, M. Piciotti, M. Debray, B. Boval, and F. Roux. Modulation of P-glycoprotein activity by glial factors and retinoic acid in an immortalized rat brain microvessel endothelial cell line. *Neurosci. Lett.* **236**:107–111 (1997).
19. W. F. Elmquist and R. J. Sawchuk. Application of microdialysis in pharmacokinetic studies. *Pharm. Res.* **14**:267–288 (1997).
20. J. H. Hooijberg, H. J. Broxterman, M. Kool, Y. G. Assaraf, G. J. Peters, P. Noordhuis, R. J. Scheper, P. Borst, H. M. Pinedo, and G. Jansen. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Res.* **59**:2532–2535 (1999).
21. D. W. Miller, K. L. Audus, and R. T. Borchardt. Application of cultured endothelial cells of the brain mirovasculature in the study of the blood-brain barrier. *J. Tissue Cult. Meth.* **14**:217–224 (1992).
22. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randal. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
23. H. Yang, Q. Wang, and W. F. Elmquist. Fluconazole distribution to the brain: a crossover study in freely-moving rats using *in vivo* microdialysis. *Pharm. Res.* **13**:1570–1575 (1996).
24. Y. Wang, S. L. Wong, and R. J. Sawchuk. Microdialysis calibration using retrodialysis and zero-net flux: application to a study of the distribution of zidovudine to rabbit cerebrospinal fluid and thalamus. *Pharm. Res.* **10**:1411–1419 (1993).
25. R. E. Galinsky, K. K. Flaharty, B. L. Hoesterey, and B. D. Anderson. Probenecid enhances central nervous system uptake of 2′,3′-dideoxyinosine by inhibiting cerebrospinal fluid efflux. *J. Pharmcol. Exp. Ther.* **257**:972–978 (1991).
26. A.H. Dantzig, R.L. Shepard, K.L. Law, L. Tabas, S. Pratt, J.S. Gillespie, S.N. Binkley, M.T. Kuhfeld, J.J. Starling, and S.A. Wrighton. Selectivity of the multidrug resistance modulator, LY335979, for p-glycoprotein and effect on cyctochrome P-450 activities. *J. Pharmcol. Exp. Ther.* **290**:854–862 (1999).
27. B. H. Norman, A. H. Dantzig, K. L. Hauser, J. S. Kroin, K. L. Law, A. D. Palkowitz, R. L. Shepard, J. P. Sluka, J. J. Starling, L. L. Tabas, and M. A. Winter. Novel inhibitors of the multidrug resistance-associated protein (MRP). *Annu. Meet. Am. Assoc. Cancer Res.* (1997).
28. M. P. Draper, R. L. Martell, and S. B. Levy. Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressed MRP, but not P-glycoprotein. *Br. J. Cancer.* **75**:810–815 (1997).
29. H. Sun, P. Bungay, and W. F. Elmquist. The influence of capillary efflux inhibition on microdialysis probe recovery. *J. Pharmacol. Exp. Therap.* **297**:991–1000 (2001).
30. I. Leier, G. Jedlitschky, U. Buchholz, S. P. Cole, R. G. Deeley, and D. Keppler. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J. Biol. Chem.* **269**:27807–27810 (1994).
31. H. Sun, D. R. Johnson, R. A. Finch, A. C. Sartorelli, D. W. Miller, and W. F. Elmquist. Transport of fluorescein in MDCKII-MRP1 transfected cells and mrp1-knockout mice. *Biochem. Biophys. Res. Commun.* **284**:863–869 (2001).
32. B. M. Emanuelsson and L. Paalzow. Dose-dependent pharmacokinetics of probenecid in the rat. *Biopharm. Drug Disposit.* **9**:59–70 (1988).
33. J. Wijnholds, E. C. M. de Lange, G. L. Scheffer, D. J. van den Berg, C. A. A. M. 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).