Valproic Acid Is Not a Substrate for P-glycoprotein or Multidrug Resistance Proteins 1 and 2 in a Number of in Vitro and in Vivo Transport Assays

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ABSTRACT

The antiepileptic drug valproic acid (VPA) is widely used in the treatment of epilepsy, bipolar disorders, and migraine. However, rather high doses are required for the clinical effects of VPA, which is due to its relatively inefficient delivery to the brain. The poor brain distribution of VPA is thought to reflect an asymmetric transport system at the blood-brain barrier (BBB). Based on recent data from in vitro experiments, multidrug resistance proteins (MRPs) have been proposed to be involved in the efflux transport of VPA at the BBB. In the present study, we used different experimental in vitro and in vivo strategies to evaluate whether VPA is a substrate for MRPs or the efflux transporter P-glycoprotein (Pgp). In contrast to known Pgp or MRP substrates, such as cyclosporin A or vinblastine, no directional transport of VPA was observed in cell monolayer efflux assays using the kidney cell lines Madin Darby canine kidney II and LLC-PK1, which had been transfected with either human or mouse cDNAs for the genes encoding Pgp, MRP1, or MRP2. Likewise, no indication for efflux transport of VPA was obtained in a rat microdialysis model, using inhibitors of either Pgp or MRPs. Furthermore, a significant role of MRP2 in brain efflux of VPA was excluded by using MRP2-deficient rats. Our data do not support the hypothesis that MRP1 or MRP2 is involved in the efflux of VPA from the brain. Thus, the molecular identity of the putative transporter(s) mediating the active efflux of VPA from the brain remains to be elucidated.

Valproic acid (VPA) is one of the major antiepileptic drugs (AEDs) for treatment of generalized or partial epilepsy but is also increasingly been used for treatment of other brain diseases, including migraine and bipolar disorders (Rogawski and Löscher, 2004). Because of their physicochemical properties, most AEDs are thought to penetrate into the brain by passive diffusion, whereas the bidirectional movement of VPA across the blood-brain barrier (BBB) is believed to be mediated jointly by passive diffusion and carrier-mediated transport, with efflux transport being much more efficient than influx transport (Shen, 1999). As a consequence, even though VPA is highly ionized (>99%) at physiological pH, it rapidly enters into the brain, but brain concentrations of VPA remain significantly lower than its unbound fraction in plasma (Shen, 1999; Löscher, 2002). Almost 30 years ago, we reported that the cerebrospinal fluid (CSF)/plasma ratio of VPA in dogs can be markedly enhanced by probenecid, an inhibitor of organic acid transport carriers, indicating that a probenecid-sensitive efflux transporter is involved in the elimination of VPA from the CSF (Frey and Löscher, 1978). Subsequently it was shown that probenecid also increased the concentration of valproate in the brain (Adkison et al., 1994). The probenecid-sensitive transporter that mediates the efflux of VPA from the CSF and brain is not known, but likely drug efflux transporters in this respect would be the multidrug resistance proteins (MRPs), some of which are located at the blood-CSF and blood-brain barriers (Sun et al., 2003; Löscher and Potschka, 2005a). MRPs, which belong to the superfamily of ATP-binding cassette (ABC) transporters,
have been first identified in chemotherapy-resistant cancers but were subsequently shown to be expressed in various normal tissues, where they serve excretory and protective roles (Borst et al., 2000). Several MRPs, including MRP1 to MRP5, can be modulated by probes (Lösch and Potschka, 2005a). Some in vitro studies have indicated that VPA may be a substrate for MRPs in brain capillary endothelial cells (Hua-Yun et al., 1998; Gibbs et al., 2004; Bachmeier and Miller, 2005), which raises the possibility that MRPs may serve as the efflux transporters of VPA at the BBB and blood-CSF barrier and would explain the previously described effects of the nonselective MRP inhibitor probenecid on brain and CSF levels of valproate.

MRPs have not only been implicated in drug resistance of cancer cells but also in drug resistance of brain disorders such as epilepsy (Lösch and Potschka, 2005a,b). Overexpression of MRP1 and MRP2 has been determined in epileptogenic brain tissue of patients with medically refractory epilepsy (Sisodiya, 2003; Schmidt and Lösch, 2005). Furthermore, some major AEDs, including phenytoin, seem to be substrates of MRPs (Lösch and Potschka, 2005a). Thus, overexpression of MRPs in brain capillary endothelial cells that form the BBB is likely to limit the brain access of AEDs that are MRP substrates (Sisodiya, 2003). Therefore, it is important to know which AEDs are transported by MRPs. In addition to MRPs, such as MRP1 and MRP2, which both are expressed at the apical membrane of brain capillary endothelial cells (Lösch and Potschka, 2005a), the drug efflux transporter P-glycoprotein (Pgp) is overexpressed in the BBB of epileptogenic brain tissue and thought to restrict the brain access of several AEDs (Sisodiya, 2003; Lösch and Potschka, 2005a,b; Schmidt and Lösch, 2005). Using bovine brain microvessel endothelial cells (BBMECs) as a model of the BBB, Bachmeier and Miller (2005) recently reported that VPA does not seem to exhibit any interactions with Pgp-related transport.

In the present study, we used different experimental in vitro and in vivo strategies to evaluate whether VPA is a substrate for the first two members of the MRP family or Pgp. First, cell monolayer efflux assays were performed using the kidney cell lines MDCKII and LLC-PK1, which had been transfected with either human or mouse cDNAs for the genes encoding Pgp (MDR1 in humans and mdr1a in rodents), MRP1, or MRP2, respectively. Wild-type cell lines were used for comparison. Second, we used a rat microdialysis model with microdialysis probes in both brain hemispheres and local (cerebral) inhibition of either Pgp or MRPs in one hemisphere to determine whether modulation of these transporters affects the brain disposition of VPA in vivo. We have used this model previously to study the role of Pgp and MRPs in brain access of various AEDs (for review, see Lösch and Potschka, 2005a). In addition to cerebral administration of Pgp or MRP inhibitors, the MRP inhibitor probenecid was also administered systemically in the present study. Third, based on previous experiments with other AEDs (Potschka et al., 2003a,b), we used the MRP2-deficient (TR−) rat strain to investigate the role of MRP2 in brain efflux of VPA. In this way, we examined transport of VPA by Pgp or MRPs from three species: mouse, rat, and human.

### Materials and Methods

**Monolayer Efflux Studies.** MDCK type II cells transfected with either human MRP1 (MDCKII-MRP1) or human MRP2 (MDCKII-MRP2) and respective wild-type cells (MDCKII/wt) were kindly provided by Prof. P. Borst (National Cancer Institute, Amsterdam, The Netherlands). Furthermore, Prof. Borst kindly provided LLC-PK1 cells transfected with either human MDR1 (LLC-MDR1) or mouse mdr1a (LLC-mdr1a) and respective wild-type LLC cells (LLC/wt). After obtaining the cells, they were grown in the absence of any selection agent (for details, see Baltes et al., 2006).

For the transport assays, cell monolayers cultured in the Transwell system, i.e., permeable filter membrane inserts in multilwell culture plates were used that allow the study of drug transport between an apical (luminal) and basolateral (abluminal) compartment (for more details, see Baltes et al., 2006). Before starting the transport experiments, the medium was replaced with Opti-MEM (Invitrogen, Eggenstein, Germany), and the transwells were preincubated for 1 h. This reduced serum medium was used without any additives according to the protocol of the laboratory that provided the cell lines (Prof. P. Borst) to minimize protein binding of the drugs. At the beginning of the experiment (t = 0), the preincubation medium was replaced by fresh Opti-MEM containing the drug either in the apical or the basolateral chamber (donor chamber). VPA was diluted as aqueous solution of its sodium salt in the medium. Samples were taken at 60, 120, 240, and 360 min from the respective opposite (receiver) chamber of the well. The concentrations of VPA were chosen on the basis of its “therapeutic plasma concentration range” (40–100 μg/ml or 280–690 μM) in patients with epilepsy (Lösch, 2002). In addition, because higher concentrations of VPA are needed to induce anticonvulsant effects in other species, e.g., rodents (Lösch, 2002), such higher concentrations were also tested in the transport assays. The transport assays including preincubation were performed at 37°C in a humidified incubator with shaking the transwells gently at 50 rpm. The integrity and tightness of the monolayers as well as their transporter expression were checked as described recently (Baltes et al., 2006).

The results of the individual transport assays are presented as the percentage of the initial drug concentration in the donor chamber versus time. In addition, several calculations were performed. Apical-to-basal (P_{app aB}) and basal-to-apical (P_{app bA}) permeabilities were determined according to Artursson (1990) using the following equation:

$$P_{app} = \frac{dQ}{dt} \times (A \times C_0 \times 60)$$  \hspace{1cm} (1)

where dQ/dt [μg/min] is the permeability rate of the drug, A is the surface area of the monolayer, and C_0 is the initial drug concentration in the donor chamber. For apically expressed transporters (Pgp, MRP2), a transport ratio (TR) was calculated by dividing P_{app bA} by P_{app aB}. For MRP1, which is expressed at the basolateral membrane of kidney cells (Borst et al., 2000), TR was calculated in the reverse fashion, i.e., by dividing P_{app aB} by P_{app bA}. Corrected transport ratios (cTRs) were calculated by division of the TR obtained in MDR1-, mdr1a-, MRP1-, or MRP2-transfected cells by the TR obtained in the respective wild-type/parental cells (Schwab et al., 2003). The substrate classification is suggested by these authors to be performed according to the following scheme: cTR < 1.5, −; 1.5 to 2.5; +; 2.5 to 3.4; ++; >3.5, +++.

As positive controls for directional transport in the different cell lines, we used the prototype Pgp substrate cyclosporin A (Schinkel et al., 1995; Fromm, 2004) and [3H]vinblastine, which is transported by Pgp, MRP1, and MRP2 (Évers et al., 1998; Goh et al., 2002; Tang et al., 2002; Yang et al., 2004). Cyclosporin A was dissolved in dimethyl sulfoxide (2 mg/ml) before dilution in the medium to give concentrations of 0.1 to 4 μM in the assay. [3H]Vinblastine (9.8 Ci/ml) was diluted with unlabeled vinblastine sulfate (which was dissolved in the medium) to give an activity of 0.25 μCi/ml and a final concent
tion of 2 µM in the assay. To inhibit any Pgp-mediated transport by expression of Pgp in MDCKII wild-type cells or MRP2/MRP1-transfected MDCKII cells (see Goh et al., 2002), the selective Pgp inhibitor tariquidar (0.2 µM) was included in all transport experiments with vinblastine in MDCKII cells. The transport experiments with vinblastine in these cells were essentially performed as described by Evers et al. (1998). Likewise, the selective MRP1/2 inhibitor MK571 (50 µM) was included in all experiments with vinblastine in LLC cells to inhibit any MRP-mediated transport in these cells. MK571 and tariquidar were also used to check the specificity of the MRP2- or Pgp-mediated directional transport of vinblastine in MDCKII or LLC cells, respectively. To examine whether transport of VPA in transfected cell lines can be altered by inhibition of MRPs or PgP, we performed additional experiments with VPA in MDCKII and LLC cells with addition of tariquidar (0.2 µM), MK571 (50 µM), probenecid (100 µM), or verapamil (100 µM) or combinations of these inhibitors.

**Studies in Animals**. The following rat strains were used for in vivo transport experiments: adult Wistar rats (Harlan-Winkelmann, Borchen, Germany) weighing 200 to 230 g; adult TR– Wistar rats, either bred in our laboratory (see below) or obtained from Harlan Netherlands (Horst, The Netherlands); and age-matched rats of the background strain of TR– rats (Harlan Netherlands). Rats were housed under controlled conditions (ambient temperature, 24–25°C; humidity, 50–60%; lights on from 6:00 AM to 6:00 PM). Animals were purchased from the breeder at least 1 week before being used in the experiments. Food (Altromin 1324 standard diet) and water were freely available. All efforts were made to minimize both the suffering and the number of animals. The procedures used in this study were conducted in accordance with the German Animal Welfare Act and were approved by the responsible governmental agency in Hannover.

**Microdialysis in Normal Wistar Rats**. These experiments were essentially performed as described recently by us in detail (Potschka and Löschner, 2001a). In short, two guide cannulae (Carnegie Medicine, Stockholm, Sweden) were implanted into the left and right frontal (motor) cortex of adult Wistar rats (Harlan-Winkelmann). After a recovery period of at least 7 days and 18 to 20 h before the experiment (to allow the BBB to reseal; Benveniste and Hansen, 1991), 3-mm microdialysis probes (diameter, 0.5 mm; CMA/12; Carnegie Medicine) were inserted through the guide cannulae into the frontal cortex of conscious, freely moving rats. The probes were perfused with dialysate buffer (mock CSF or Ringer’s solution) at a flow rate of 2 µl/min. Each 60-µl outflow sample was collected for drug analysis. One hour after onset of perfusion, VPA was administered i.p., and dialysate samples (2 per h) were collected over the next 2 h. It should be noted that the first sample (0–30 min after VPA) may not reflect exactly the brain extracellular VPA concentration kinetics because the sample is diluted with residual drug-free dialysate buffer in the microdialysis tubing system. The dose of VPA (200 mg/kg of its sodium salt) was chosen on the basis of previous experiments in Wistar rats (Löschner et al., 1989), showing that this dose resulted in anticonvulsant effects in the kindling model. Together with each dialysate sample, a blood sample was collected for drug analysis in plasma and dialysate (see below). For inhibition of MRP1 and MRP2, local application of probenecid (10 mM) via one (either the right or left) microdialysis probe was started 30 min before VPA injection. For inhibition of Pgp, verapamil (5 mM of the hydrochloride) was applied. The contralateral microdialysis probe was perfused with the respective dialysate buffer without inhibitor and served as individual control in each rat. Concentrations of the inhibitors in the dialysate buffer were based on previous experiments with phenytoin, in which these concentrations of verapamil and probenecid increased the BBB penetration of phenytoin (Potschka and Löschner, 2001a,b). For systemic application, probenecid was dissolved in NaCl (by means of dilute NaOH) and injected 15 min before VPA at a dose of 50 mg/kg i.p. This dose has been proven to potentiate the anticonvulsant efficacy of phenytoin in the rat kindling model (Potschka et al., 2003a). Using a crossover design, the same animals were used for control experiments, in which an equivalent amount of vehicle was injected 15 min before VPA. These control experiments were performed at least 4 weeks either before (two rats) or after (three rats) the probenecid experiment. For each microdialysis probe used for the in vivo experiments, the in vitro drug recovery was determined before and after the in vivo experiment and used for correction of the in vivo dialysate concentrations as described previously (Potschka and Löschner, 2001b). Mean VPA recovery in vitro was 17.98 ± 5.62% (mean ± S.D. of 55 determinations). For in vivo experiments in Wistar rats, two probes were used in combination (for left and right cortex), with recovery values not differing by more than 5%.

**Microdialysis in TR– Rats**. In addition to microdialysis experiments in normal Wistar rats, adult TR– rats of the Wistar strain were used as described above for normal Wistar rats, except that microdialysis was performed from only one hemisphere. Five TR– rats were bred in our department. The breeding pairs were kindly provided by Prof. R. P. J. Oude Elferink (Academic Medical Center, University of Amsterdam, The Netherlands). Four TR– rats were obtained from Harlan Netherlands. TR– rats from Prof. Oude Elferink and Harlan were genetically identical. For comparison with TR– rats, age-matched rats of the background strain (Harlan) were used.

**Experiments with Determination of VPA in Brain Tissue**. For these experiments, 200 mg/kg VPA was administered i.p., and groups of Wistar rats were decapitated either 15 or 120 min after drug injection. The treated groups received 50 mg/kg probenecid, which was administered i.p. 15 min before VPA. The control groups received an equivalent amount of vehicle instead of probenecid. At the time of decapitation, blood was sampled for drug analysis in plasma. After decapitation, the brains were dissected and weighed. Subsequently, the brain tissue was homogenized in the same amount of distilled water and immediately stored at −20°C until analysis. VPA analysis in brain tissue was additionally performed in TR– rats. For these experiments, four of the normal Wistar rats and six of the TR– rats used for microdialysis (see above) were decapitated directly after the end of the period of dialysate sampling (i.e., 120 min after administration of VPA).

**Drug Analysis**. The VPA concentration in plasma, in dialysate, in brain tissue, and in transport assay samples was measured by high-performance liquid chromatography with ultraviolet detection as described earlier (Potschka and Löschner, 2001a) with a few modifications. Instead of acetonitrile, methanol was used for extraction of VPA from plasma and transport assay samples. For drug analysis in brain tissue, the homogenate was spiked with heptanoic acid as an internal standard, precipitated with ethanol, vortexed, and centrifuged. The supernatant was used for high-performance liquid chromatography analysis. Dialysate samples were injected without further purification into the high-performance liquid chromatography apparatus. The detection wavelength of the UV detector was 210 nm. The limit of detection was 25 µg/ml VPA in plasma or dialysate, 6.4 µg/ml VPA in transport assay samples, and 10 µg/g brain tissue. Variation coefficients from repeated analysis of the same samples were 1.3% for plasma, 1.6% for dialysate, 2.5% for transport assay samples, and 1.5% for brain samples, respectively.

Cyclosporin A was measured by liquid chromatography-mass spectrometry as described elsewhere (Koal et al., 2004). The radioactivity in samples from experiments with [3H]vinblastine was quantified using a scintillation counter.

**Drugs.** VPA (used as its sodium salt) was diluted from a commercial aqueous solution (Orfrril; Desitin, Hamburg, Germany). Cyclosporin A was kindly provided by Novartis (Basel, Switzerland) and tariquidar (XR9576) by Xenova Ltd. (Slough, Berkshire, UK). (-)-Verapamil and probenecid were obtained from Sigma-Aldrich (Deisenhofen, Germany). Vinblastine sulfate and MK571 (sodium salt) were obtained from Calbiochem (Merck Biosciences, Darmstadt, Germany) and [3H]vinblastine sulfate (9.8 Ci/mmol) from Amersham (Buckinghamshire, UK).
Statistics. In the in vitro transport experiments, the statistical significance of differences between apical to basolateral and basolateral to apical drug transport was calculated by two-way analysis of variance (ANOVA) followed by post hoc testing with the Bonferroni test. In the microdialysis experiments, the statistical significance of differences between left and right cortex or between TR- rats and normal Wistar rats was calculated by two-way ANOVA followed by post hoc testing with the Bonferroni test. The statistical significance of differences between plasma area under the curve (AUC) values was calculated by Student’s t test. In experiments with determination of VPA in brain tissue, the statistical significance of differences between groups was calculated by Student’s t test. P < 0.05 was considered significant in all tests.

Results

Monolayer Efflux Studies. The prototype Pgp substrate cyclosporin A exhibited significant directional transport in the Pgp-overexpressing cell lines with average cTRs of 3.42 (LLC-MDR1) and 1.96 (LLC-mdr1a), respectively (Fig. 1; Table 1). Likewise, vinblastine (in the presence of MK571) exhibited significant directional transport in these cell lines with average cTRs of 3.37 (LLC-MDR1) and 4.26 (LLC-mdr1a), respectively (Fig. 1; Table 1). This Pgp-mediated transport of vinblastine could be suppressed by the Pgp inhibitor tariquidar (Fig. 1). In MRP2-transfected MDCKII cells, vinblastine (in the presence of tariquidar) exhibited significant directional transport with an average cTR of 5.3 (Fig. 1; Table 1). In MRP1-transfected MDCKII cells, vinblastine (in the presence of tariquidar) showed a significant directional transport in the basolateral direction, resulting in a cTR of 1.64, thus meeting the criteria for a substrate (cTR of at least 1.5; Schwab et al., 2003). Transport of vinblastine by MRP2 could be inhibited by MK571 (50 μM) but not probenecid (100 μM) (Fig. 1; Table 1).

In contrast to vinblastine, with VPA, at concentrations within the “therapeutic range” (40–100 μg/ml; 280–690 μM), no polarized efflux was observed in MDCK/wt, MDCK-MRP1, or MDCK-MRP2 cell lines (Fig. 2; Table 1). When studying VPA at 330 μM (47 μg/ml) in the LLC-MDR1 and LLC-mdr1a cell lines, we obtained cTRs of 1.47 (LLC-MDR1) and 1.6 (LLC-mdr1a), which just met the criteria for a weak Pgp substrate (TR 1.5–2.5) (Schwab et al., 2003) but were mostly due to unusually low TR in the LLC-wt cell line (Fig. 3; Table 1). When higher concentrations of VPA were tested, no po-

![Fig. 1. Representative experiments with cyclosporin A (CsA) and vinblastine (VBL) in MDCKII and LLC cells. Data are given as the percentage of the initial drug concentration (as indicated in the figure) in the donor chamber versus time. Triangles, basolateral-to-apical (b-A) transport; squares, apical-to-basolateral (a-B) transport. All experiments were performed in triplicate, and values are shown as means ± S.D. When no error bar is visible, the deviation was within the size of the symbols. Note that different percentage scales at the y-axis were used for different drugs. For each experiment, TRs are given. Note that the TR for VBL in MRP1-overexpressing cells was calculated by dividing $P_{app_{aB}}$ by $P_{app_{bA}}$ because VBL was transported in the basolateral direction in these cells. The significance of differences between transport in the basolateral or apical direction was analyzed by two-way ANOVA; in case of a significant difference (P < 0.05), results from post hoc testing, i.e., significant differences between the two curves at individual time points of the experiment, are indicated by an asterisk (P < 0.05). The data illustrate that CsA displays transport in the apical direction in MDR1- and mdr1a-transfected LLC cells, whereas VBL exhibits transport in the apical direction in Pgp- and MRP2-overexpressing cell lines but transport in the basolateral direction in the MRP1-overexpressing line. Directional transport of VBL in LLC-MDR1 or LLC-mdr1a cells can be inhibited by the Pgp inhibitor tariquidar (0.2 μM), whereas transport of VBL in MDCKII-MRP2 cells can be inhibited by the MRP inhibitor MK571 (50 μM). Probenecid (100 μM) does not affect transport of VBL in MDCKII-MRP2 cells.}
also examined whether probenecid (100 μM) was always around 1. Using MDCKII-MRP2 cells, we analyzed transport of VPA in MDCKII or LLC cell lines, but verapamil nor probenecid or MK571 resulted in any direction. Therefore, we performed experiments in which various MRP or Pgp inhibitors were included in the monolayer efflux assay either alone or in combination. As shown in Figs. 2 and 3 and Table 1, neither addition of tariquidar or the MRP inhibitor probenecid via the microdialysis probe did not result in any significant difference in the 2nd h of the experiments.

Lack of asymmetry in transport as observed in most experiments with VPA may be either an indication of passive transport or an indication of equal active transport in both directions. Therefore, we performed experiments in which various MRP or Pgp inhibitors were included in the monolayer efflux assay either alone or in combination. As shown in Figs. 2 and 3 and Table 1, neither addition of tariquidar or verapamil nor probenecid or MK571 resulted in any direction. Therefore, we performed experiments in which various MRP or Pgp inhibitors were included in the monolayer efflux assay either alone or in combination. As shown in Figs. 2 and 3 and Table 1, neither addition of tariquidar or the MRP inhibitor probenecid via the microdialysis probe did not result in any significant difference in the 2nd h of the experiments.

### Summary of transport assay results

The table shows TRs and cTRs from individual transport experiments with cyclosporin A, vinblastine, or VPA as shown in Figs. 1 to 3. cTRs were calculated as the TR obtained in transfected cells, divided by the TR obtained in respective wild-type (parental) cells in the same experiment. Transport by wild-type cells was not repeated in every experiment with a given cell line and drug, explaining that there are different numbers of TRs and cTRs for some experiments. Note that the TR for VBL in MRP1-overexpressing cells was calculated by dividing $P_{app,ab}$ by $P_{app,ba}$, because VBL was transported in the basolateral direction in these cells. Substrate classification was based on the proposal of Schwab et al. (2003) according to the following scheme: cTR < 1.5, +; 1.5 to 2.5, ++; 2.6 to 3.4, +++; and $>3.5$, ++++. 

<table>
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<th>Concentration of Substrate</th>
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<th>MDCKII-MRP2</th>
<th>LLC/wt</th>
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### Microdialysis Experiments in Normal Wistar Rats

During the microdialysis experiments in normal Wistar rats, VPA plasma levels were determined at 30, 60, 90, and 120 min after the i.p. administration of 200 mg/kg of the drug. As shown in Figs. 4 and 5, VPA plasma levels reached peak concentrations within 30 min and then steadily declined thereafter to ~50% of the initial concentration within the 2-h duration of the experiment. VPA dialysate levels usually reached peak levels at the second determination (sample collected from 30–60 min) and then steadily declined like the plasma concentration. The actual brain entry of VPA through the blood-brain barrier is displayed in Figs. 4, C and G, and 5C. As shown by the dialysate/plasma ratios, the extracellular VPA concentration reached almost constant levels of approximately 10 to 35% of the plasma concentration during the 2nd h of the experiments.

Local administration of the Pgp inhibitor verapamil or the MRP inhibitor probenecid via the microdialysis probe did not exert any significant effect on the extracellular brain concentration of VPA compared with the untreated contralateral site (Fig. 4). In addition to administering probenecid via the microdialysis probe, we also performed experiments in which the MRPI inhibitor was administered systemically (50 mg/kg i.p.) 15 min before VPA. As shown in Fig. 5, coadministration of probenecid did not result in any significant difference in dialysate VPA concentrations or in the dialysate/plasma ratio compared with control experiments without probenecid. Furthermore, the plasma kinetics of VPA were not affected by probenecid (Fig. 5). In contrast to VPA, local administration of the Pgp inhibitor verapamil or the MRPI inhibitor...
probenecid significantly increased the dialysate/plasma ratio of the AED phenytoin (Fig. 4, D and H), indicating that Pgp and MRPs are involved in the regulation of brain uptake of this drug.

**Microdialysis Experiments in MRP2-Deficient Wistar Rats.** To validate the data from the experiments with MRP inhibition by probenecid, extracellular VPA brain concentration was compared between MRP2-deficient TR/H11002 rats and wild-type rats of the Wistar background strain following i.p. administration of 200 mg/kg VPA. If MRP2 is involved in VPA efflux at the BBB, lack of MRP2 should result in an increased VPA brain concentration. However, the comparison of the MRP2-deficient and background Wistar strains did not result in any significant difference in the VPA concentration time diagrams (Fig. 5, D–F). The plasma and dialysate VPA concentrations of TR rats tended to be lower than those of the wild-type Wistar rats, which prompted us to calculate the AUC for the VPA concentrations in the two strains. This resulted in a significant difference ($P = 0.0343$) between the strains in plasma AUC (TR rats, $18,420 \pm 1080$; wild-type rats, $22,890 \pm 1602$; mean $\pm$ S.E.M., $n = 9$), whereas the AUC for the VPA dialysate concentrations did not differ significantly between TR rats and controls. The slightly lower VPA dialysate concentrations in TR rats (Fig. 5) are probably a consequence of the lower plasma concentrations in this strain compared with normal Wistar rats. In contrast to VPA, the dialysate/plasma ratio of the AED phenytoin was significantly higher in TR rats than normal controls (Fig. 5G), indicating that MRP2 is involved in the regulation of brain uptake of this drug.

**Brain Tissue Levels of VPA.** The lack of probenecid treatment or MRP2 deficiency to affect brain dialysate levels of VPA prompted us to perform additional experiments in which VPA was determined in brain tissue after treatment with probenecid. Two time points were investigated, 15 and 120 min after administration of 200 mg/kg i.p. VPA with or without coadministration of 50 mg/kg i.p. probenecid (15 min before VPA). Data are shown in Fig. 6. Fifteen minutes following VPA, a brain/plasma ratio of 0.15 was determined in the absence of probenecid (Fig. 6A). After treatment with probenecid, plasma and brain levels of VPA were about the same as in the absence of probenecid. Accordingly, the brain/plasma ratio of VPA was not affected by probenecid in rats (Fig. 6A). Likewise, no significant differences in plasma or brain concentrations of VPA between probenecid-treated rats and controls were seen 120 min after administration of VPA (Fig. 6B).

In a further experiment, VPA was determined in brain tissue of TR rats and controls 120 min following i.p. administration of 200 mg/kg VPA. As shown in Fig. 6C, there was a tendency for reduced VPA levels in plasma and brain in TR rats, but the difference to control was not statistically

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**Fig. 2.** Representative experiments with VPA in MDCKII cell lines. For further details, see legend to Fig. 1. VPA does not show any directional transport under the conditions of the experiments. Furthermore, addition of the MRP inhibitor MK571 or the MRP modulator probenecid did not affect the penetration of VPA through the cell monolayers.
significant. Furthermore, the brain/plasma ratio of TR rats and controls was comparable.

Discussion

In contrast to our expectations, we did not obtain any significant evidence for transport of VPA by MRPs in the present study. In addition, VPA did not seem to be transported by Pgp in the model systems used here. Although the present data on Pgp are in line with a recent study in which lack of Pgp-related transport interactions was reported for VPA by using monolayers of bovine BMECs (Bachmeier and Miller, 2005), our data on MRPs are in apparent contrast to several previous reports indicating that VPA may be a substrate for MRPs. Using bovine BMECs, Huai-Yun et al. (1998) reported that VPA (100 μM) increased the monolayer uptake of the MRP substrate fluorescein by 50%, suggesting that VPA may be a substrate and competitive inhibitor of MRPs expressed by bovine BMECs. In a subsequent study by Gibbs et al. (2004), the nonselective MRP inhibitors indomethacin and probenecid were shown to increase VPA uptake by bovine BMECs. Similar results were obtained in the MRP-overexpressing human lung adenocarcinoma cell line A549 (Gibbs et al., 2004). Bachmeier and Miller (2005) showed that VPA significantly enhanced the accumulation of the mixed Pgp and MRP probe, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, with an IC50 of 769 μM. However, these studies did not demonstrate which MRPs were involved, and they did not exclude that transporters other than MRPs were involved in these findings. In the present study, by using well defined cell lines overexpressing Pgp, MRP1, or MRP2, no indication of directional transport of VPA was obtained, although VPA was tested over a large range of concentrations (40–500 μg/ml or 280–3500 μM, respectively). In this respect, it is important to note that the therapeutic plasma concentration range of VPA in patients with epilepsy is approximately 40 to 100 μg/ml (280–690 μM), but peak plasma levels after VPA administration may be even higher (Löschner, 2002). In rodent seizure models, minimal plasma concentrations of VPA associated with anticonvulsant activity are approximately 1000 μM (Löschner, 2002). At such high concentrations, transporters in monolayer transport assays may be saturated. However, directional transport of VPA at concentrations far below its therapeutic concentration range would not explain the low brain exposure of this compound seen in vivo after administration of anticonvulsant doses in laboratory animals or patients with epilepsy.

When comparing the therapeutic plasma concentration of VPA in epilepsy patients or rodent models of epilepsy with the VPA concentrations tested in the present study, the plasma protein binding of VPA needs to be considered because it is the free concentration on both sides of the
membrane (brain and plasma) that drives transport processes. In humans, plasma protein binding of VPA is high but dependent on the drug concentration (Löschter, 1985; Shen, 1999). The average plasma free fraction of VPA (slightly below 10%) remains constant up to a plasma concentration of 75 µg/ml and increases to 15% at 100

Fig. 4. Effect of verapamil or probenecid on brain dialysate levels of VPA. The figure shows VPA concentration time profiles in plasma (A and E) and brain dialysate (B and F). VPA was injected i.p. at a dose of 200 mg/kg. All values are mean ± S.E.M. of seven (verapamil) or five (probenecid) rats. Dialysates were obtained 0 to 120 min after VPA from the right and left frontal cortex of the same rats. Each dialysate sample was collected over a period of 30 min (i.e., from 0–30, 30–60, 60–90, and 90–120 min, respectively). C and G, ratio between dialysate and plasma concentrations. Verapamil (5 mM) or probenecid (10 mM) were administered via one (either left or right) microdialysis probe, beginning 30 min before VPA, over the course of the experiment. The treatment did not result in any significant difference either in the dialysate VPA or in the dialysate/plasma ratio. For comparison, data from respective experiments with phenytoin are illustrated in D and H. These data are shown as mean ± S.E.M. of five (verapamil) or eight (probenecid) rats, respectively. Significance of differences is indicated by an asterisk (P < 0.05). The data with phenytoin are from previous publications of our group (Potschka and Löschter, 2001a; Potschka et al., 2003a).
μg/ml, 22% at 125 μg/ml, and 30% at 150 μg/ml, indicating saturation of plasma albumin (Löschler, 1985; Shen, 1999). In rodents, plasma protein binding of VPA is much lower than in humans, with average values of 60% in the rat and only 10% in the mouse (Löschler, 1978). Despite these marked species differences in plasma protein binding, sim-
ilar low brain/plasma concentration ratios of VPA have been determined in humans, rats, and mice (Löschler, 1985). Thus, in contrast to other AEDs, brain (and CSF) concentrations of VPA in different species do not reflect the plasma free fraction of VPA. Furthermore, in patients, the average brain/free plasma concentration ratio of VPA is only approximately 0.5, also suggesting asymmetric transport of VPA (i.e., efflux rate exceeds influx rate) across the BBB (Shen, 1999).

Lack of asymmetric transport as observed with VPA in the present in vitro assays may indicate lack of active transport but could also be explained by equal active transport in both directions. Therefore, we performed experiments in which various MRP or Pgp inhibitors were included in the monolayer efflux assays either alone or in combination. Neither the MRP inhibitors probenecid and MK571 nor the Pgp inhibitors verapamil and tariquidar disclosed any directional transport of VPA in the assays. Probenecid is known to inhibit different transporter molecules, which transport endogenous and exogenous anionic compounds. The affected transporter systems include not only transporter molecules of the MRP family but also different members of the organic anion transporting-polypeptide and the organic anion transporter (OAT) families (Sugiyama et al., 2001; Shitara et al., 2005). Furthermore, although probenecid can inhibit transport by some MRPs, including MRP1 and MRP2, in certain cases, it has also been shown in vitro that probenecid can stimulate transport of substrates by MRP2 (Huisman et al., 2002; Zelcer et al., 2003). The effect of probenecid on MRP2-mediated transport depends on the substrate transported, which has been explained by two interacting ligand binding sites in MRP2 (Zelcer et al., 2003). At the concentration of probenecid (100 μM) chosen for the present experiments, it neither inhibited nor stimulated transport of VPA. Furthermore, in contrast to the more selective MRP inhibitor MK571, probenecid, at 100 μM, did not affect the directional transport of vinblastine by MRP2.

Although transport assays using monolayers of immortalized kidney cells from dog (MDCK) or pig (LLC) are widely used to identify substrates or inhibitors of Pgp and MRPs, lack of measurable transport of a drug in such cell lines does not necessarily exclude that this drug is transported at the level of the BBB (Löschler and Potschka, 2005a). The efficacy of drug transport by efflux transporters in a tissue depends on a number of factors, such as the nature and physical state of the surrounding lipids, interactions with other tissue-specific proteins, and tissue-specific regulation of the transporters (Löschler and Potschka, 2005a). Furthermore, it has been
demonstrated recently in in vitro assays, including LLC-MDR1 and a mouse in vivo model, that Pgp in the BBB differs strikingly in its sensitivity to Pgp inhibitors from Pgp in other tissues (Choo et al., 2006). Thus, despite the negative in vitro results with VPA in kidney cell lines, we decided to examine the in vivo role of Pgp and MRPs in efflux of VPA from the brain.

In this second series of experiments, we used a microdialysis model in rats to examine whether brain uptake of VPA can be increased by inhibiting Pgp or MRPs. Neither the Pgp inhibitor verapamil nor the nonselective MRP inhibitor probenecid increased brain uptake of VPA, which is in contrast to previous experiments with various other AEDs in this model (Loscher and Potschka, 2005a). These in vivo data from rats supported our in vitro findings that VPA is not a substrate for Pgp or MRPs. The lack of probenecid to increase the penetration of VPA into either extracellular fluid or brain tissue of rats is in contrast to several previous reports in which probenecid was shown to elevate brain, CSF, or brain dialysate levels. However, after our initial study in dogs (Frey and Loscher, 1978), most subsequent studies on probenecid’s effects on VPA brain distribution were done in rabbits (e.g., Cornford et al., 1985; Adkison et al., 1994, 1996; Scism et al., 2000), so that interspecies differences could be involved in the apparent discrepancies between the present and previous studies. This possibility is also suggested by a study of Golden et al. (1993), in which probenecid was shown to decrease VPA concentrations in the CSF of rats, whereas we found an increase in CSF concentrations of VPA following probenecid in dogs (Frey and Loscher, 1978). Species differences in substrate recognition by efflux transporters have been described for various drugs (Loscher and Potschka, 2005a) and would be a likely explanation for the apparent discrepancy between the present and previous data on VPA.

In this respect, it is important to note that we studied transport of VPA by efflux transporters from three species, mouse, rat, and human.

A problem when using probenecid, a wide-spectrum inhibitor of organic anion transporters, for studying the efflux of VPA from the CSF or brain is that probenecid may exert an inhibitory effect on both influx and efflux transporters at the blood-CSF barrier and BBB. In a recent paper by Gibbs et al. (2004), a biphasic effect of the nonselective transport inhibitors probenecid and indomethacin on VPA uptake into bovine BMECs was observed. Low inhibitor concentrations increased VPA uptake, whereas the opposite effect was observed at high inhibitor concentrations, which indicates the potential for dual actions of these inhibitors (Gibbs et al., 2004). Thus, the negative results with probenecid in the present study in rats could be the fortuitous result of nearly comparable inhibition of influx and efflux of VPA. Inhibition of VPA influx by probenecid could also explain the finding of the rat study of Golden et al. (1993), in which probenecid was shown to decrease VPA concentrations in the CSF. Unfortunately, there is a lack of more selective MRP inhibitors to test this hypothesis, but the fact that similarly high doses of probenecid increased brain dialysate and brain tissue concentrations of VPA in rabbits (Scism et al., 2000) but exerted no effect in rats (present study) argues in favor of interspecies differences in the transporters involved in efflux of VPA from the brain.

In a third series of experiments, we used MRP2-deficient TR− rats to study the involvement of MRP2 in brain uptake or extrusion of VPA. The TR− rat, a mutant strain of the Wistar rat, was first described by Jansen et al. (1985) as a hepatic anion transport-deficient (“TR−”) rat strain. It exerts a single-nucleotide deletion in the MRP2 gene resulting in the absence of the gene product from the canalicular membrane of hepatocytes (Paulusma et al., 1996), so that this mutant rat is widely used to study the role of MRP2 in hepatobiliary excretion of organic anions (Konig et al., 1999).

Furthermore, Miller et al. (2000) reported that, in contrast to nonmutant controls, MRP2 is absent in brain capillary endothelial cells of TR− rats. Thus, the negative results with probenecid in the present experiments, VPA concentrations in dialysates or brain tissue were not different between TR− rats and wild-type controls, which seems to exclude any important role of MRP2 in brain distribution of VPA, at least in rats. The lack of MRP2 to transport VPA in transfected kidney cell lines (MDCKII-MRP2) and the in vivo rat BBB model is in line with a recent study of Wright and Dickinson (2004) with TR− rats, indicating that MRP2 does not play a significant role in biliary excretion of VPA. As in the study of Wright and Dickinson (2004), the mean VPA plasma concentration time profiles were not significantly different between Wistar and TR− rats in the present experiments, but lower VPA plasma AUC were observed in TR− rats. The reasons for this observation are not clear but may involve the enhanced excretion of VPA as VPA-acyl glucuronide via the urine observed by Wright and Dickinson (2004) in TR− rats.

In contrast to VPA, the major AED phenytoin reaches higher extracellular brain levels in TR− rats than controls, indicating that MRP2 is involved in the brain efflux of phenytoin (Potschka et al., 2003a). However, the present data do not exclude that probenecid-insensitive MRPs or other, non-Pgp/MRP-related organic anion transporters at the BBB may be involved in efflux transport of VPA. An interesting candidate transporter in this respect may be the ABC transporter breast cancer resistance protein (ABCG2), which, like Pgp and MRP2, is expressed at the apical membrane of brain capillary endothelial cells, is involved in brain uptake of certain drugs and is up-regulated by seizures (van Vliet et al., 2005; Bredeld et al., 2006). However, in a recent study using MDCKII cells transfected with breast cancer resistance protein, no directional transport of VPA was observed (Cerveny et al., 2006).

In our hands, VPA is the first AED that seems to be neither transported by Pgp or MRPs in the in vivo and in vitro models used. Because the present study also included cells overexpressing human Pgp, it is tempting to speculate that our data can be extrapolated to patients with epilepsy. Although there is no evidence that VPA has advantages toward other AEDs in patients with drug-resistant epilepsy, VPA has been reported to exert efficacy in patients with drug-resistant status epilepticus (Rosenow et al., 2002; Kalviainen et al., 2005; Limdi et al., 2005). Status epilepticus is known to induce overexpression of Pgp and MRPs in brain capillary endothelial cells of rats and humans (Sisodiya and Thom, 2003; Loscher and Potschka, 2005a), so that the efficacy of VPA in status epilepticus could relate to the present finding suggesting that VPA is not a substrate for these efflux transporters. This suggestion is, of course, speculative and needs to be further investigated using rat models of drug-resistant
status epilepticus. An important observation in this respect is the finding that resistance of status epilepticus to the Pgp/MRP substrate phenytoin can be counteracted by inhibition of Pgp (Mazarati et al., 2003). Thus, lack of transport by Pgp or MRPs could be an important advantage for treatment of drug-resistant status epilepticus.

In conclusion, our data do not support the hypothesis that MRPs, particularly MRP1 and MRP2, are involved in the efflux of VPA from the brain. Furthermore, in contrast to previous studies in rabbits, brain levels of VPA were not affected by probenecid in rats. Thus, the molecular identity of the putative transporter mediating the active efflux of VPA from the brain remains obscure. Because of the lack of selectivity of probenecid in inhibiting MRPs and its effects on various other efflux and influx transporters at the BBB (see above), we cannot exclude that MRPs, particularly MRPs other than MRP1 or MRP2, in vivo are involved in VPA transport at the BBB of rats. For instance, MRPs is apically expressed at the BBB and could in theory be involved in efflux transport of VPA (Loscher and Potschka, 2005a), which needs to be examined further. As described in the Introduction, the bidirectional movement of VPA across the BBB is believed to be mediated jointly by passive diffusion and carrier-mediated transport, with efflux transport being more efficient than influx transport (Shen, 1999). The uptake of VPA from blood into the brain is facilitated by a medium- and long-chain fatty acid selective anion exchanger at the brain capillary epithelium, which accounts for two-thirds of the barrier permeability (Shen, 1999). The mechanism(s) governing the efficient transport of VPA in the reverse direction, i.e., from brain to blood, may involve bidirectionally operating (non-ABC related) organic anion transporters at the BBB. For instance, the multispecific OAT2 has been shown to transport VPA in mOAT2-expressing oocytes (Kobayashi et al., 2002). However, it is not known whether OAT2 plays any role in drug transport in the brain. Thus, the mechanisms responsible for asymmetric VPA transport between blood and brain remain to be further elucidated. In view of the fact that compounds affecting brain permeability are often sensitive to the bulk flow of CSF (Shen et al., 2004), the low brain/plasma ratio of VPA may reflect this flow instead of transporter-mediated efflux at the BBB.

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