Multidrug Resistance Protein MRP2 Contributes to Blood-Brain Barrier Function and Restricts Antiepileptic Drug Activity

HEIDRUN POTSCHKA, MAREN FEDROWITZ, and WOLFGANG LÖSCHER
Department of Pharmacology, Toxicology, and Pharmacy, School of Veterinary Medicine, Hannover, Germany
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ABSTRACT
The blood-brain barrier (BBB) is a physical and metabolic barrier between the brain and the systemic circulation, which functions to protect the brain from circulating drugs, toxins, and xenobiotics. ATP-dependent multidrug transporters such as P-glycoprotein (Pgp; ABCB1), which are found in the apical (luminal) membranes of brain capillary endothelial cells, are thought to play an important role in BBB function by limiting drug penetration into the brain. More recently, the multidrug resistance protein MRP2 (ABCC2) has been found in the luminal surface of brain capillary endothelium of different species, including humans. In endothelial cells from patients with drug-resistant epilepsy, MRP2 was shown to be overexpressed, indicating that it may be critically involved in multidrug resistance of such patients. However, the role of MRP2 in drug disposition into the brain is defined poorly. Herein, we used different strategies to study the contribution of MRP2 to BBB function. First, the MRP inhibitor probenecid was shown to increase extracellular brain levels of the major antiepileptic drug phenytoin in rats, indicating that phenytoin is a substrate of MRP2 in the BBB. This was substantiated by using MRP2-deficient TR−/H11002 rats, in which extracellular brain levels of phenytoin were significantly higher compared with the normal background strain. In the kindling model of epilepsy, coadministration of probenecid significantly increased the anticonvulsant activity of phenytoin. In kindled MRP2-deficient rats, phenytoin exerted a markedly higher anticonvulsant activity than in normal rats. These data indicate that MRP2 substantially contributes to BBB function.

Transport by ATP-dependent efflux pumps, such as P-glycoprotein (Pgp; ABCB1) and multidrug resistance proteins (MRPs), influences bioavailability and disposition of many drugs (Ayrton and Morgan, 2001). These efflux pumps are expressed widely in various tissues and serve as defense mechanisms limiting tissue accumulation of naturally occurring toxins, xenobiotics, and drugs (Silverman, 1999). Of the current 12 members of the MRP family, MRP2 (ABCC2) is considered the major mammalian membrane transporter responsible for the secretion of bilirubin glucuronides from liver to bile (Gerkm and Vore, 2002; Meier and Stieger, 2002). More recently, MRP2 has been found in the apical (luminal) membrane of brain capillary endothelial cells in fish, rats, pigs, and humans, implicating MRP2 in drug transport from brain to blood (Miller et al., 2000; Dombrowski et al., 2001; Miller et al., 2002). Endothelial cells of brain capillaries, containing tight junctions and bipolar differential expression of transporters, maintain the blood-brain barrier (BBB) (Lee et al., 2001). However, although the role of Pgp as a gatekeeper in the BBB is well established, limiting entry of drugs and toxins into the brain (Schinkel, 1999), the role of MRP2 in regulating drug permeability across the BBB is defined poorly. Using cDNA arrays, a 225% increase in MRP2 gene expression was found in brain capillary endothelial cells from patients with drug-resistant temporal lobe epilepsy (TLE), suggesting that MRP2 expression changes may play an important role in resistance to antiepileptic drugs (AEDs) by decreasing the permeability of AEDs across the BBB (Dombrowski et al., 2001). However, there is no direct evidence that AEDs or other centrally acting drugs are transported by MRP2 in the BBB.

In the present study, we used three different strategies to study the contribution of MRP2 to BBB function in rats. First, the MRP1/MRP2 inhibitor probenecid (Gerkm and Vore, 2002; Scheffer and Schepfer, 2002) was used to study whether MRP2 inhibition in the BBB increases extracellular brain levels of AEDs as determined by microdialysis via probes in the cerebral cortex, which contains high levels of MRP2 mRNA (Cherrington et al., 2002). Second, we used MRP2-deficient TR−/H11002 rats (Jansen et al., 1985; Paulusma et al., 1996) to determine whether the absence of MRP2 in the BBB of these rats (Miller et al., 2000) leads to an increased penetration of AEDs into the brain. Third, the kindling model of
TLE (Sato et al., 1990) was used to study whether inhibition of MRP2 or lack of MRP2 in the BBB enhance anticonvulsant activity of major AEDs.

Materials and Methods

In Vivo Microdialysis. Two guide cannulae (Carnegie Medicine, Stockholm, Sweden) were implanted with a stereotaxic apparatus into the left and right frontal (motor) cortex (AP, +3.2; L, ±3.2; V, 2.0) of adult Wistar rats (Harlan-Winkelmann, Borchen, Germany) as described previously (Potschka and Löscher, 2001a). After a recovery period of at least 3 days (to allow the BBB to reseal; Benveniste and Hansen, 1991) and 14 to 16 h before the experiment, a 3-mm microdialysis probe (diameter 0.5 mm; CMA/12, Carnegie Medicine) was inserted through the guide cannula to a depth of 5.0 mm from bregma into the frontal cortex of conscious rats. Each rat was placed in a freely moving system, consisting of a plastic cylinder with counterbalancing arm carrying a two-channel swivel (Carnegie Medicine). The probe was perfused with mock cerebrospinal fluid (25 mM NaHCO3, 122 mM NaCl, 1.2 mM MgSO4, 3 mM KCl, 1.3 mM CaCl2 and 0.4 mM H2KPO4, pH 7.4) at a flow rate of 2 μl/min with a syringe pump (Carnegie Medicine). Each 40-μl outflow sample was collected for drug analysis. One hour after onset of perfusion, an AED (either phenytoin or phenobarbital) was administered i.p. and dialysate samples (2/h) were collected over the next 2 h. The doses of phenytoin (50 mg/kg) and phenobarbital (30 mg/kg) were chosen on the basis of previous experiments in female Wistar rats (Potschka and Löscher, 2001a; Potschka et al., 2002), showing that these doses in rats result in plasma levels similar to the “therapeutic range” known from patients with epilepsy. Together with each dialysate sample, a blood sample was collected from the periorbital plexus (Carnegie Medicine). Each 40-μl outflow sample was collected for drug analysis. One hour after onset of perfusion, an AED (either phenytoin or phenobarbital) was administered i.p. and dialysate samples (2/h) were collected over the next 2 h. The doses of phenytoin (50 mg/kg) and phenobarbital (30 mg/kg) were chosen on the basis of previous experiments in female Wistar rats (Potschka and Löscher, 2001a; Potschka et al., 2002). For inhibition of MRP2 in the BBB, local administration of probenecid (10 mM dissolved in the perfusion solution) via one microdialysis probe was started 30 min before AED injection. The other microdialysis probe was perfused with mock cerebrospinal fluid only and served as individual control in each rat. For each microdialysis probe used for the in vivo experiments, 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 Löscher, 2001a). 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%. On conclusion of each experiment, the correct location of the microdialysis probe in the frontal cortex was verified histologically as described previously (Potschka and Löscher, 2001a). In all rats used in this article, the probes were correctly located in the left and right cortex. Furthermore, there was no indication for any obvious neurodegeneration around the tip of the probe other than that caused by the probe itself.

In addition to microdialysis experiments in normal Wistar rats, adult MRP2-deficient TR−/− Wistar rats were used for microdialysis of phenytoin and phenobarbital as described above, except that microdialysis was performed from only one hemisphere. The TR−/− rats were bred in our department; breeding pairs were kindly provided by Prof. R. P. J. Oude Elferink (Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands). For comparison with TR−/− rats, age-matched rats of the Wistar background strain (Harlan Netherland, Horst, The Netherlands) were used.

The statistical significance of differences between left and right cortex in normal Wistar rats was calculated by two-way analysis of variance (ANOVA) for repeated measurements followed by post hoc testing with the Wilcoxon test for paired replicates. Significance of differences between TR−/− rats and background strain was calculated by two-way ANOVA followed by the Mann-Whitney U test.

Kindling Model. Adult Wistar rats (nonmutant or MRP-deficient TR−/− rats) were chronically implanted with a bipolar electrode into the right basolateral amygdala (AP, −2.2; L, 4.8; V, 8.5 in Wistar rats from Harlan-Winkelmann; AP, −2.2; L, 5.0; V, 8.3 in TR−/− rats and Wistar rats from Harlan Netherland) as described previously (Potschka and Löscher, 2002). Electrical stimulation of the amygdala was initiated after a recovery period of 2 weeks after surgery. Kindling was performed by amygdala stimulations which were applied once daily for 1 s with a stimulus strength of 330 μA (using 1-ms monophasic square-wave pulses, 50 Hz) until at least 10 consecutive fully kindled stage 5 seizures according to Racine (1972) were elicited. In these fully kindled rats, the anticonvulsant effect of phenytoin and phenobarbital was assessed either alone or after pretreatment with probenecid (administered 15 min before the AEDs) by determining the threshold for generalized (stage 4/5) seizures (GST) 2 to 3 days before (vehicle control) and 60 min after AED administration in the same groups of rats. The GST is a sensitive measure of drug effects in individual kindled rats and often used for determining the activity of anticonvulsant drugs (Croucher and Bradford, 1991; Attwell et al., 1998; Löscher et al., 2000; Gernert and Löscher, 2001).

For dose selection of probenecid, different doses (50, 100, and 200 mg/kg) were administered alone and GST was determined after 75 min. Because 100 and 200 mg/kg, but not 50 mg/kg, probenecid induced significant GST increases, a dose of 50 mg/kg was used for combination experiments with AEDs. For phenytoin, different doses (6.25, 12.5, 25, and 50 mg/kg) were administered, and dose selection was based on these dose-response experiments (see Results). Similarly, different doses of phenobarbital (2.5, 5, 10, and 30 mg/kg) were tested for dose selection (see Results). GST was determined by a staircase procedure as described previously (Potschka and Löscher, 2001). Blood was sampled immediately after GST determination for AED analysis in plasma (Potschka and Löscher, 2001a; Potschka et al., 2002). In addition to GST determinations, rats were observed for drug adverse effects, including the rotarod test, as described previously (Gernert and Löscher, 2001). The statistical significance of differences between drug treatments in the same group of rats was calculated by the Wilcoxon test for paired replicates. Significance of differences between TR−/− rats and background strain was calculated by the U test.

Results

Effect of MRP2 Inhibition on BBB Permeability in Vivo. The MRPI/MPR2 inhibitor probenecid was used to select two AEDs whose penetration into the brain can or cannot be affected by MRP2 inhibition in the BBB. Because, in contrast to MRP2, MRPI is located in basolateral rather than apical (luminal) cell membranes (Borst et al., 1999) and is thought to be predominantly expressed in brain parenchyma and not in endothelial cells of the BBB (Regina et al., 1998; Decleves et al., 2000), inhibition of MRPI by probenecid was not considered a bias for the present experiments. Using in vivo brain microdialysis in conscious rats, extracellular brain levels of phenytoin have recently been shown to be significantly increased by local application of probenecid via the dialysis probe (Potschka and Löscher, 2001b), so that phenytoin was chosen as a drug apparently being subject to transport by MRP2. From preliminary experiments with various AEDs (carbamazepine, phenobarbital, lamotrigine, and felbamate), phenobarbital was chosen because its brain penetration was not affected by probenecid. The comparison between phenytoin and phenobarbital is shown in Fig. 1. The intracerebral administration of probenecid did not alter the plasma levels of phenytoin or phenobarbital, but significantly increased the extracellular brain level and the dialysate/plasma ratio of phenytoin, but not of phenobarbital.
Fig. 1. Drug concentration-time profiles in plasma and brain dialysate (extracellular fluid) after systemic (i.p.) administration of phenytoin (left column) or phenobarbital (right column) in Wistar rats from Harlan-Winkelmann. Drug doses were 50 (phenytoin) or 30 (phenobarbital) mg/kg, respectively, i.e., doses within the anticonvulsant dose range of these drugs in Wistar rats. All values are means ± S.E.M. of eight (phenytoin) or five (phenobarbital) rats. The group data on phenytoin include data from five rats, which were published previously (Potschka and Löscher, 2001b). Dialysates were obtained 30 to 120 min after drug administration from the right and left frontal cortex. The lower panels show the ratio between dialysate and plasma concentrations. The MRP inhibitor probenecid was administered via one of the two microdialysis probes, beginning 30 min before AED administration, over the course of the experiment, resulting in a significant increase in dialysate levels of phenytoin compared with the vehicle-treated control hemisphere, whereas dialysate levels of phenobarbital were not affected by probenecid. A significant difference between hemispheres was also found for dialysate-plasma ratios of phenytoin, but not phenobarbital. Results from comparisons of vehicle-treated and probenecid-treated hemispheres by ANOVA were as follows: phenytoin, dialysate levels, \( P = 0.0088 \); dialysate/plasma ratio, \( P = 0.0118 \); phenobarbital, dialysate levels, \( P = 0.1909 \); and dialysate/plasma ratio, \( P = 0.1658 \). Results from post-hoc testing of individual differences to the vehicle-treated site are indicated by asterisk (\( P < 0.05 \)).

**BBB Permeability in MRP2-Deficient TR− Rats.** To validate the data from the experiments with MRP2 inhibition by probenecid, brain penetration of phenytoin and phenobarbital was compared between MRP2-deficient TR− Wistar rats and the normal Wistar background strain. Phenytoin levels in plasma or decay of plasma phenytoin levels did not
differ significantly between mutant rats and background strain, whereas phenobarbital levels in plasma of TR− rats were slightly lower compared with normal rats (Fig. 2). Phenytoin reached higher extracellular brain levels in TR− rats compared with nonmutant rats (analysis by ANOVA indicated a significant difference with $P = 0.0237$), resulting in a significantly higher dialysate/plasma ratio in MRP2-deficient rats. No such difference was seen for phenobarbital, thus substantiating the results from MRP2 inhibition by probenecid.

**Fig. 2.** Drug concentration-time profiles in plasma and brain dialysate (extracellular fluid) after systemic (i.p.) administration of phenytoin (left column) or phenobarbital (right column) in MRP2 deficient TR− rats and rats of the normal Wistar background strain from Harlan Netherland. Drug doses were 50 (phenytoin) or 30 (phenobarbital) mg/kg, respectively. Data are means ± S.E.M. of five (phenytoin) or six (phenobarbital) rats. Dialysates were obtained 30 to 120 min after drug administration from the right and left frontal cortex. The lower panels show the ratio between dialysate and plasma concentrations. Similar to the data from MRP inhibition by probenecid in Fig. 1, MRP2-deficient TR− rats reached significantly higher phenytoin dialysate levels than controls ($P$ value from ANOVA was 0.0237) and a significantly higher dialysate/plasma ratio for phenytoin ($P = 0.0237$) but not for phenobarbital. Results from post hoc testing of individual differences between TR− rats and controls are indicated by asterisk ($P < 0.05$). Other results from comparisons of TR− rats and controls by ANOVA were as follows: phenytoin, plasma levels, $P = 0.808$; phenobarbital, plasma levels, $P = 0.0316$; dialysate levels, $P = 0.3125$; and dialysate/plasma ratio, $P = 4102$. 

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Effect of MRP2 Inhibition on AED Efficacy in Kindled Rats. An increase in extracellular brain levels of phenytoin by inhibition of MRP2 should be associated with increased anticonvulsant activity because the extracellular level that is thought to be relevant for phenytoin's anticonvulsant action (Rogawski and Porter, 1990). Fully kindled rats were used to study whether probenecid potentiates phenytoin's anticonvulsant effect. First, different i.p. doses of probenecid (ranging from 50 to 200 mg/kg) were tested in kindled rats to select a dose that does not exert a significant effect on seizure threshold when given alone. A dose of 50 mg/kg was chosen from these experiments (Fig. 3A). This dose of probenecid was then injected i.p. 15 min before phenytoin to study whether this results in an enhanced anticonvulsant activity of the AED. Phenytoin was administered at a dose (6.25 mg/kg), which alone did not exert any significant anticonvulsant effect (Fig. 3A). As shown in Fig. 3A, the combination of probenecid and phenytoin resulted in a marked anticonvulsant effect, in that the seizure threshold was significantly increased by 90%. To obtain such a threshold increase with phenytoin alone, the dose of phenytoin had to be more than doubled, as determined by dose-response experiments (data not shown). Higher doses of probenecid alone (100 or 200 mg/kg) significantly increased GST by only about 60% without any clear dose-response (data not shown).

Thus, it is highly unlikely that the large GST increase obtained after combined treatment with subanticonvulsant doses of probenecid and phenytoin (Fig. 3A) was just a result of an additive effect apart from any effect on brain phenytoin transport. Furthermore, plasma levels of phenytoin were not affected by i.p. administration of probenecid (Table 1), so that the significant increase of anticonvulsant efficacy was most likely related to enhanced penetration of phenytoin into the brain because of MRP2 inhibition in the BBB. No behavioral adverse effects were observed at the combination of probenecid and phenytoin, and all rats passed the rotarod test. The finding that probenecid increased the anticonvulsant efficacy but not the toxicity of phenytoin can be explained by the fact that behavioral adverse effects, including rotarod failures, are only observed after much higher doses or brain levels of phenytoin. Thus, even at 12.5 or 25 mg/kg phenytoin, no central nervous system toxicity is observed in the Wistar rats used in this study (data not shown).

In contrast to phenytoin, probenecid did not increase the anticonvulsant efficacy of phenobarbital in the kindling model (Fig. 3B). Plasma levels of phenobarbital were not affected by probenecid (Table 1).

AED Efficacy in MRP2-Deficient TR− Rats. To substantiate the effect of MRP2 inhibition in the kindling model, MRP-2 deficient TR− rats were kindled, and the anticonvulsant effect of phenytoin and phenobarbital was compared in kindled TR− Wistar rats and kindled rats of the normal Wistar background strain. Although plasma levels of phenytoin were slightly lower in TR− compared with nonmutant rats (Table 1), phenytoin was much more effective to increase seizure threshold in TR− rats (Fig. 4A), substantiating that inhibition or lack of MRP2 in the BBB results in a functionally relevant increase in brain levels of this major AED. Adverse effects of phenytoin (slight ataxia) were not different between TR− rats and controls, and all rats passed the rotarod test. In contrast to findings with phenytoin, no significant difference in anticonvulsant activity of phenobarbital was found between MRP2-deficient and normal Wistar rats (Fig. 4B).

Discussion

Our results show that MRP2 participates in a permeation barrier for selected drugs crossing the BBB barrier. Both inhibition of MRP2 and lack of MRP2 result in a significant increase of drug levels in the brain that is not secondary to alterations in peripheral drug pharmacokinetics. As demonstrated by the present data, the MRP2-deficient TR− rat is a
suitable model to study the involvement of MRP2 in the BBB in drug penetration into the brain. The TR− rat, which was first described by Jansen et al. (1985) as a hepatic anion transport-deficient ("TR−") rat strain, has 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 (König et al., 1999). However, recently Miller et al. (2000) reported that MRP2 is also absent in brain capillary endothelial cells of TR− rats, which prompted us to use these rats for the present experiments.

To our knowledge, the present data are the first to demonstrate a role of MRP2 in BBB function in vivo. In line with the present results on phenytoin, recent in vitro experiments in isolated capillaries from rat, pig, and fish brain using MRP2 modulators such as leukotriene C4 have indicated that MRP2 contributes substantially to the active barrier function of the endothelium (Miller et al., 2000, 2002; Fricker et al., 2002). The localization of MRP2 to the luminal surface of the brain capillary endothelium (Miller et al., 2000), and the wide spectrum of drugs accepted as substrates by MRP2 (Borst et al., 1999; Leslie et al., 2001; Gerk and Vore, 2002) implicate that this transporter may be as important as Pgp in BBB function. Furthermore, the finding that phenytoin is subject to transport by Pgp (Potschka and Löschler, 2001a; Rizzi et al., 2002) and MRP2 in the BBB demonstrates that MRP2 and Pgp have an overlapping substrate spectrum, which is known from substrate recognition studies on Pgp and MRPs (Lee et al., 2001). The limitation of phenytoin’s access to the brain by Pgp and MRP2 might explain why phenytoin reaches its peak anticonvulsant effect less rapidly than most other AEDs, at least in rodents (Löschler et al., 1991).

In addition to a role of MRP2 in normal functioning of the BBB, it has been suggested that overexpression of MRP2 may be involved in the drug refractoriness of patients with TLE (Dombrowski et al., 2001), which was the reason to chose AEDs and the kindling model of TLE for the present experiments. Absence of MRP2 in the BBB led to increased penetration of phenytoin into the brain and significantly enhanced anticonvulsant activity compared with rats with intact MRP2 function. Similar results were obtained when phenytoin was combined with probenecid to inhibit MRP2. A
significant increase of drug penetration into the brain by probenecid has previously been reported for the major AEDs valproate (Frey and Löschner, 1978) and carbamazepine (Potschka et al., 2001) and has been attributed to inhibition of MRP2 in the BBB (Löscher and Potschka, 2002). These data and the potentiating effect of probenecid on phenytoin’s anticonvulsant effect in the kindling model suggest novel options for treatment of refractory epilepsy, such as addition of a MRP2 inhibitor to current treatment with AEDs. Even though probenecid is not specific for MRP2, but also inhibits MRP1 and organic anion transports systems, it is often used as an inhibitor of MRP2 in drug transport studies, especially in tissues, such as for instance brain capillary endothelial cells, in which MRP1 is not predominantly expressed (Gerk and Vore, 2002; Löscher and Potschka, 2002). The use of probenecid as an inhibitor of MRP2-mediated drug transport in the BBB was substantiated by the present experiments because similar findings to MRP inhibition by probenecid were obtained in MRP2-deficient rats. Nevertheless, new, more selective MRP2 inhibitors will hopefully soon become available and should be used instead of probenecid in experiments on the role of MRP2 in BBB function (Löscher and Potschka, 2002).

In contrast to phenytoin, phenobarbital’s brain distribution or anticonvulsant activity were not affected by probenecid or lack of MRP2 in the BBB, indicating that not all AEDs are substrates for this transporter. However, phenobarbital is a substrate for Pgp (Potschka et al., 2002) so that both MRP2 and Pgp act in concert to restrict the brain penetration of AEDs.

MRP2 is not the only MRP that is located in brain capillary endothelial cells and is overexpressed in epileptogenic brain tissue of patients with pharmacoresistant epilepsy (Dombrowski et al., 2001; Lee et al., 2001). In addition to the MRP2 gene, the genes encoding for MRP3 and MRP5 were found to be significantly overexpressed in patients with refractory epilepsy, whereas expression of the MRP1 gene was not significantly altered (Dombrowski et al., 2001). Of these MRP genes, the largest overexpression in brain capillary endothelial cells was seen for the MRP2 gene (Dombrowski et al., 2001). With respect to the role of MRPs in BBB function, differences in the cellular location of MRPs have to be considered. Whereas MRP2 is located in apical cell membranes, which is the appropriate position for a protective role, other MRPs, including MRP1, MRP3, and MRP5, are located basolaterally (Borst et al., 1999), so that overexpression of the latter MRPs in brain capillary endothelial cells would not reduce entry of drugs into the brain.

In addition to AEDs, brain pharmacokinetics of drugs comprising the multidrug resistance phenotype, including anticancer drugs, selected antibiotics, analgesics, antidepressants, anxiolytics, and human immunodeficiency protease inhibitors (Fromm, 2000; Litman et al., 2001; Taylor, 2002), are likely to be impacted by the coordinated expression of Pgps and MRPs such as MRP2 in the BBB. Our results with AEDs suggest that maximizing the brain penetration of these therapeutic agents may require at least concurrent blockade of both MRP2 and Pgp.

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References


Address correspondence to: Dr. W. Lösch, Department of Pharmacology, Toxicology, and Pharmacy, School of Veterinary Medicine, Bünteweg 17, D-30559 Hannover, Germany. E-mail: wolfgang.loescher@tiho-hannover.de