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Evaluation of transport of antiepileptic drugs by efflux transporters (multidrug transporters) of the blood-brain barrier

Thesis

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Chapter 3: Topiramate is a substrate for human P-glycoprotein

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Gott,

meiner Familie,

allen, die in Costa Rica meinen wissenschaflichen Geist

aufgeweckt haben,

dem deutschen Volk

Contents

Abbreviations:

ABC	ATP-bindig cassette superfamily
ABC-T	ABC transporters
AED	Antiepileptic drug
ANOVA	Analysis of variance
AUC	Area under curve
BBB	Blood-brain barrier
BCEC	Brain capillary endothelial cell
BCRP	Breast cancer related protein
bFGF	Basic fibroblast growth factor
CETA	Concentration equilibrium transport assay
CNS	Central nervous system
DMSO	Dimethyl sulphoxide
FCS	Fetal calve serum
FDA	Food and drug administration of the U.S.A.
GABA	Gamma amino-butyric acid
HPLC	High performance liquid chromatography
ILAE	International League Against Epilepsy
MDT	Multidrug transporter
MRP	Multidrug resistance-associated proteins
NBF	Nucleotide binding fold
Papp	Apparent permeability
Pgp	P-glycoprotein
TEER	Trans-epithelial/ endothelial electrical resistance
TJ	Tight junctions
TLE	Temporal lobe epilepsy
TMD	Transmembrane domain
TR	Transport ratio
cTR	Corrected transport ratio
WHO	World Health Organization

Chapter 1

Introduction

Chapter 1: Introduction

1.1. Epilepsy and pharmacoresistance in epilepsy

Epilepsy is a chronic, pathologic condition of the central nervous system (CNS) that "implies a persistent epileptogenic abnormality of the brain that is able to spontaneously generate paroxysmal activity", according to the definition of the International League against Epilepsy (ILAE) (Engel, 2006).

Also according to ILAE, an epileptic seizure is defined as "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Fisher et al., 2005). It is relevant to consider that the presentation of an isolated or transient epileptic seizure does not mean the patient is epileptic. Epilepsy is, hence, a chronic condition that is manifested after a period of epileptogenic changes took place in the brain (Engel, 2006).

The World Health Organization (WHO) estimates about 50 million people worldwide being afflicted with epilepsy, which accounts for about 1% of the global burden of disease (WHO, 2005). The prevalence of this illness in Europe was reported from 4.3 to 7.8 per 1000 in 2004 (Pugliatti et al., 2007).

One of the main concerns regarding epilepsy is the relative high proportion of patients who do not respond to the treatment, i.e., they continue experiencing recurrent seizures in spite of receiving antiepileptic drug (AED) therapy and having adequate serum drug-levels. This condition has been termed pharmacoresistance.

Although a unique definition of pharmacoresistance does not exist, most definitions usually include some basic common criteria for categorization of pharmacoresistance, such as the number of AEDs used without success, a minimal reduction in seizure frequency, and a specific period of treatment (Schmidt & Löscher, 2005). In general, it has been proposed that when patients do not become seizure free during a treatment period of 12 months after receiving several suitable AEDs at maximal tolerated doses, they can be considered pharmacoresistant (Schmidt & Löscher, 2005).

The proportion of patients suffering from this condition also varies according to the type of epilepsy syndrome and the location of epileptic focus (Schmidt & Löscher, 2005). Regarding the epilepsy syndrome, a prospective Finnish study showed that 7 of 9 patients with symptomatic generalized epilepsy and 5 of 10 with symptomatic partial epilepsy were pharmacoresistant, while only 1 of 8 patients affected by idiopathic generalized epilepsy was considered to be drug refractory (Sillanpää et al, 1999). When

the epileptic focus is located in the temporal lobe (temporal lobe epilepsy [TLE]), the most common type in adult epileptic patients, the prevalence of pharmacoresistance has been mentioned to reach 75 % (reviewed by Schmidt & Löscher, 2005), but varies greatly from about 40% to 60% (Holmes, 2004). This variation can be at least partially explained by the different criteria for definition of pharmacoresistance used in the studies, and the follow-up period of the patients (Holmes, 2004).

Independently of the prevalence, the consequences of pharmacoresistant epilepsy can imply social, economical, familiar and psycho-physical deleterious impacts for affected patients. Cognitive impairment and physical injury are common consequences of untreatable epilepsy (Holmes, 2004), but also those people suffering from it have an increased risk of death, whether because of accidents and suicide or because of sudden unexplained death (Nashef et al., 1995; Sperling et al., 1999). On the other hand, patients with uncontrolled seizures are subject to social exclusion, social stigma, and have less possibilities to be employed and enrolled in full-time educational programs (Baker et al., 1997). Van Hout et al. (1997) found a parallel between number of seizures and worse quality of life (assessed by means of a Functional Status Questionnaire).

Regarding economic cost of epilepsy, an initial estimate in Europe reported a total cost of €15.5 billion in 2004, with total costs per case ranging from €2,000 to €11,500 (prices were adjusted for purchasing power parity for each EU member-country) showing a high variability among countries. The overall number of cases in western countries and the quality of their health system are believed to increase costs, in relation to eastern ones (Pugliatti et al., 2007).

On the other hand, how much the drug-refractory epilepsy can increase the cost per patient is not exactly known, but one study that involved patients from Great Britain, Germany and France, which was conducted in the nineties, reported an increase in total costs as the frequency of seizures increased. Total costs per patient during a 3-month period went up from $780 in the seizure-free group to $2,171 in the group with daily seizures. Indirect costs in the later group were more than three times higher than those in the former one (van Hout et al., 1997).

When considering the reported prevalence of epilepsy in the world, the negative impacts it has on the patients' life and the economical cost it represents for health systems, it is evident that epilepsies are a major public health problem, which is of greater concern in case of drug-refractory epilepsy (Schmidt & Löscher, 2009). Special efforts should be directed to better understand this condition, in order to improve its

management and treatment. The ILAE proposed the topic “Improving treatment in epilepsy” as one of the six major priorities in epilepsy-research (Baulac & Pitkänen, 2009).

Albeit the many efforts exerted to comprehend the phenomenon of pharmacoresistance in epilepsy, its cause hasn’t been still unveiled. With a view to addressing this issue, two main hypotheses have been proposed and explored in the last years, namely the target hypothesis and the multidrug resistance hypothesis. A third one, the inherent severity model of pharmacoresistance, was also recently proposed (Rogawski & Johnson, 2008).

The target hypothesis lies on the findings about alterations in several AED targets, which were reported to occur in brain tissue from pharmacoresistant patients and from animal models of epilepsy. It basically proposes that intrinsic or acquired alterations of target molecules lead to an impairment of the drug-target relationship, because of a change in the later element, i.e. the pharmacodynamics are altered (Schmidt & Löscher, 2005; Remy & Beck, 2006).

Among the main neuronal targets in epilepsy are the voltage-gated sodium channels, which are responsible for rising phase of neuronal action potentials and account for the ability of brain neurons to fire with high frequency, a required condition not only for normal neuronal function, but also for epileptic activity (Rogawski & Löscher, 2004). The ability to modulate these channels explains a large proportion of the antiepileptic effects of several major AEDs, such as phenytoin, carbamazepine, lamotrigine and, possibly, topiramate a.o. (Rogawski & Löscher, 2004). A reduced sensitivity of these channels to the AED carbamazepine was reported to occur in tissue from patients undergoing surgical resection of epileptic foci (Remy et al., 2003; Jandova et al., 2006). It has also been reported that *in vitro* sodium channel phosphorylation by protein kinase C (which is activated in epileptic activity) can alter the action of topiramate on sodium currents (Curia et al., 2007).

Another common target for AEDs, such as benzodiazepines and phenobarbital, are the $GABA_A$ receptors (Rogawski & Löscher, 2004). These receptors are activated by one of the CNS inhibitory neurotransmitters, the gamma aminobutiric acid (GABA). The activation of these fast chloride-permeable ionotropic $GABA_A$ receptors leads to an influx of chloride anions in the neurons and, hence, induces a state of hyperpolarization, which in turn reduces the ability of neurons to fire at high rates. A change in $GABA_A$ receptor subunits occurs because of status epilepticus (Goodkin et al., 2008), and $GABA_A$ currents are kinetically altered in drug resistant epileptic brain (Ragozzino et al.,

2005). Alterations in $GABA_A$ receptors have been associated with resistance to phenobarbital in an animal model of TLE (Volk et al., 2006; Bethmann et al., 2008), and are probably the cause of pharmacoresistance to benzodiazepines during status epilepticus (Macdonald & Kapur, 1999; Chen & Wasterlain, 2006). As pointed out by Schmidt and Löscher (2009), it is interesting that the target hypothesis has not been investigated more deeply in human epileptic patients.

The inherent severity model of pharmacoresistance was recently introduced by Rogawski and Johnson (2008). It postulates that the relative response to medication depends on the severity of the disease, and is particularly related to the frequency of seizures during the early stages (Rogawski and Johnson, 2008). In other words, according to this hypothesis, the pharmacoresistance is related to the magnitude of pathophysiological changes occurred, which manifest a more severe form (i.e. higher seizure-frequency) of epilepsy. This, in term, would imply that pharmacological strategies available so far are inherently (*per se*) ineffective for those patients.

The multidrug transporters hypothesis is the mostly investigated hypothesis at time (Schmidt and Löscher, 2009), and is the one we explore in this scientific work. Multidrug transporters (MDT) are membrane-bound carrier proteins that are expressed in several tissues including the blood-brain barrier (BBB). MDTs belong to the ATPase –binding cassette (ABC) super-family of transporters that includes P-glycoprotein (Pgp) and multidrug-resistance-associated proteins (MRPs) among its main representatives (Löscher & Potschka, 2005a,b). They are able to transport actively their substrates (most of them lipophilic substances) against a concentration gradient. Those luminal MDT of the BBB are one of the main defense components of the barrier and are involved in the protection of the neural tissue from a wide range of xenobiotics (Löscher & Potschka, 2005a,b [for more details about MDTs see page 13]). According to the MDT hypothesis, the over-expression of these transporters at the luminal side of the BBB endothelial cells accounts for an increased extrusion of AEDs from the brain tissue back to the blood stream, what in turn leads to decreased concentrations of AEDs in the target tissue (Löscher & Potschka, 2005a; Schmidt & Löscher, 2009 [see Fig. 1.1). Hence, it proposes a model where the drug-target relationship is altered because of impaired pharmacokinetics. Since the main goal of the present work was to investigate a specific aspect of this hypothesis, it is discussed in more detail in the next paragraphs.

First evidences regarding the MDT hypothesis were the observation of Pgp (for more details see page 17) over-expression and the up-regulation of MDR1, its regulatory gene, in brain tissue of pharmacoresistant epileptic patients (Tishler et al., 1995;

Sisodiya et al., 1999). These observations were confirmed by other investigators (Dombrowski et al., 2001, Sisodiya et al., 2002; Aronica et al., 2003; Aronica et al., 2004). Other MDTs were also reported to be up-regulated and/or over-expressed in BBB endothelial cells, such as some MRPs, with MRP2 (Dombrowski et al., 2001; Aronica et al., 2003) and MRP5 (Dombrowski et al., 2001) among them.

Those findings in human epileptic patients have been paralleled in animal models of TLE. One of the cornerstones in these investigations has been the possibility to select pharmacoresistant epileptic animals (nonresponders) and responsive ones (Brandt et al., 2004). An increased Pgp expression has been determined in brain tissue of epileptic rats, and it was shown to be higher in nonresponders (Löscher & Potschka, 2005a; Volk & Löscher, 2005). Furthermore, it has also been described that this Pgp over-expression involves seizure-relevant brain structures (Seegers et al., 2002; Volk et al., 2004; van Vliet et al., 2007). Similarly, the expression of MRP1, MRP2 and breast cancer related protein (BCRP) was found to be higher in rats with frequent daily seizures (van Vliet et al., 2005).

Investigations also demonstrated that glutamate, an excitatory neurotransmitter that is increased during seizures (Holmes, 2002; Beart & O'Shea, 2007), induces Pgp expression in brain microvessel endothelial cells *in vitro* (Zhu & Liu, 2004) and *in vivo* (Bankstahl et al., 2008a). Bauer et al. (2008) also demonstrated that the Pgp over-expression mediated by glutamate can be localized in epileptogenic-relevant areas (Bauer et al., 2008).

Those micro-anatomical and *in vitro* changes set the question whether the over-expression of Pgp and other MDTs over-expression is an epiphenomenon without active role in pharmacoresistance, or a main, active phenomenon which mediates pharmacoresistance (Oby & Janigro, 2006; Löscher & Sills, 2007; Robey et al., 2008). Hence, a demonstration that Pgp and other MDTs are able to transport AEDs was needed, in order to show a parallel between structural and functional changes. Transport of several AEDs has been demonstrated *in vivo* in a rat model of TLE by means of microdialysis (Löscher & Potschka, 2005b) and *in vitro* for mouse-Pgp using the bidirectional transport assay (Baltes et al., 2007a). In contrast to human patients, for whom it hasn't been shown yet, it was demonstrated that the localized Pgp over-expression can influence the brain uptake of the major AED phenytoin in epileptic rats (van Vliet et al., 2007). But more importantly, in rat models of TLE a change of non-responder status to responder status was successfully achieved by inhibition of Pgp with tariquidar, a specific, third generation, alosteric modulator (Brandt et al., 2006, van

Vliet et al, 2006), what constitutes a proof of principle for the MDT hypothesis in animal models of epilepsy.

Sisodiya (2003) suggested that, in order its role in pharmacoresistance to be accepted, the mechanism proposed by any hypothesis for drug refractory epilepsy should satisfy at least the following criteria:

1) The mechanism must be detectable in epileptogenic brain tissue.

2) It must have appropriate functionality mediating drug resistance.

3) It must be active in drug resistance.

4) When overcome, it must affect drug resistance.

If these criteria are applied, one can certainly say that the MDT hypothesis can be accepted, since the criteria has been fulfilled, but only in case of animal models of TLE (Schmidt & Löscher, 2009).

In case of human epilepsy, those criteria haven't been met completely as yet. A parallel between micro-anatomical changes and functional-pharmacological alterations altogether linked to the phenomenon of pharmacoresistance and its reversal in human epileptic patients is missing. It is not known so far, or at least not clear, neither *in vitro* nor *in vivo,* whether the AEDs are transported by epilepsy-linked, over-expressed human MDTs. Even more challenging, a recent study showed an apparent lack of AED transport by human Pgp *in vitro*, while mouse Pgp was able to transport several AEDs (Baltes et al., 2007a).

This panorama led us to investigate whether major AEDs are substrates of human MDTs, specifically Pgp and some MRPs, by means of methods that consider the special pharmacokinetic characteristics of the AEDs, i.e. their relatively high cellular permeability.

In order to ease the comprehension of our investigation and specially the rationale of the methods and approaches used in it, which deal with drug efflux transporters of the BBB and their role in pharmacoresistance, a general overview of the BBB and some of the epilepsy-relevant MDTs and their influence in CNS pharmacokinetics is offered next.

1.2. The blood-brain barrier

The BBB is a cellular barrier located at the interface between blood and neural tissue and regulates the molecular exchange between blood and brain interstitial fluid. Its main functions are to maintain the electrolytic balance in the brain interstitial fluid, to select

those nutrients important for brain tissue, to avoid blood-transported peripheral neurotransmitters and neuroactive substances from influencing CNS neurons, and to protect the brain tissue from harmful substances, whether endogenous or exogenous; all of that being crucial for proper neuronal function (Abbott et al., 2006). Other barriers that also influence this delicate balance are the choroid plexuses in the lateral, third and fourth ventricles, which mediate molecular exchange between cerebrospinal and brain interstitial fluids; and the arachnoid epithelium, between blood and subarachnoid cerebrospinal fluid (Abbott et al., 2006).

Cellular and molecular constituents of the barrier

Several cellular types constitute the BBB: the endothelial cells, the surrounding pericytes, the astrocytes, whose end-processes cover the microvasculature; the peripheral interneurons and microglia (Abbott et al., 2006). The concept of neurovascular unit is wider, and relates to those cellular elements that influence the BBB and the cerebral blood flow, i.e. it includes the formerly mentioned BBB-elements, plus the blood-borne elements (Abbott et al., 2006). Figure 1.1 shows a general schema of the BBB structure in normal and altered conditions.

The endothelial cells directly face the blood tissue and are tightly attached to each other through the intercellular connections (tight junctions [TJ], and adherens junctions). Their high specialization leads to the characteristic barrier phenotype of the BBB (discussed in detail below).

The pericytes partially surround the brain capillary endothelial cells (BCECs) and share the same basal lamina. They confer structural support to the BBB and induce the BCECs to organize in capillaries *in vitro*, are involved in defense responses, and are able to contract and change the diameter of the vessel lumen in response to several stimuli (Thomas, 1999; Ramsauer et al, 2002; Haseloff et al., 2005; Peppiatt et al., 2006). Pericytes also seem to play an important role in the induction of BBB-phenotype by improving the formation of TJ *in vitro* (Dohgu et al., 2005).

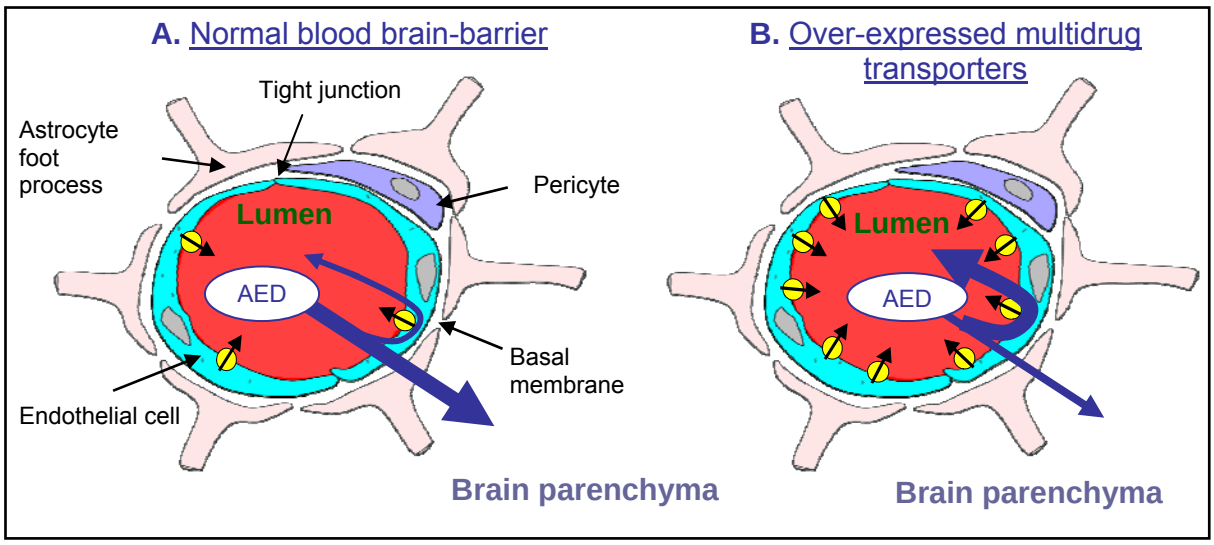

Figure 1.1 Graphical representation of normal and altered blood-brain barrier (BBB), and the possible role of the multidrug transporters (MDTs) in the phenomenon of pharmacoresistance in epilepsy. **A:** The main cellular components of the BBB are the endothelial cells, which are joined one another by intercellular tight junctions, and are polarized cells that show luminal and abluminal faces. The pericytes are embedded in the same basal lamina as the endothelial cells. Astrocytes end-feet surround the capillaries. MDTs (here represented as yellow points with arrows) that are expressed at the luminal part of the endothelial cells account for extrusion of several xenobiotics to the capillary lumen, and avoid them reaching the brain tissue. Antiepileptic drugs (AEDs) are highly permeable compounds that are able to cross the BBB and to reach the brain parenchyma when the MDTs are expressed in normal levels (thickness of blue arrows represent AED concentration moving from blood to brain). This occurs, in virtue of their high permeability, so that they can overcome the influence of MDT. **B:** Over-expression of MDT leads to a particular situation, where most molecules of AEDs are being extruded back to the capillary lumen (represented as a thicker, curved blue arrow), and, as a consequence, the amount of molecules crossing the barrier is reduced (represented as a thinner, straight blue arrow). Adapted from: Löscher & Potschka, 2005b

Astrocytes, whose end-processes cover almost entirely the endothelium, have a central role in maintaining the water-electrolyte homeostasis during neural activity, what can be accomplished because of their spatial relationship to the brain vessels and, hence, to the perivascular space where the volume regulation takes place (Abbott et al., 2006). Astrocytes seem to exert vital influence on the expression of BCEC's barrier phenotype, such as formation of competent TJ (Dehouck, et al., 1990; Rubin et al., 1991; Wolburg et al, 1994; Cecchelli et al., 1999) and the expression of polarized MDTs (Gaillard et al., 2000; Kido et al., 2002; Hawkins et al., 2002; Willis et al., 2007).

The role of neurons as modulators of the BBB-phenotype has not been so thoughtfully investigated as for astrocytes. There is some discrepancy regarding their influence on TJ formation in vitro (Roux & Couraud, 2005; Lim et al., 2007), while other authors have shown they act synergistically with astrocytes to induce BCEC differentiation (Schiera et al., 2003). Neural precursor cells can influence the expression of Pgp *in vitro* (Lim et al., 2007).

Endothelial cells phenotype and CNS-pharmacokinetics

The above mentioned cellular elements constitute the BBB and, hence, contribute directly or indirectly to maintaining the microenvironment of the brain tissue in vertebrates, but the barrier phenotype in itself is given mainly by the endothelial cells of brain microvessels (Abbott et al., 2006). Although the concept of a BBB exists since over a century as an inductive concept deriving from the lack of CNS staining with bilirubin (in case of icterus) and intravenously injected dyes (Ehrlich, 1887; Wislocki & Leduc, 1952), the role of BCECs in the BBB was initially understood in the sixties. Evidence obtained from microscopical analyses demonstrated that the inter-endothelial-cell connections, the TJ, prevented peroxidase from entering the brain (Reese & Karnowsky, 1967, and reviewed by Møller et al., 1978). Permeability studies comparing cerebral and non-cerebral endothelium, and BCECs of brain structures lacking an intact BBB, such as the pineal gland, let clear that the BCECs of the BBB show a particular cellular phenotype (Møller et al., 1978), which is described below.

The special characteristics of brain endothelium provide the chemical and physical barrier properties of the BBB (Abbott et al., 2006). The *physical barrier* properties of the brain capillary endothelia result from their lack of fenestrae, their relatively low number of pinocytic vesicles, their reduced degree of transcytosis (whether receptor-mediated or adsorptive-mediated) for large hydrophilic molecules, and, most importantly, the complexity of their intercellular connections (Abbott et al., 2006; de Boer et al., 2003).

The intercellular connections are formed mainly by TJ and adherens junctions. Figure 1.2 resumes the basic structure of those subcellular components. Occludin is a membrane protein with two external loops and four integral membrane domains, and has been implicated in the regulation of barrier properties. Claudins are a family of integral membrane proteins that share the four transmembrane domains of occludin. External loops of claudins are longer and more complex than occludin's ones. Claudin species are the proteins that determine barrier function, regulating both transendothelial electrical resistance (TEER) and paracellular permeability. Claudins 1, 5 and 3 have

been described in BCECs. Members of the junctional adhesion molecule family have been involved in organizing the tight junctional structure and in leukocyte extravasation. Finally, several sub-membrane tight junction-associated proteins, such as zonula occludens proteins 1, 2, and 3 are important in anchoring the transmembrane tight junction proteins to the cytoskeleton (Wolburg & Lippoldt, 2002).

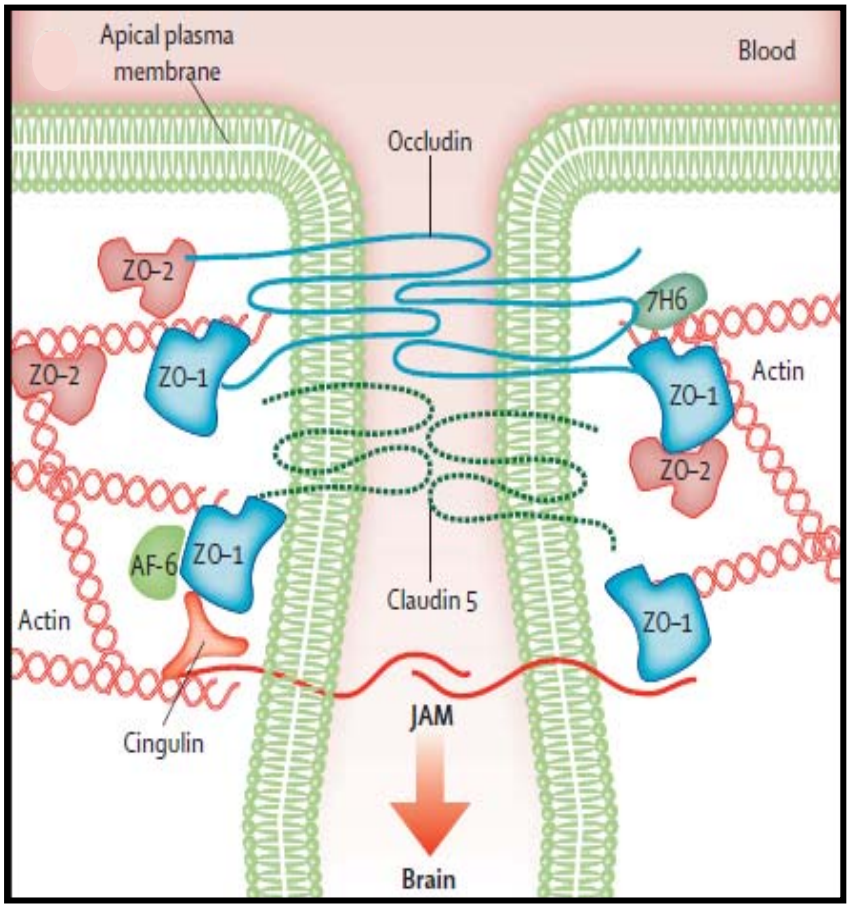

Figure 1.2 Basic structure of intercellular tight junctions (TJ) and adherens junctions between two adjacent endothelial cells. The intercellular space is the route for paracellular movement of molecules between blood and brain. This is restricted by extracellular loops of complex integral proteins such as occludins and claudins, but especially by the later ones. Together with their intracellular anchor proteins they constitute the TJ complex. Extracellular domains of the junctional adhesion molecules (JAM) do not play a major role in paracellular permeability restrictions, but in the regulation of TJ formation, and in leukocyte extravasation. Zonula occludens (ZO) proteins anchor the TJ and JAM to the cytoskeleton. Source: Neuwelt et al., 2008.

Remarkably, the TJ are particularly complex in the BCECs. In freeze-fracture images they appear as a network of strands formed by intramembranous particles, and they can occlude the intercellular cleft very effectively, especially in the so called “kissing points”

(Nagy et al., 1984; Wolburg et al., 1994; Wolburg & Lippoldt, 2002). The TJ play an important role in limiting the paracellular permeability between adjacent BCECs. A proper closure of the intercellular cleft is of crucial relevance for the physical barrier. The fact that TJ relate to the compartmentalization or polarization of cell monolayers (i.e., the ability to separate apical and basolateral compartments or luminal and abluminal sides) was firstly described for epithelial cells, for which a logarithmic correlation between number of TJ strands and the TEER was demonstrated (Claude, 1978), and this function of the TJ is dependant on the phosphorylation states mediated by different subfamilies of protein kinases C (Andreeva et al., 2006). Butt et al. (1990) showed that the TEER can reach 1100 to 1500 Ωcm^2 in pial vessels. Currently, the TEER is widely considered a surrogate parameter for predicting the proper formation of TJ and the consequent paracellular permeability of epithelial and endothelial cell monolayers *in vitro*.

Another characteristic of TJ contributes to the limitation of paracellular permeability: They are apparently interlinked with lipid structures, forming a protein-lipid backbone (Wolburg & Lippoldt, 2002).

The phenotypic properties of BCECs, i.e. their polarization, their lack of fenestrae, their low number of transport- and pinocytic- vesicles, and, most importantly, their intercellular connections with highly complex TJ join together to confer the BBB on exceptional *physical barrier* properties. One can imagine this as a continuous lipid layer facing the blood tissue, which leaves only two possibilities to blood-carried molecules for crossing it: crossing between cells (paracellular route) through aqueous phase, which is extremely limited by TJ and adherens junctions of adjacent BCEC; and crossing across cells (transcellular route), the forced route for most molecules going through the BBB (Abbott et al., 2006).

Hence, molecules require some characteristics for *crossing passively* this physical barrier:

1) Very low molecular size, where the molecules can diffuse by paracellular route and/or through the plasmalema (e.g., O_2, CO_2).

2) Lipophilicity, where relatively larger molecules (usually under 500 Da [Partridge, 2003]) can partition onto the lipid bilayer and continue displacing to the brain tissue by diffusion gradient (e.g., most CNS-bioavailable drugs).

3) A combination of both (e.g., ethanol, barbiturates). Of particular importance is to mention that most CNS-bioavailable drugs developed up to now are characterized by

their lipophilicity combined with their low molecular mass, which is usually below 500 Da (Pardridge, 2003).

In principle, this physical barrier leaves no chance for hydrophilic molecules for crossing passively the luminal lipophilic layer formed by the BCEC. On the other hand, it would allow many lipophilic molecules, including toxins, crossing the endothelium. But contrarily to both, it does allow selected hydrophilic substances crossing the BBB, and it does avoid some lipophilic ones from entering the brain tissue. These properties are part of the other barrier phenotype the BCEC displays: the *chemical barrier*.

In the first case, some hydrophilic molecules can cross selectively the BCEC in a concentration-dependant manner, which is mediated by specific carriers that move substrates whether Na^+-dependently of Na^+-independently (e.g., GLUT1 that carries glucose, and LAT1 and L-system for large neutral amino acids) (Tsuji & Tamai, 1999). Some of these carriers are located at the luminal and abluminal membranes, facilitating particle-movement in both senses. These carriers can also transport some drugs that otherwise, only by virtue of their hidrophilicity, could never cross the BBB; e.g., L-DOPA and gabapentin which are carried by the L-system (Tsuji & Tamai, 1999). Hydrophilic molecules, which are important for brain metabolism, and lack a carrier-mediated influx, are *carried actively* through the BCEC by means of receptor-mediated transcytosis (e.g., insulin, transferrin) or by adsorptive transcytosis (e.g., albumin), yet the degree of endocytosis/transcytosis in the BCEC is much lower than in peripheral endothelia (Tsuji & Tamai, 1999; Abbott et al, 2006).

In the second case, several efflux transporters provide an effective mechanism for extruding lipophilic substances (e.g. toxins and drugs) from brain tissue to the blood, thus avoiding endogenous or exogenous, lipophilic substances from entering the brain parenchyma. An obvious pre-requisite for this phenotype is the polarization of the BCEC, with transporters being able to keep lipophilic substances in the blood tissue being located at the luminal membrane of BCEC (Pardridge, 2003). Since the main goal of our work relates to the role of some of these active efflux transporters in CNS pharmacokinetics, they will be described in more detail in the next paragraphs.

Multidrug transporters of the blood-brain barrier: The ABC transporters

The ATP-binding cassette transporters (ABC-T) are multidomain integral membrane proteins that utilize the energy of ATP hydrolysis to translocate solutes across cellular membranes (Jones & George, 2004; Rees et al., 2009). They constitute the largest transmembrane protein families (also referred as superfamily), with many highly

evolutionary conserved members being expressed in prokaryotic and eukaryotic organisms (Jones & George, 2004).

The differentiation of ABC transporters from other ATP-binding proteins is based on the sequence and organization of their ATP-binding domains, also known as nucleotide-binding folds. The nucleotide-binding folds contain the highly conserved motifs Walker A and B, separated by approximately 90–120 amino acids. ABC genes contain an additional element, the signature (C) motif, located just upstream of the Walker B site. The functional protein typically contains two nucleotide-binding folds and two trans-membrane domains (TMD). The TMD typically contain 6–11 membrane-spanning α-helices and provide the specificity for the substrate. The nucleotide-binding folds are located in the cytoplasm and transfer the energy to transport the substrate across the membrane (Allikmets et al., 1996; Dean et al., 2001; Dean, 2002). Figure 1.3 shows the basic structure of ABC transporter superfamily.

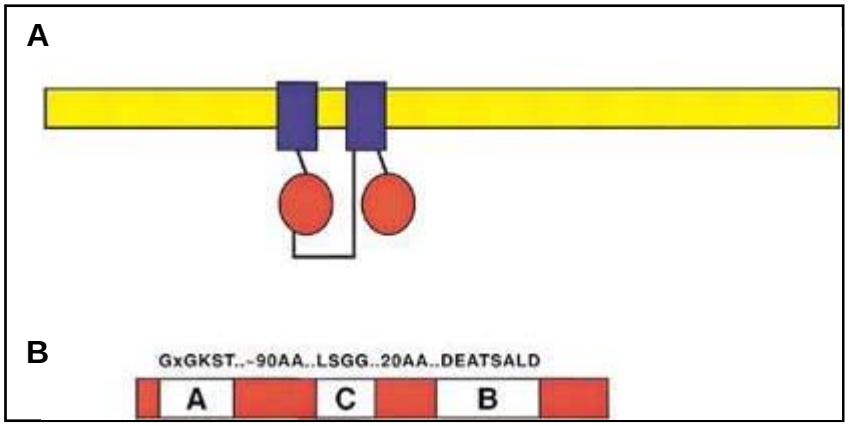

Figure 1.3 Diagram of the basic, common structure of ABC transporter superfamily. **A:** The structure of a representative ABC protein. The transmembrane domains (TMD, blue) span the lipid bilayer (represented as a horizontal, yellow bar), while the nucleotide-binding folds (NBF, red) are located in the cytoplasm. Most commonly, a full transporter show motifs arranged N-terminus-TMD-NBF-TMD-NBF-C-terminus, as shown here, but other arrangements are also found. **B:** The NBF of an ABC gene contains the Walker A and B motifs found in all ATP-binding proteins. In addition, a signature or C motif is also present. Above the diagram are the most common amino acids found in these motifs; subfamilies often contain characteristic residues in these and other regions. Source: Dean, 2002.

The ABC-T superfamily includes 7 subfamilies which are named as follows: “ABC” for the superfamily, followed by the characters from “A” to “G” which designate each subfamily, and the subfamily-members are identified by consecutive numbering

(http://www.genenames.org/genefamily/abc.html, retrieved on May 10, 2009), but the currently used nomenclature is much wider (see Table 1.1).

Their ability to transport a wide range of chemically dissimilar molecules against a concentration gradient, and most of them in a unidirectional sense, points out two basic roles they play in diverse organisms from bacteria to superior mammals: 1) protection against noxious compounds and secretion of internally produced substances –efflux function; and b) internal incorporation of vital elements –influx function; with both functions being mutually exclusive, i.e. they are carried out by different transporters, with a particular orientation on the plasma membrane (Dean et al., 2001; Dean, 2002).

Particularly important in pharmacology is the efflux ability of some members of the ABC-T superfamily that have been implicated in pharmacoresistance of cancer and pathogenic microbes to drugs (Löscher & Potschka, 2005b). ABC-T are expressed in a wide variety of tissues in mammals and, hence, play a major role in drug pharmacokinetics (Szakács et al., 2008). For instance, Pgp, the mostly investigated ABC-T by far, is expressed in intestinal epithelium, hepatocytes, renal epithelium and BBB-endothelium of mammals (Thiebaut et al., 1989; Huls et al., 2009) meaning that it limits the diffusion of a variety of lipophilic drugs on the one hand, and promotes the elimination of drugs on the other hand. Yet, several ABC-T restrict the access of many drugs into the target tissue, i.e. the extracellular space, if they are expressed at biological barriers (e.g., restrained drug access to brain parenchyma when the transporters locate on the luminal membrane of the BBB-endothelium), and/or to the intracellular target molecules if the expression takes place on the plasmalemma of target cells (e.g., tumor cells, bacteria). In other words, they can influence pharmacokinetics at several biological, compartmental levels.

At the BBB the ABC-T play a major role in the conformation of the chemical barrier phenotype, since they can transport lipophilic xenobiotics towards the lumen of the vessels and can keep them back from the brain-parenchymal compartment. This means they can protect the neural tissue against potential toxins, that otherwise could be able to reach the neuronal environment; but additionally, they can influence pharmacokinetics of many potentially CNS-active drugs (Fricker & Miller, 2004; Schinkel & Jonker, 2003; Sun et al., 2003; Löscher & Potschka, 2005a,b).

All the seven ABC-T subfamilies are represented in the BBB, where a total of 25 specific transporters' genes have been demonstrated to be up-regulated in BCECs of different mammalian species including humans (Warren et al., 2009). However, only ABCB1 (Pgp), ABCC1 (MRP1), ABCC2 (MRP2), ABCC4 (MRP4), ABCC5 (MRP5) and

ABCG2 (BCRP) have been previously well characterized at the BBB at gene and protein expression levels (Fricker & Miller, 2004; Löscher & Potschka, 2005a,b; Roberts et al., 2008). In the present work a total of four members belonging to two subfamilies were investigated for their ability to transport AEDs, namely Pgp (subfamily ABCB) and MRP1, MRP2 and MRP5 (subfamily ABCC). Some comparative characteristics of these MDT in rats and humans are summarized in Table 1.2.

Table 1.1 Alternate nomenclatures of selected ABC transporters

Official nomenclature[1]	ABCB1	ABCC1	ABCC2	ABCC5
Alternate names[2]	P-glycoprotein Pgp or P-gp MDR1* ABC20 GP170 PGY1	MRP1 ABC29 GS-X DKFZp781G125 DKFZp686N04233	MRP2 CMOAT DJS cMRP ABC30 KIAA1010	MRP5 MOATC MOAT-C SMRP ABC33 pABC11 EST277145 DKFZp686C1782
Alternate subfamily name[3]	MDR	---------- MRP ----------		

(*) The name *MDR1* is preferentially used to refer to the gene and not to the protein

(1) Dean, 2002; NCBI Entrez Gene database

(2) NCBI Entrez Gene database; Schinkel & Jonker, 2003

(3) http://nutrigene.4t.com/humanabc.htm

(Websites retrieved on May 2009)

The bulk of information related to the mechanism of drug transport by ABC-T derives from different experiments performed with the prototype Pgp (ABCB1). It appears that drug binding occurs at the level of the inner (cytoplasm) leaflet of the cell membrane. The substrate collides at this phospholipid interphase with a high-affinity site of the transporter, what stimulates ATP hydrolysis on the NBF. This way, the energy for drug translocation is provided. A change in transporter tridimentional configuration modifies the affinity of the drug binding pocket, causing the release of the drug; but whether the drug is released to the aqueous phase or to the outer leaflet of the membrane is a debated issue (Seeger & van Veen, 2009). The drug binding described in this model would explain the observations that ABCB1 function is quite sensitive to the lipid-microenvironment (Ferté, 2000; Orlowski et al., 2006).

P-glycoprotein (ABCB1)

This 170 kDa glycoprotein was first time described in 1976, and received its name in virtue of the observation that its expression altered drug permeability (Juliano & Ling, 1976). This is also the mostly investigated ABC-T so far, and the prototype on which much research about MDT has been carried out (Dean et al., 2001; Löscher & Potschka, 2005a,b; Seeger & van Veen, 2009). It is recognized as an efflux transporter limiting drug permeation through several biological barriers such as intestine, placenta and BBB (Schinkel & Jonker, 2003; Fricker & Miller, 2004; Löscher & Potschka, 2005a,b). A large list of chemically dissimilar, lipophilic drugs constitutes the substrate-spectrum of this transporter, including anticancer drugs (e.g., doxorubicin, daunorubicin, idarubicin, vinblastine, vincristine, etoposide, and methotrexate); immunosupresive agents (e.g., cyclosporin A, tacrolimus); corticoids (e.g., dexamethasone, hydrocortisone); analgesics (e.g., morphine, fentanyl); human-immunodeficiency-virus protease inhibitors (e.g., amprenavir, saquinavir, ritonavir), among many other drugs (Löscher & Potschka, 2005b).

Pgp is expressed on the apical membrane of polarized kidney epithelial cells that are used as models for investigation of substrate specificity (Schinkel & Jonker, 2003).

Regarding expression and polarized localization at the BBB, in a recent comparative study it was shown that MDR1 (the Pgp encoding gene) was the ABC-T gene mostly expressed in brain microvessels of five different species including humans (Warren et al., 2009) and it has been shown to be particularly enriched in brain microvessels in comparison to brain tissue (Dauchy et al., 2008). The luminal localization of Pgp at the BCEC has been widely corroborated in human and non human mammals (Schinkel & Jonker, 2003; Sun et al., 2003; Fricker & Miller, 2004; Löscher & Potschka, 2005a,b). Furthermore, the up-regulation and over-expression of this transporter have been observed in case of pharmacoresistant epileptic patients (Tishler et al., 1995; Sisodiya et al., 1999; Dombrowski et al., 2001, Sisodiya et al., 2002; Aronica et al., 2003) and in animal models of TLE (Seegers et al., 2002; Volk et al., 2004; Volk & Löscher, 2005; van Vliet et al., 2007). *In vitro* and *in vivo* evidence shows that phenytoin and phenobarbital are substrates for mouse/rat Pgp (Mdr1a) (Löscher & Potschka, 2005a; van Vliet et al., 2006; Brandt et al., 2006; Baltes et al., 2007; Yang & Liu, 2008), but it is not clear whether these compounds are transported by human Pgp. Hence, Pgp is one of the main candidates that may efflux AEDs from human brain tissue in case of intractable epilepsy.

Multidrug resistance-associated proteins 1, 2 and 5 (ABCC1, ABCC2, ABCC5)

At present the MRP subfamily (ABC subfamily C) contains 13 members, but 4 of them do not mediate drug transport. MRP1 and MRP2, but not MRP5 contain an additional TMD (TMD_0), with the first NBD located between TMD_0 and TMD_1 (Dallas et al., 2006). This subfamily of ABC-T is one of the largest subfamilies that are able to affect drug disposition, and show a particular affinity for organic anions (Dallas et al., 2006; Borst et al., 2007).

The MRP1 gene was first cloned in 1992 from a human lung cancer cell line (H69AR) that was resistant to chemotherapeutics, but did not express Pgp (Cole et al., 1992). Unlike Pgp, MRP1 shows preferential transport for anionic compounds such as glucoronide, glutathione, and sulfate conjugates (e.g., aflatoxin B1-epoxide-glutathione); and confer resistance to a variety of natural-derived anticancer drugs such as vinca alkaloids and anthracyclines (Dallas et al., 2006). Cytokines, such as leukotrienes C4, D4 and E4; antiviral drugs (e.g., ritonavir and saquinavir), and toxins (e.g., acetanilide pesticides, arsenic and some tobacco-derived carcinogens), among others, complement the substrate-spectrum of MRP1 (Löscher & Potschka, 2005b; Dallas et al., 2006).

MRP2 is known to play an important role in the hepatobiliary excretion of mono- and bis-glucoronidated bilirrubin, and its homozygous deficiency causes Dubin-Johnson syndrome, a pathology characterized by conjugated hyperbilirubinemia with observable jaundice, but with mild clinical affection (Schinkel & Jonker, 2003). There are many coincidences, but not complete overlap in the substrate-spectrum of MRP2 and MRP1. It includes anticancer drugs (e.g., methotrexate, anthracyclines, vincristine, cisplatin, and etoposide), antiviral drugs (e.g., indinavir, ritonavir, and saquinavir), hormones (estradiol-3-glucoronide), and diverse amphipathic anionic drugs and endogenous compounds (e.g., leukotriene C4) (Schinkel & Jonker, 2003; Löscher & Potschka, 2005b; Dallas et al., 2006).

MRP5 is ubiquitously expressed, but in low levels. It has been difficult to characterize its tissue distribution since several monoclonal antibodies failed to give clear identification (Borst et al., 2007). Several MRP1 and/or MRP2 substrates, such as vincristine, leukotriene C4, etoposide or daunorubicin are not transported by MRP5. Its substrate-spectrum includes monophosphorylated compounds such as the antiviral agent and AMP analog 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and the antiviral agent stavudine monophosphate (Schinkel & Jonker, 2003; Dallas et al., 2006). MRP5 transports the cyclic nucleotides 3', 5'-cAMP and c-GMP, and confers resistance to nucleotide analogs (e.g., cladribine) in MRP5 transfectants (Borst et al., 2007).

Concerning the expression of MRPs in polarized kidney epithelial cells that serve as models for investigation of substrate specificity, MRP2 and MRP4 are expressed on the apical membrane; while MRP1, MRP3 and MRP5 localize on the basolateral membrane (Schinkel & Jonker, 2003; Dallas et al., 2006).

In relation to the expression of several members of the ABC subfamily C at the BBB, Dauchy et al. (2008) found no difference in the relative expression of MRP1 or MRP5 mRNA between brain microvessels and brain tissue, while MRP2 mRNA was not expressed. They analyzed healthy tissue adjacent to pathological brain areas obtained from epilepsy-surgery or tumor resection, but unfortunately they do not report on comparison with pathological regions. Notwithstanding, these observations are in agreement with those recently reported by Warren et al. (2009), who did analyze mRNA expression from epileptogenic-resected focus, and who also did not find clear expression of MRP2 in human tissue, but high expression of MRP1 and MRP5 mRNAs, among other transporters. MRP2 seems to be rarely or not expressed in normal rat BBB (Sugiyama et al., 2003; Warren et al., 2009). Contrarily, other authors have reported the luminal expression and/or functionality of MRP2 protein in non-human BBB (Miller et al, 2000; Fricker et al, 2002; Potschka et al, 2003a). Strikingly, MRP2 gene or protein appears to be up-regulated or over-expressed in case of brain tissue of epileptic rats (van Vliet et al., 2007; Hoffmann et al., 2006) and humans (Dombrowski et al., 2001; Aronica et al, 2004; Kwan and Brodie, 2005; Kubota et al., 2006), a situation of particular relevance for the issue we are focused in; that is, the pharmacoresistance in epilepsy. It seems that MRP2 up-regulation and expression occur as a response of pathologic processes, and for this reason, is a particularly interesting ABC-T that could alter drug pharmacokinetics in such conditions.

MRP1 protein has been reported to be expressed at the luminal side of bovine (Zhang et al., 2004) and human BCEC (Nies et al., 2004). Contradictorily, some authors reported its expression whether ambiguous or mainly abluminal in case of rats (Roberts et al., 2008), mice (Soontornmalai, 2006) and humans (Kubota et al., 2006).

On the other hand, MRP5 protein seems to be expressed on the luminal part of bovine (Zhang et al., 2004), mice (Soontornmalai, 2006) and human BCEC (Nies et al., 2004; Kubota et al, 2006), but abluminal in rat BCEC (Roberts et al., 2008). This situation may obey to species differences, the precision of the methodology used and/or environmental factors affecting MRP expression (Kubota et al., 2006; Roberts et al., 2008). Additionally, problems with the available anti-MRP5 antibodies have been presumed to influence those differences in results (Borst et al., 2007).

The up-regulation and over-expression of MRP5 has been reported in epileptogenic tissue of human patients (Dombrowski et al., 2001; Kwan & Brodie, 2005). It is less clear whether MRP1 is up-regulated in such conditions, but its expression was found to be relatively high in both epileptogenic and control tissues (Dombrowski et al., 2001; Aronica et al., 2004).

Table 1.2 Comparative characteristics of some ABC transporters of the blood-brain barrier in rat and human

Official symbol	H: ABCB1 R: Abcb1a Abcb1b*		H: ABCC1 R: Abcc1		H: ABCC2 R: Abcc2		H: ABCC5 R: Abcc5	
	H	R	H	R	H	R	H	R
Size (aa)	1280	1a: 1272 1b: 1277	1531	1532	1545	1541	1437	1436
Other notations for genes	MDR1	Mdr1a; Mdr1b	MRP1	Mrp1	MRP2	Mrp2	MRP5	Mrp5
Location at BCEC	lum	lum (Mdr1a)	lum (mainly)	ablum ?	lum	lum	lum	ablum ?
Transport of AEDs	No	Yes *in vitro* *in vivo*	No	ND	No	Yes *in vivo*	No	ND

H = human; R = rat; ND = not determined as yet; BCEC: brain capillary endothelial cells; lum = luminal; ablum = abluminal.

(*) ABCB1 is encoded by two genes in rat: Abcb1a and Abcb1b

References: Miller et al., 2000; Potschka et al., 2003a; Schinkel & Jonker, 2003; Sun et al., 2003; Fricker & Miller, 2004; Nies et al., 2004; Löscher & Potschka, 2005a,b; Kubota et al., 2006; Baltes et al., 2007; Borst et al., 2007; Roberts et al., 2008, UniProt FTP <http://genome-mirror.binf.ku.dk/> retrieved May, 2009.

In addition to their expression at the BBB, these transporters are able to efflux a wide range of chemically dissimilar drugs including anticancer drugs, antidepressants, analgesics and antipsychotic agents, among many others (Löscher & Potschka, 2005a). Therefore, the above commented MDTs are potential candidates for transport of AEDs.

1.3. Models for the investigation of substrate specificity of human ABC transporters and of drug permeation through the blood-brain barrier

In vivo and *in vitro* models for the investigation of substrate specificity of ABC-T from different mammal species, and their reciprocal correlations have been a subject of intense research, and have been revised by several authors. We base the following brief and basic introduction to some frequently used models on some notable reviews: Polli et al., 2001; Schinkel & Jonker, 2003; Fricker & Miller, 2004; Garberg et al., 2005; Löscher & Potschka, 2005b; Dallas et al., 2006; Balaz, 2009.

Frequently used *in vitro* (direct) transport experiments are performed whether on lipidic vesicles, on cells or on cell monolayers. Transport in lipidic vesicles utilize inside-out, membranous vesicles that are purified from cells which were previously transfected for the expression of a particular transporter. The transporters are directed to the inner part of the vesicle, so that the substrates are translocated to the interior of this lipidic complex, i.e., they are accumulated inside. Incubation with a standard inhibitor of the transporter allows for discriminating between transport and absence of transport.

Amongst common *in vitro* assays that utilize whole cells, but that do not require the formation of a tight monolayer are the (direct) uptake assay of the drug in question, and the calcein uptake assay in combination with the investigated drug (indirect method). In both cases, cells are either chemically selected or transfected for the expression of ABC-T, and compared for drug transport with non-selected (non-resistant) or parental cells, and with cells incubated with a standard inhibitor. In the first case, the direct accumulation of the drug in the cells is measured after an appropriate incubation period. In principle, drugs that are substrates of the transporter are actively effluxed, and thus, less accumulated within the cells, and this condition can be reversed with the inhibitor. A disadvantage of this assay is that a highly lipophilic drug can diffuse quickly through cell membrane, limiting the concentration inside the cell. As a consequence, negative results are not to be discarded when highly permeable compounds are investigated. In the second case, the calcein uptake assay, the transport of a drug is indirectly measured. Calcein acetoxymethyl ester (calcein-AM) is a non-fluorescent, lipophilic substance that rapidly diffuses into the cells, where it is cleaved by intracellular esterases resulting in the fluorescent, impermeable calcein, which is trapped within the cell unless actively extruded by Pgp, or MRP1 (Szakács et al., 1998; Dogan et al., 2004). This assay relies on the theory that if the tested drug is a substrate for Pgp, for instance, it will compete with calcein for affinity sites in the transporter. Hence, a Pgp

substrate can decrease calcein efflux, and as a consequence, it can increase calcein accumulation. This assay has the advantage that no high-cost analytic equipment other than a fluorometer is needed. The biggest disadvantage of the calcein uptake assay is that if a drug does not inhibit calcein efflux, it does not necessarily mean that the drug is not transported. No necessary correlation exists between substrates and inhibitors (Feng et al., 2008).

In the last years the use of cell monolayers has been largely extended, especially because they may better simulate the *in vivo* situation (Liu et al., 2008). In this case, polarized cell monolayers are grown on the microporous membrane of inserts, which are located within a larger compartment (i.e., a well), so that a tridimensional, two-compartment (i.e., apical and basolateral) model is created (Fig. 1.4). For the widely used bidirectional transport assay, the drug is diluted in the medium and applied in one of both compartments, and then samples are taken from the opposite compartment at given time intervals. This way, the permeability of a drug can be assessed from basolateral to apical and vice versa. An obvious requisite for this model is that the transporter must have a polarized expression. For instance, Pgp is expressed on the apical membrane in the kidney epithelial cell lines MDCK II and LLC-PK1. In this example, it is expected that a substrate will penetrate more quickly from basolateral to apical direction, than from apical to basolateral. A ratio of the permeabilities in both directions is easily calculated, and the ratio obtained in the transfectants (e.g., MDR1 transfected cells) is corrected with the ratio obtained in the parental cells, in order to find the difference that is attributable to the overexpressed transporter. This model has been used not only for drug screening of ABC transporter substrates (typically using Caco-2, MDCK II and/or LLC-PK1 cells), but also for the investigation of drug permeability across the BBB (including the former cell lines, and/or primary cultures of rat, porcine or bovine brain capillary endothelial cells). One of the biggest disadvantages of this Transwell® model is that the only available cells so far display relatively high paracellular permeability, because of suboptimal expression of TJ (Liu et al., 2008). For monitoring the tightness of the monolayer, the permeability of polar substances, such as fluorescein, mannitol or sucrose, can be measured. This parameter indicates the rate of drug diffusion via the paracellular route (paracellular markers). Another important parameter to be tested is the transepithelial or transendothelial electrical resistance (TEER) which has a direct relation with the formation of TJ; i.e., tighter monolayers show higher TEER values, but the values vary for each cell line. The safest method is a combination of TEER measurement and the use of paracellular markers.

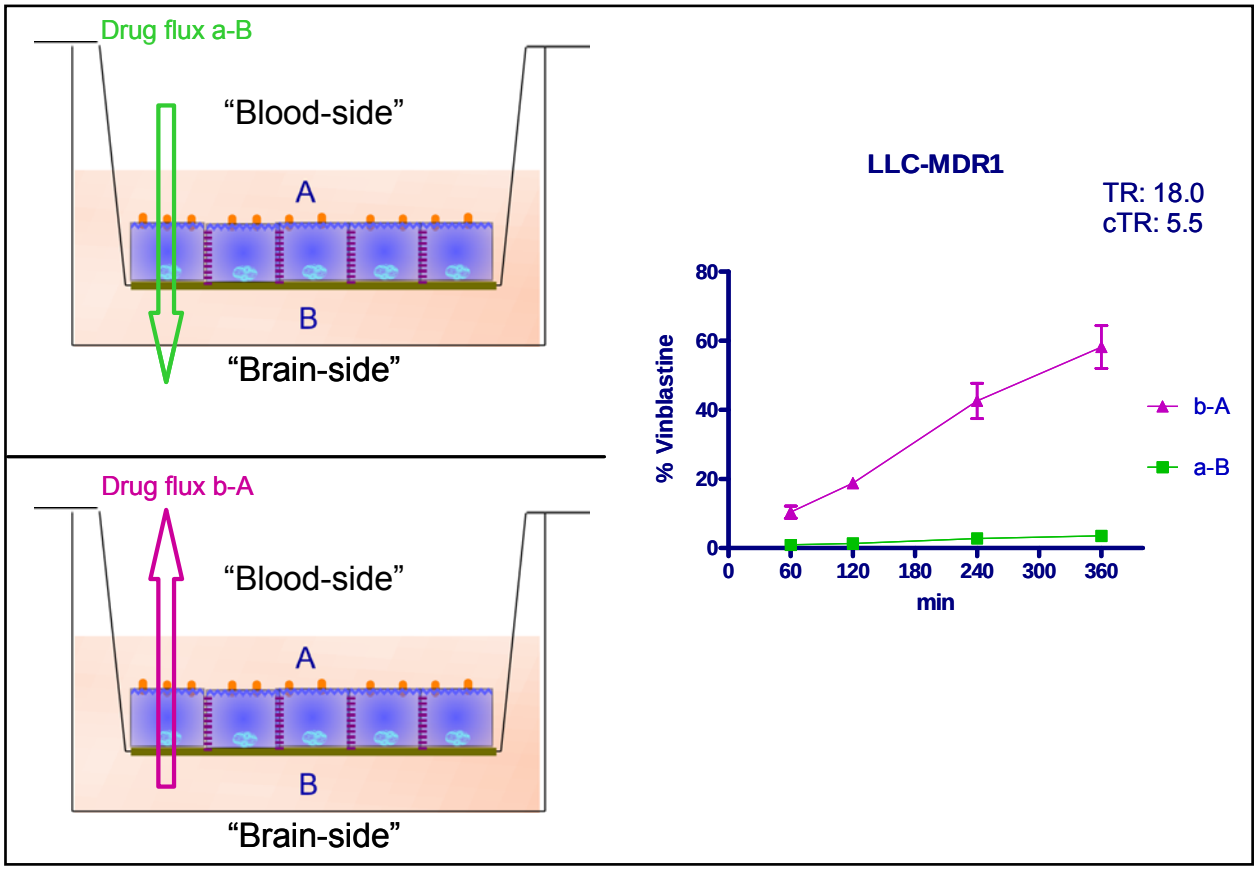

Figure 1.4 Transwell® model for bidirectional transport assay. Polarized cell monolayers that express apical transporters (orange dots) are grown on the microporous membrane of an insert (apical [A]), which is located within a basolateral compartment (B). Given the polarization of the cells and their transporters, the basolateral chamber represents the "brain side" and the apical chamber, the "blood side" in this model. The permeability of a drug from basolateral to apical (purple arrow and ploted line), and from apical to basolateral (green arrow and ploted line) are measured, and a transport ratio between them (TR) is obtained. The corrected transport ratio (cTR) of a drug is the ratio of the TR from Pgp over-expressing cells divided by the TR from parental cell lines, and indicates the magnitude of transport mediated by the over-expressed transporter. The graph shows typical results for the standard Pgp substrate vinblastine sulphate. A drug is considered to be a good substrate for Pgp, if the cTR is higher than 2.5 in this model. A cTR ≥ 1.5 is considered as the cut-line to classify the drug as substrate.

One of the most destacable *in vitro* systems for BBB permeability is the dynamic model described by Santaguida et al. (2006) and Cucullo et al. (2007) which consists of sets of capillaries. Endothelial cells are cultured onto the lumen of the capillaries, while astrocytes are grown on the abluminal part of them. But most importantly, the medium can flow through the system, recapitulating the physiological shear stress, and

improving the formation of tight junctions. Endothelial cells from human beings have been used for drug permeability assays across this *in vitro* BBB (Cucullo et al., 2007).

In vivo studies of brain permeability and impact of MDTs at the BBB are obviously limited, and the approach of this topic is beyond the scope of this work, but some illustrative examples are mentioned next. A feasible technique for *in vivo* studies are inhibitory assays, where MDTs are systemically inhibited and the increment in the brain permeation of the drug is measured indirectly by using a specific end-point; i.e., a drug-specific pharmacological effect. One of the few available clinical examples is an inhibitory assay with the opioid drug loperamide, which was performed in healthy volunteers. Loperamide can not permeate into the brain tissue because it is a substrate for Pgp. In order to investigate this, human beings were administered with the opioid, while the Pgp was inhibited with quinidine. This resulted in increased brain availability, with the concomitant opioid effect. The end-point measured in this test was the respiratory rate, with respiratory depression observed after Pgp inhibition, because of the activity of loperamide on the respiratory center (Sadeque et al., 2000). On the other hand, a promising tool for the evaluantion of CNS drug permeation are imaging techniques, such as positron emission tomography, where ^{11}C-radiolabeled drugs can be measured non-invasively in human beings. With this approach, inhibitory studies for drug permeation across the BBB *in vivo* can be carried out. This technique is still under development.

The most remarkable animal model for studies of drug transport mediated by ABC-T is the knockout-mice model, in which brain concentrations of the investigated drug are compared between wildtype mice and knockout-mice. An increase of drug brain-uptake in the latter indicates involvement of the silenced transporter in the efflux of such a drug (Rizzi et al., 2002; Doran et al., 2005).

1.4. General characteristics of antiepileptic drugs

AED therapy is the main option to treat epileptic patients (Schmidt, 2009). AEDs are intended to be used as sustained therapy to prevent occurrence of seizures, but not to cure epilepsy, meaning that they must be administered chronically (Rogawski & Löscher, 2004; Schmidt, 2009). AEDs also show therapeutic benefits to treat migraine headache, neuropathic pain and bipolar affective disorder, among other CNS conditions (Rogawski & Löscher, 2004).

Approved AEDs in Europe and U.S.A include: acetazolamide, carbamazepine, clonazepam, clorazepate, ethosuximide, ethotoin, felbamate, gabapentin, lacosamide, lamotrigine, levetiracetam, mephenytoin, methsuximide, oxcarbazepine, phenobarbital, phenytoin, pregabalin, primidone, tiagabine, topiramate, trimethadione, valproate, vigabatrin, and zonisamide, for a total of 24 molecules (Schmidt, 2009). Notwithstanding, old AEDs such as phenobarbital, carbamazepine, phenytoin and valproic acid are the mostly used AEDs worldwide, especially because of their lower cost in comparison to more modern molecules (WHO, 2005).

The pharmacodynamics of AEDs relate to their ability to modify the bursting properties of neurons and to reduce synchronization in localized neuronal ensembles. This is accomplished by acting on one or more molecular targets such as ion channels, neurotransmitter transporters and neurotransmitter metabolic enzymes. In general, the mechanisms of action include: 1) modulation of voltage-gated ion channels; 2) enhancement of synaptic inhibition; and 3) inhibition of synaptic excitation (Rogawski & Löscher, 2004).

Concerning drug-drug interactions, several AEDs such as carbamazepine, phenobarbital and phenytoin are potent inducers of cytochrome p450 system; while others inhibit enzymes involved in glucoronidation (e.g., valproate). Modern AEDs (e.g., levetiracetam, topiramate) do not show such interactions, and are less likely to be involved in enzyme-based drug-drug interactions (Schmidt, 2009).

Regarding their pharmacokinetics, most AEDs are lipophilic and very small molecules (i.e. <500 Da) that can gain access to most compartments including brain. Some relevant pharmacokinetic characteristics of AEDs are detailed in Table 1.3 while their chemical structures are depicted in Figure 1.5.

In contrast to AEDs, good Pgp substrates have no or poor access to brain parenchyma, although they are lipophilic drugs, what theoretically would mean they can overcome the physical barrier of the BBB. They are also slightly larger molecules (i.e., >500 Da) than most CNS-reaching compounds (Pardridge, 2003), and their permeability through the BBB is strongly affected by Pgp (i.e., the BBB-chemical barrier). Some pharmacokinetic characteristic of selected MDT substrates are included in table 1.3.

The high permeability of AEDs and their ability to reach adequate CNS concentrations points out the relevance of MDT over-expression at the BBB. According to the MDT hypothesis of pharmacoresistance in epilepsy, the higher amount of

transporters expressed at the luminal side of the BBB is a critical point that makes the switch to a non-responder status of the patient, given altered drug pharmacokinetics and less concentration in the site of action, with the consequent alteration of the drug-target relationship (see Figure 1.1). Under this perspective, it was our aim to investigate whether the human MDTs Pgp, MRP1, MRP2 and MRP5 are able to transport several AEDs *in vitro*.

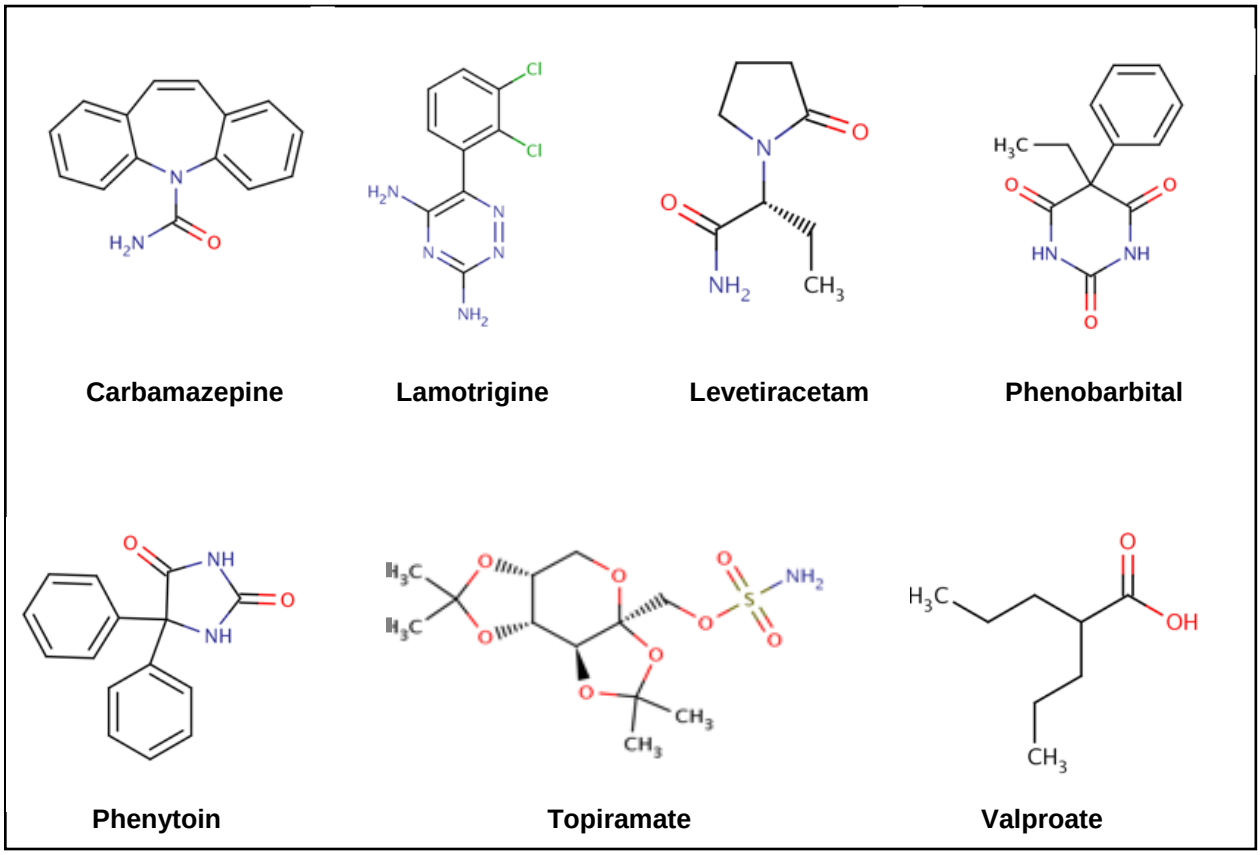

Figure 1.5 Molecular structures of antiepileptic drugs that were investigated in the present work. Source: http://www.drugbank.ca; retrieved in June, 2009.

Table 1.3 Some pharmacokinetic characteristics of selected antiepileptic drugs and Pgp substrates

Drug	MW	LogP [e]	LogD	PB (%)	h-ABC transport	CYP induction	CYP inhibition	B/P ratio rodents[e]	LogPS rodents[e]
Carbamazepine	236.3	2.3	2.67 [c]	76	n.k.	2C9; 3A families	No	0.8 [m]	
Lamotrigine	256.1	2.5	-0.19 [c]	55	n.k.	No	No	1.1 [m]	
Levetiracetam	170.2	-0.6	-0.67 [c]	<10	n.k.	No	No		
Phenobarbital	232.2	1.5	1.5 [e]	20-45	n.k.	2C; 3A families	No	0.7 [r]	
Phenytoin	252.3	2.2	2.5 [c]	73	Pgp	2C; 3A families	No	0.6 [m]	-2.2 [m]
Topiramate	339.4	-0.7	2.97 [c]	15-41	n.k.	No	No		
Valproic acid	144.2	2.7	-0.02 to 0.16 [c]	10-18.5	n.k.	No	Yes		-2.5 [m]
Multidrug transporter substrates									
Cyclosporine A	1202.6	2.9	5.0 [c]	90	Pgp, MRP1	No	No	0.3 [m]	-1.9 [m]
Digoxin	780.9	2.2	2.2 [c]	25	Pgp	No	No	0.1 [m]	-4.3 [m]
Vinblastine	811.0	3.9	4.1 [c]	98-99	Pgp, MRP1, MRP2	No	3A4		-3.5 [m]

MW: molecular weight; LogP: octanol:water partition coefficient at isoelectric point; LogD: octanol:water partition coefficient at physiological pH; PB: protein binding; h-ABC: The human ABC transporter the drug is substrate for; n.k.: not known; CYP: cytochrome P450; B/P ratio: brain to plasma concentration ratio; LogPS: logarithm of BBB permeability-surface area product, that represents the uptake clearance across the BBB (theoretically, unlike B/P, PS is a direct measure, i.e., it is not confounded by plasma and brain tissue binding [Liu et al., 2004]).

(e) Experimental; (c) calculated; (m) mouse; (r) rat

References: Leppik & Sherwin, 1979; Schinkel et al., 1995; Fromm et al., 1999; Murakami et al., 2000; Liu et al., 2004; Garberg et al., 2005; Löscher & Potschka, 2005a; Brunton et al., 2006; Cucullo et al., 2007; Schmidt, 2009; http://www.drugbank.ca; http://www.chemspider.com; both retrieved in June, 2009.

Chapter 2

Several major antiepileptic drugs are substrates for human Pgp

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Several major antiepileptic drugs are substrates for human P-glycoprotein

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ABSTRACT

One of the current hypotheses of pharmacoresistant epilepsy proposes that transport of antiepileptic drugs (AEDs) by drug efflux transporters such as P-glycoprotein (Pgp) at the blood–brain barrier may play a significant role in pharmacoresistance in epilepsy by extruding AEDs from their intended site of action. However, several recent *in vitro* studies using cell lines that overexpress efflux transporters indicate that human Pgp may not transport AEDs to any relevant extent. In this respect it has to be considered that most AEDs are highly permeable, so that conventional bi-directional transport assays as used in these previous studies may fail to identify AEDs as Pgp substrates, particularly if these drugs are not high-affinity substrates for Pgp. In the present study, we used a modified transport assay that allows evaluating active transport independently of the passive permeability component. In this concentration equilibrium transport assay (CETA), the drug is initially added at identical concentration to both sides of a polarized, Pgp-overexpressing cell monolayer instead of applying the drug to either the apical or basolateral side for studying bi-directional transport. Direct comparison of the conventional bi-directional (concentration gradient) assay with the CETA, using *MDR1*-transfected LLC cells, demonstrated that CETA, but not the conventional assay, identified phenytoin and phenobarbital as substrates of human Pgp. Furthermore, directional transport was determined for lamotrigine and levetiracetam, but not carbamazepine. Transport of AEDs could be completely or partially (>50%) inhibited by the selective Pgp inhibitor, tariquidar. However, transport of phenobarbital and levetiracetam was also inhibited by MK571, which preferentially blocks transport by multidrug resistance transporters (MRPs), indicating that, in addition to Pgp, these AEDs are substrates of MRPs. The present study provides the first direct evidence that several AEDS are substrates of human Pgp, thus further substantiating the transporter hypothesis of pharmacoresistant epilepsy.

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1. Introduction

Resistance to antiepileptic drugs (AEDs) is a major, unresolved problem in epilepsy therapy, affecting about 30–40% of all patients (Kwan and Brodie, 2000; Chang and Lowenstein, 2003). Most patients with AED-resistant epilepsy are resistant to several, if not all, AEDs, despite the fact that these drugs act by different mechanisms. The consequences of uncontrolled epilepsy can be severe, and include shortened lifespan, bodily injury, neuropsychologial and psychiatric impairment, and social disability (Sperling, 2004). Consequently, there is a pressing need to develop new and more effective treatment strategies to counteract or prevent pharmacoresistance. For this goal, we need to understand the mechanisms underlying AED resistance. One of the candidate mechanisms that has attracted growing interest is the limitation of AED access to epileptogenic brain region(s) by localized overexpression of drug efflux transporters such as P-glycoprotein (Pgp) at the blood–brain barrier (BBB) (Löscher and Potschka, 2005a). A prerequisite for this transporter hypothesis of drug resistance is that AEDs are substrates of human Pgp.

However, several recent reports, including studies by our group, have indicated that, in contrast to rodent Pgp, human Pgp may not transport AEDs to any relevant extent (Schinkel et al., 1996; Mahar Doan et al., 2002; Crowe and Teoh, 2006; Baltes et al., 2007a,b). These recent reports have used conventional (bi-directional) transport assays with polarized intestinal (Caco-2) or kidney (MDCKII, LLC) cell lines expressing the human *multidrug resistance-1* (*MDR1*; *ABCB1*) gene that encodes Pgp. The transport assays were performed in a conventional manner with the Transwell® system that allows studying drug transport between an apical and basolateral compartment separated by a polarized cell monolayer on a polyester filter membrane, applying the AED to either the apical or basolateral chamber for studying bi-directional transport. However, because most AEDs are very lipophilic, passive transcellular diffusion could form a bias in such assays by concealing

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active transport. Thus, the conventional bi-directional transport assay may fail to identify highly permeable compounds as Pgp substrates, particularly if they are not high-affinity substrates for this efflux transporter (FDA, 2006). In line with this possibility, Robey et al. (2008) recently suggested that it may be that AEDs are Pgp substrates but are not so well transported that they can be detected by the model systems used in previous studies.

This prompted us to modify the transport assay in a way that allows evaluating active transport independently of the passive permeability component. For this purpose, we adapted a method recently described for measuring Pgp-mediated transport of highly permeable antibiotics in the Caco-2 model (Pachot et al., 2003). Instead of applying the drug to either the apical or basolateral chamber for studying bi-directional transport, the drug is initially added at identical concentration to both chambers, resulting in concentration equilibrium conditions (Pachot et al., 2003). This concentration equilibrium transport assay thus minimizes the problem of drug concentration gradients that is known to affect identification of highly permeable compounds as Pgp substrates. In the present study, the concentration equilibrium transport assay was used to determine whether major AEDs are transported by human Pgp, using kidney cell lines transfected with *MDR1*. The known high-affinity Pgp substrates vinblastine and digoxin were included in the study for comparison.

2. Materials and methods

2.1. Cell lines and cell cultures

LLC-PK1 cells transfected with human *MDR1* (LLC–MDR1) and respective wildtype (Wt) LLC cells were kindly provided by Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands). Some experiments were also performed with MDCK type II cells transfected with human *MDR1* (MDCKII–MDR1) and respective wildtype cells, which were also kindly provided by Prof. P. Borst. After obtaining the cells, they were cultured as described in detail recently (Baltes et al., 2007a,b). Cells were used within 10 passages or less after thawing from liquid nitrogen, and at a maximum of 13 passages after receiving them from Prof. Borst. Because transfected LLC cells may lose the transporter cDNA in the absence of a selection agent such as vincristine, they were regularly tested for vincristine resistance (640 nmol) before being used for transport experiments (for details see Baltes et al., 2007a).

2.2. Transcellular transport assays

Cells were seeded on transparent polyester membrane filters (Transwell-Clear®, 24 mm diameter, 0.4 μm pore size, Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.3×10^6 cells/cm^2 (LLC) or 0.4×10^6 cells/cm^2 (MDCKII), cultured for 1–2 days to confluence and used for transport assays between days 5 and 7 after confluence (for details see Baltes et al., 2007a,b). Transport studies were performed with the filter inserts in Transwell® multiwell culture plates that allow studying drug transport between an apical and basolateral compartment. For the present experiments, 6-well plates were used. Before starting the transport experiments, the medium was replaced with Opti-MEM® (Gibco™/Invitrogen Corporation, Eggenstein, Germany) and the transwells were pre-incubated for 1 h (with or without transport inhibitor, respectively; see below). This reduced serum medium was used without any additives according to the protocol of the laboratory that provided the cell lines (Prof. P. Borst) in order to minimize protein binding of the drugs. At the beginning of the experiment ($t = 0$), the pre-incubation medium was replaced by fresh Opti-MEM® containing the drug either in the apical or the basolateral chamber (donor chamber) or in both chambers, depending on the protocol (see below). The volumes in the upper and lower compartment were 2000 and 2700 μl, respectively. For drug analysis, samples were taken at 60, 120, 240, and 360 min (in some experiments also 480 and 600 min). The transport assays including pre-incubation were performed at 37 °C in a humidified incubator (5% CO_2) with shaking the transwells gently at 50 rpm. Monolayers were checked for integrity before and after each transport experiment (see below).

In a first series of experiments, two different protocols were compared:

1. Concentration gradient conditions, which were used to determine passive and active transport across cell monolayers either in the apical or basolateral direction;
2. Concentration equilibrium conditions, which were used to evaluate active transport independently of passive transport.

2.2.1. Concentration gradient conditions

In these experiments, bi-directional permeability studies were initiated by adding the drug to either the apical (for apical to basolateral transport, a–B) or the basolateral (for basolateral to apical, b–A) side of the monolayer. For drug analysis, 100 μl aliquots were collected from the receiver compartment (which contained medium with the same concentration of the drug solvent as the medium in the donor chamber). The volume of the donor chamber was adjusted after each sampling to avoid "transport" effects by hydrostatic pressure. Each experiment was performed in triplicate.

2.2.2. Concentration equilibrium conditions

In these experiments, transport study was initiated by adding the drug to both (apical and basolateral) sides of the monolayer, so that initial drug concentration was the same in both compartments. In experiments with transport inhibitors, the respective inhibitor was also added to both chambers. For drug analysis, aliquots were collected from both compartments over the course of an experiment (100 μl basolateral and 130 μl apical, in order to avoid influences by hydrostatic pressure). Each experiment was performed in triplicate and, except for vinblastine, repeated at least once. After we established that concentration equilibrium conditions were more sensitive to measure AED transport than concentration gradient conditions (see Section 3), all subsequent experiments were performed under concentration equilibrium conditions.

In all experiments, the integrity of the monolayers was checked by measuring transepithelial electrical resistance (TEER) of the polarized cells before and after each experiment. Only monolayers with a TEER of at least 100 Ω cm^2 were used for analysis of drug transport, as recommended in the FDA guidance for such studies (FDA, 2006). Experiments in which TEER values decreased by more than 15% compared to initial readings were discarded. In most experiments, [^{14}C]-mannitol was used as an additional integrity marker (in separate wells). As generally known from such monolayers (Flanagan et al., 2002; Taub et al., 2002; Baltes et al., 2004; FDA, 2006), high TEER values ($\geq$100 Ω cm^2) were correlated with low flux of mannitol, indicating absence of any relevant paracellular penetration as a result of tight junctions between adjacent cells. Less than 1% of mannitol diffusion per hour, and an apparent permeability (P_{app}) of [^{14}C]-mannitol lower than 12 nm/s were used as indicators of integrity of the monolayer. To check for functional Pgp in the apical membrane of cell monolayers, transport of the Pgp substrate digoxin was tested in separate wells in each experiment with AEDs. Experiments with no clear basolateral to apical (B/A) transport of digoxin were discarded.

In an additional series of experiments, we evaluated whether pre-incubation of LLC–MDR1 cells with the Pgp substrate vincristine enhances the transcellular transport of AEDs. Typically, transfected (Pgp-overexpressing) LLC cells are resistant to the cytotoxic vinca alkaloid vincristine (up to 640 nM), while LLC wildtype cells do not survive such treatment (Schinkel et al., 1995), so that treatment with vincristine can be used as a selection test to prove whether transfected LLC cells overexpress functional Pgp (Baltes et al., 2007a). Treatment with vincristine further induces *MDR1* expression (Brügger et al., 2002), which was the rationale to use this treatment in the present experiments. LLC–MDR1 cells were seeded on plates and selected with 640 nmol vincristine overnight, then cultured in vincristine-free medium up to 6 days after confluence. Finally, cells were treated with vincristine (320 nmol) 15 h before the transport assay. To determine whether this treatment enhanced the membrane expression of Pgp, immunofluorescent labeling of Pgp (using the monoclonal Pgp antibody C219) followed by confocal laser-scanning microscopy was used as described recently (Baltes et al., 2007a). For determining whether treatment with vincristine enhanced the functional activity of Pgp, a rhodamine-123 uptake assay was performed essentially as described elsewhere (Decorti et al., 2001; Yasuda et al., 2002; Perriere et al., 2007). Uptake of the fluorescent Pgp substrate rhodamine-123 was measured over 2 h (in the absence or presence of the Pgp inhibitor tariquidar) and was calculated as absolute fluorescence in the cell lysate per mg protein. Experiments were performed in triplicate.

2.3. Drugs

Levetiracetam was kindly provided by UCB Pharma (Brussels, Belgium), phenytoin and carbamazepine by Desitin (Hamburg, Germany), lamotrigine by GlaxoSmithKline (Stevenage, Herts, U.K.), and tariquidar (XR9576) by Xenova Ltd. (Slough, Berkshire, U.K.). Phenobarbital (as sodium salt) was obtained from Serva (Heidelberg, Germany), vinblastine sulphate and MK571 [(*E*)-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[(3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid, sodium salt] from Alexis Biochemicals (Axxora, Lörrach, Germany), and [^{3}H]-vinblastine sulphate (9.8 Ci/mmol) and [^{14}C]-mannitol (61.0 mCi/mmol) from Amersham (Buckinghamshire, U.K.). Digoxin was obtained from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany), and [^{3}H]-digoxin from PerkinElmer LAS GmbH (Rodgau-Jügesheim, Germany). Vincristine sulphate and rhodamine-123 were obtained from Sigma–Aldrich (Taufkirchen, Germany).

Phenytoin, lamotrigine and rhodamine-123 were dissolved in ethanol ($\leq$0.5% ethanol in final solution) and carbamazepine in methanol (0.1% methanol in final solution) and then diluted in the cell culture medium. Phenobarbital and levetiracetam were directly dissolved in medium. Vinblastine sulphate and vincristine sulphate were dissolved in purified water. Digoxin and tariquidar were dissolved in DMSO ($<$0.1% DMSO in final solution) and MK571 was dissolved in medium.

The choice of drug concentrations was based on therapeutic plasma concentrations of AEDs in epilepsy patients, previous transport studies with vinblastine, digoxin, tariquidar and MK571 (Smith et al., 1998; Baltes et al., 2007a,b) and FDA recommendations (FDA, 2006). The following concentrations were used: phenytoin, 5 and 50 μM; phenobarbital, 50 μM; levetiracetam, 120 and 210 μM; lamotrigine, 20 μM; carbamazepine, 30 μM. [^{3}H]-vinblastine sulphate (9.8 Ci/mmol) was diluted with unlabeled vinblastine sulphate to give an activity of 0.25 μCi/ml and a final concentration of 2 μM in the assay (for equilibrium conditions, concentration of [^{3}H]-vinblastine was 0.025 μCi/ml). Because vinblastine is a substrate for Pgp and multidrug resistance protein (MRP) 2 (Evers et al., 1998; Tang et al., 2002), the MRP inhibitor MK571 (50 μM) was included in all transport experiments with vinblastine. [^{3}H]-digoxin (40 Ci/mmol) was diluted with unlabeled digoxin to give an activity of 0.05 μCi/ml and a final concentration of 10 nM in the assay. For inhibition of Pgp, tariquidar was used at concentrations of 0.2 or 0.5 μM. For inhibition of MRPs, MK571 was used at 50 μM.

2.4. Drug analysis

Concentrations of AEDs were measured by high performance liquid chromatography (HPLC) with UV-detection as described earlier (Potschka and Löscher, 2001; Potschka et al., 2001, 2002, 2004). Limits of quantification were as follows: carbamazepine, 25 ng/ml; lamotrigine, 25 ng/ml; levetiracetam, 400 ng/ml; phenytoin, 40 ng/ml; and phenobarbital, 25 ng/ml, respectively. The radioactivity in samples from experiments with [^{3}H]vinblastine or [^{3}H]digoxin was quantified using a scintillation counter. Rodamine-123 was quantified using a Fluoroscan II (LabSystems® Oy, Helsinki, Finland) with 485 nm excitation and 538 nm emission filters.

2.5. Analysis of transport data

For experiments with concentration gradient conditions, the results of the individual transport assays are presented as the percentage of the initial drug concentration in the donor chamber vs. time. Apical-to-basal ($P_{app\ aB}$) and basal-to-apical ($P_{app\ bA}$) permeabilities were determined according to Artursson (1990) using the following equation:

$$P_{app}\ [nm/s] = (dQ/dt)/(A \times C_0 \times 60)$$

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. Transport ratio (TR) was calculated by dividing $P_{app\ bA}$ by $P_{app\ aB}$. Corrected transport ratios (cTR) were calculated by division of the TR obtained in *MDR1*-transfected cells by the TR obtained in the respective wildtype/parental cells (Schwab et al., 2003). A cTR of at least 1.5 is considered as indicator of active, asymmetrical transport (Schwab et al., 2003).

For experiments with concentration equilibrium conditions, the results of the individual transport assays are presented for each chamber as the percentage of the initial drug concentration vs. time. The statistical significance of differences between drug concentrations in the two chambers was calculated by two-way analysis of variance (ANOVA) for repeated measures, followed by Bonferroni post-tests. A representative example for an experiment under concentration equilibrium conditions is shown in Fig. 1. Note that the volumes of the apical and basolateral chambers are not identical: the volume of the apical chamber is 2.0 ml and that of the basolateral chamber 2.7 ml. Thus, even though the initial concentration (in μM or μg/ml) of drug in the two chambers is identical, the initial amount of drug is different in the two chambers, explaining that percent increase of drug concentration in the apical chamber is not identical to percent decrease in the basolateral chamber during the experiment. In other words, because the amount of drug is higher in the basolateral chamber (as a result of the larger volume of this chamber), basolateral to apical transport of drug will affect the relative (%) concentration of drug in the apical chamber more markedly than reflected by the decrease in relative (%) concentration in the basolateral chamber. To allow comparison of the magnitude of transport between drugs, the area under the drug concentration vs. time curves (AUC) in the apical chamber was calculated in percent above the initial concentration × time (see Fig. 1 for illustration), by using the trapezoidal rule. The idea behind this was that if a drug is transported actively by Pgp from the basolateral to the apical chamber, the amount of drug in the apical compartment should increase above the initial concentration (i.e., >100%), so that the AUC of the percent increase × time in the apical chamber should allow comparing the magnitude of transport between drugs (see Table 1). These calculations were carried out for *MDR1*-transfected cells as well as wildtype cells and experiments with Pgp or MRP inhibitors. Percent inhibition of transport by Pgp or MRP inhibitors was calculated by comparing AUCs in the presence or absence of inhibitors. We also performed all calculations with amount drug transported instead of relative (%) values, but this did not change the results of our study, so that all subsequent calculations were performed with relative (%) concentrations as illustrated in the figures and Table 1.

3. Results

3.1. Comparison of transport of vinblastine, digoxin, phenytoin and phenobarbital by Pgp in conventional vs. concentration equilibrium transport assays

Under concentration gradient conditions as commonly used in transcellular transport assays in Pgp-overexpressing cell lines, the prototype Pgp substrates vinblastine and digoxin exhibited directional (basolateral to apical) transport with cTRs of 3.01 and 3.41, respectively, in LLC–MDR1 cells (Fig. 2). With digoxin, directional transport was also observed in LLC wildtype cells, indicating transport by endogenous pig Pgp. Substantial basolateral to apical transport in wildtype cells was also determined with vinblastine (not illustrated), but because this drug is transported by both Pgp and MRP2, MK571 was included in all experiments with vinblastine, which prevented any vectorial transport in wildtype cells, but not LLC–MDR1 cells (Fig. 2).

In contrast to vinblastine and digoxin, the AEDs phenytoin and phenobarbital did not show any asymmetrical transport under concentration gradient conditions (Fig. 2). However, both AEDs were highly permeable, rapidly penetrating from the donor to the receiver compartment in both directions because of the concentration gradient conditions of the conventional assay. Comparison of data obtained for vinblastine and digoxin vs. phenytoin and phenobarbital (Fig. 2) indicated higher passive permeability of the AEDs in both wildtype and transfected cells, which was obviously a result of their high lipophilicity and, possibly, also their lower molecular size compared to the prototype Pgp substrates.

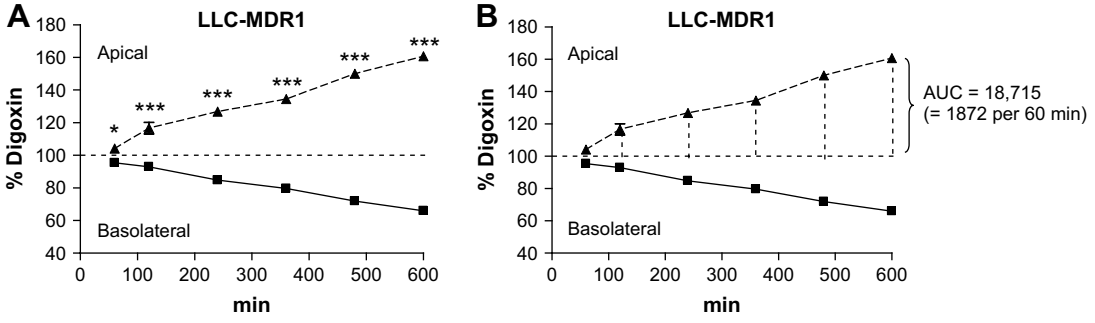

Fig. 1. Representative experiment with digoxin in LLC cells transfected with human *MDR1*, using the concentration equilibrium transport assay. In this assay, medium containing equal concentration of drug is added to both (apical and basolateral) sides of the monolayer, so that the initial drug concentration is the same in both compartments. If the drug is transported actively by Pgp, the amount of drug in the apical compartment should increase as the amount of drug in the basolateral compartment decreases (Smith et al., 1998), which is illustrated by the time course of digoxin in "A". Data are shown as the percentage of the initial drug concentration (=100%) in the apical and basolateral chamber vs. time. The triangles show values for the apical chamber and the squares respective data for the basolateral chamber. The experiment was performed in triplicate and values are shown as means ± SEM. When no error bar is visible, the deviation was within the size of the symbols. Significant differences between the two chambers are indicated by asterisk (*$P < 0.05$; ***$P < 0.001$). "B" illustrates how the area under the drug concentration vs. time curves (AUC) in the apical chamber was calculated in percent above the initial concentration × time (in min), by using the trapezoidal rule (see Section 2 for further details).

Table 1
Comparison of the magnitude of basolateral to apical transport of drugs in LLC–MDR1 cells under the conditions of the concentration equilibrium transport assay

Drug	AUC of transport from basolateral to apical chamber (% above initial concentration in apical chamber × time [in min] per 60 min)	Transport relative to digoxin (=1) in LLC–MDR1 cells	Percent inhibition of transport by tariquidar (%)	Percent inhibition of transport by MK571 (%)
Digoxin	1757 ± 281	1	92	43
Vinblastine	1674 ± 173	0.95	66	
Phenytoin	258 ± 36.7	0.15	86	
Phenobarbital	189 ± 13.6	0.11	54	89
Phenobarbital (in vincristine-pretreated cells)	294 ± 8.57*	0.17	45	95
Lamotrigine	173 ± 32.0	0.1	61	
Levetiracetam	162 ± 44.5	0.09	63	60
Carbamazepine	Not different from wildtype	0		

Transport was estimated by calculating the area under the drug concentration vs. time curves (AUC) in the apical chamber in percent above the initial concentration × time (in min) (for more details, see Section 2 and Fig. 1). Data are shown as means ± SEM. To allow comparing experiments with a duration of either 360 or 600 min, all data are expressed per 60 min. Each experiment was performed in triplicate and, except for vinblastine, repeated at least once (range 2–7 experiments per drug). Based on AUCs, transport was also calculated relative to the prototype Pgp substrate digoxin (=1). Furthermore, the average percent inhibition of transport by either tariquidar (0.2 or 0.5 μM) or, when tested, MK571 (50 μM) is shown for each drug. With phenobarbital, a transport experiment was also performed in *MDR1*-transfected MDCKII cells (see text), resulting in an AUC of 176 ± 17.6, i.e., a value very similar to that determined in LLC–MDR1 cells. Transport of phenobarbital in vincristine-pretreated LLC–MDR1 cells was significantly higher than in non-treated cells (*$P = 0.0028$).

In order to determine whether active transport can be detected when passive diffusion is minimized, concentration equilibrium conditions were chosen by adding the drug at identical concentration at both sides of the LLC–MDR1 monolayer. As a result of basolateral to apical transport by Pgp, concentrations of vinblastine and digoxin rapidly increased in the apical chamber, but decreased in the basolateral chamber under these conditions, which could be reduced (vinblastine) or prevented (digoxin) by the Pgp inhibitor tariquidar (Fig. 2). Under concentration equilibrium conditions, a significant basolateral to apical transport was also determined for phenytoin and phenobarbital, although the magnitude of this transport was below that of vinblastine or digoxin (Fig. 2). Such directional transport of phenytoin or phenobarbital was not observed in LLC wildtype cells and, in case of phenytoin, could be inhibited by tariquidar (for experiments with tariquidar and phenobarbital see below).

In order to check whether the AED transport observed in these experiments is cell specific, we repeated the experiments with phenobarbital in *MDR1*-transfected MDCKII cells (not illustrated). Comparable to the experiments with LLC cells illustrated in Fig. 2, no directional transport of phenobarbital was determined under concentration gradient conditions in MDCK wildtype cells (TR 0.98) or MDCK–MDR1 cells (TR 1.02), but significant directional transport was observed in MDCK–MDR1 cells under concentration equilibrium conditions, thus substantiating the data obtained with LLC–MDR1 cells.

In all experiments illustrated in Fig. 2, assays were run over 6 h and drug analyses were restricted to few time points (i.e., 1, 2, 4 and 6 h) during this period. We therefore evaluated whether limiting the study to 2 h and adding more time points enhances the sensitivity of the normal bi-directional assay to detect Pgp-mediated transport of phenytoin. Respective data with LLC cells are shown in Fig. 3. Results were very similar to those shown in Fig. 2 in that no clear asymmetrical transport of phenytoin was seen under concentration gradient conditions, whereas significant transport was determined in the concentration equilibrium assay. However, comparing transport data from the 6-h (Fig. 2) vs. 2-h (Fig. 3) experiments also demonstrate that prolonging the duration of the experiment enhances the sensitivity of the equilibrium assay to identify asymmetrical transport, because in the 2-h experiment significant differences between the two chambers were not observed before 100 min. This was also observed with digoxin (not illustrated; see also Fig. 4). Based on these results, all further experiments with AEDs were therefore performed with LLC cells under concentration equilibrium conditions and long study duration.

3.2. Pgp-mediated transport of AEDs determined by concentration equilibrium transport assay

In these subsequent experiments, transport was studied over 600 min instead of 120 or 360 min to enhance the sensitivity of the assay for identifying whether an AED is a Pgp substrate. As shown in Fig. 4, in LLC–MDR1 cells, the amount of digoxin in the apical compartment progressively increased while the amount of digoxin in the basolateral continuously decreased over 600 min of the experiment, indicating that digoxin was transported actively by Pgp. Transport could be completely inhibited by tariquidar (Fig. 4), independently of whether the inhibitor was used at 0.2 or 0.5 μM.

Significant, albeit less marked transport from the basolateral to the apical compartment was also determined for phenytoin (50 μM) in LLC–MDR1 cells but not in wildtype cells (Fig. 4). Transport could be inhibited by tariquidar. As already seen in the experiments with 360 min of study duration (Fig. 2), transport of phenytoin reached a plateau after 120 min, which could result from saturation of transport at the relatively high drug concentration used. Alternatively, this plateau could be a result of rediffusion of drug from the apical to basolateral compartment because of the concentration gradient resulting from asymmetrical transport. For clarification, we repeated the experiment with a 10-fold lower concentration of phenytoin (5 μM). As shown in Fig. 4, significant vectorial transport was determined, which could be completely inhibited by tariquidar. In contrast to the data with 50 μM, no plateau was observed with 5 μM of phenytoin, but the amount of phenytoin in the apical compartment progressively increased over the 600 min of the experiment.

Directional transport was also observed with phenobarbital, which was reduced but not completely antagonized by tariquidar. Average inhibition of transport by tariquidar in two experiments was 54%. In order to investigate whether induction of Pgp expression enhances the transport of phenobarbital by LLC–MDR1 cells, cells were pretreated with vincristine. This pretreatment enhanced directional transport of phenobarbital, but, again this transport could only partially be reduced by tariquidar (Fig. 4). In contrast, transport was completely inhibited by MK571 (50 μM), indicating that efflux transporters other than Pgp contributed to the observed transport of phenobarbital.

For substantiating the effect of vincristine treatment, we also studied accumulation of rhodamine-123 in vincristine-pretreated LLC–MDR1 cells (Fig. 4). Rhodamine-123 accumulation was significantly reduced by 33% by this pretreatment. In line with increased transporter expression at the apical cell membrane, TEER values of vincristine-pretreated cells were higher than those of

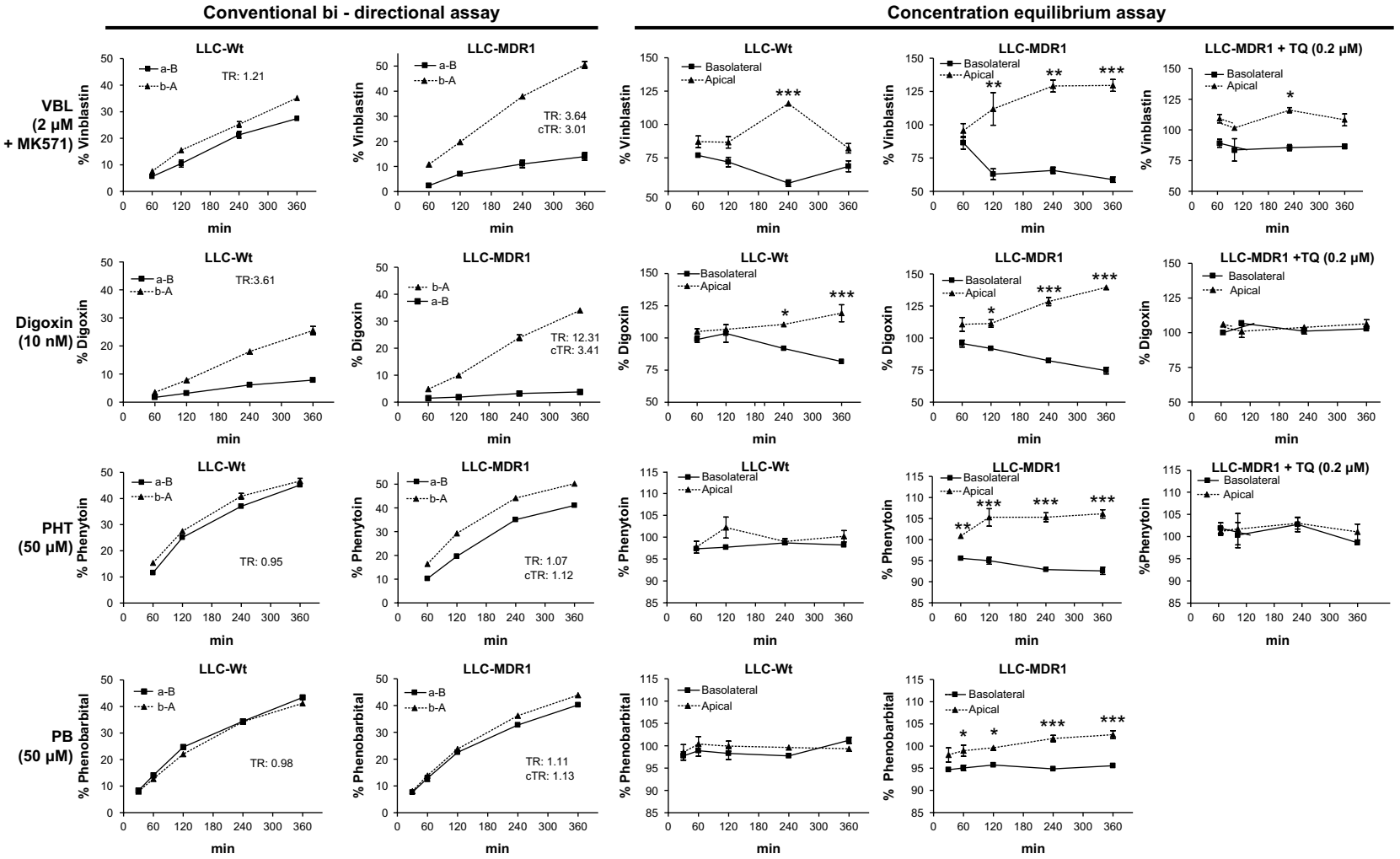

Fig. 2. Representative experiments with vinblastine (VBL), digoxin, phenytoin (PHT), and phenobarbital (PB) in LLC cells transfected with human *MDR1* and wildtype (Wt) LLC cells. Two types of assays are compared: the conventional bi-directional (concentration gradient) assay, in which the drug is added to either the apical (for apical to basolateral transport, a–B) or the basolateral (for basolateral to apical, b–A) side of the monolayer, and the concentration equilibrium assay, in which the drug is added to both (apical and basolateral) sides of the monolayer, so that the initial drug concentration is the same in both compartments. For the conventional assay, data are given as the percentage of the initial drug concentration in the donor chamber vs. time. For each experiment, transport ratios (TR) are given (for calculation, see Section 2). For LLC–MDR1 cells, transport ratios (cTR) were calculated by dividing the TR obtained in *MDR1*-transfected cells by the TR obtained in wildtype/parental cells. A cTR of $\geq$1.5 is considered as indicator of active, asymmetrical transport (Schwab et al., 2003). This criterion was obtained for vinblastine and digoxin, but not phenytoin and phenobarbital. For the concentration equilibrium assay, data are given as the percentage of the initial drug concentration (=100%) in either the apical or basolateral chamber vs. time. Significant differences between the two chambers are indicated by asterisk ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). In all experiments, except the experiment with phenobarbital (but see Fig. 4), it was tested whether directional transport can be inhibited with the selective Pgp inhibitor tariquidar (TQ). For each drug, the conventional assay and the concentration equilibrium assay were performed in parallel with the same batch of cells. In the experiment with vinblastine, both wildtype and MDR1-transfected cells contained 50 µM MK571 to inhibit transport by MRPs. All experiments were performed in triplicate and values are shown as means ± SEM. When no error bar is visible, the deviation was within the size of the symbols.

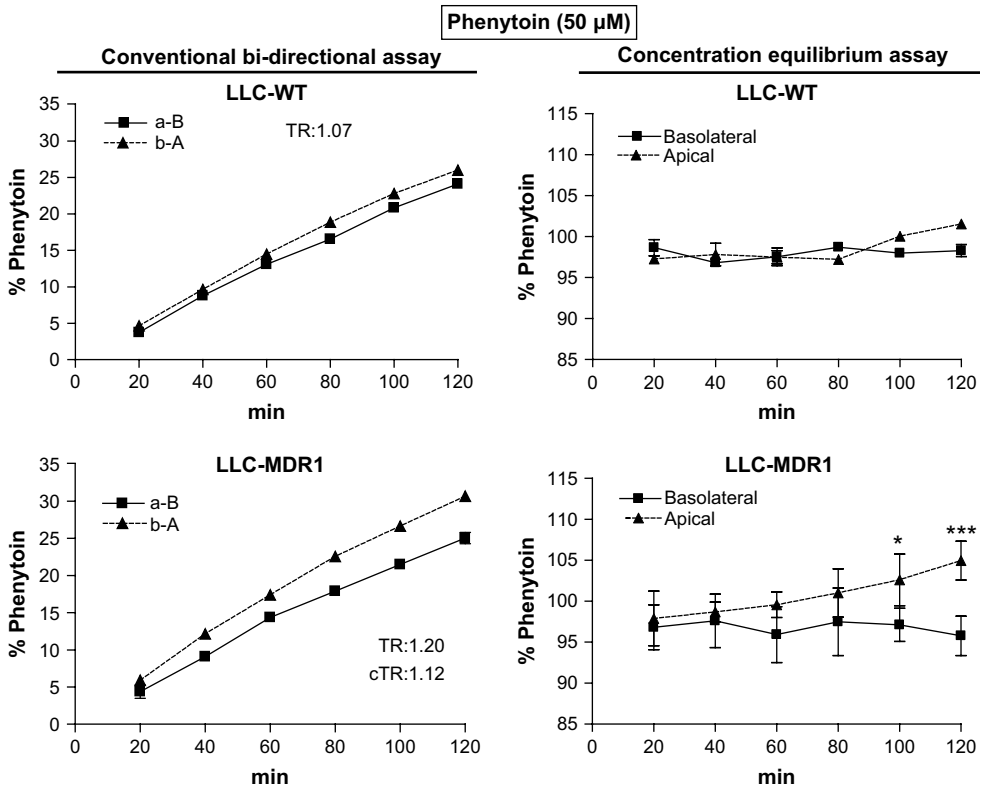

Fig. 3. Comparison of transport of phenytoin in the conventional bi-directional (concentration gradient) assay and the concentration equilibrium assay. Compared to the experiments shown in Fig. 3, study duration was reduced from 6 to 2 h and more time points were added. For further details, see Fig. 2 legend.

non-pretreated cells (not illustrated). Furthermore, laser-scanning confocal microscopy of immunofluorescently stained preparation of LLC–MDR1 cells substantiated that vincristine-pretreated cells exhibited increased expression of Pgp in the apical membrane (Fig. 4).

The fact that MK571 at the concentration (50 μM) used in our experiments almost completely inhibited vectorial transport of phenobarbital, whereas only partial inhibition was obtained with tariquidar (Fig. 4) indicates that MK571 not only inhibited MRPs but also, at least in part, Pgp. This possibility was directly addressed by testing the effect of MK571 on vectorial transport of digoxin. As shown in Fig. 4, MK571 partially inhibited transport of digoxin in LLC–MDR1 cells, suggesting an inhibitory effect of MK571 on Pgp at this concentration.

In addition to phenytoin and phenobarbital, significant basolateral to apical transport in LLC–MDR1 cells was also determined for lamotrigine (Fig. 5). Such transport was not observed in wildtype cells and could be inhibited by tariquidar in *MDR1*-transfected cells. In contrast, directional transport was not determined for carbamazepine, including experiments in which LLC–MDR1 cells were pretreated with vincristine (Fig. 5). Although some small but statistically significant differences between apical and lateral chambers were found (Fig. 5), these were not affected by treatment with tariquidar and were also observed in wildtype cells (not illustrated), indicating absence of any *MDR1*-mediated transport of carbamazepine under the conditions of the assay. Similar to phenytoin (Fig. 3), we also performed an experiment with carbamazepine, in which study duration was reduced to 2 h and additional time points were added, again without observing any significant asymmetrical transport in either the conventional (bi-directional) or modified (equilibrium) assays (not illustrated). In contrast, significant basolateral to apical transport was determined for levetiracetam, which, similar to phenobarbital, could only partially be inhibited by tariquidar (Fig. 5). Average inhibition of transport by tariquidar in two experiments was 63%. When the experiment with levetiracetam was repeated in the absence or presence of MK571, transport was inhibited by MK571 (Table 1), resembling the experiment with phenobarbital (Fig. 4) and indicating that efflux transporters other than Pgp contributed to the observed transport of levetiracetam. Transport of levetiracetam was examined at two different concentrations (120 and 210 μM) without indication that transport efficiency was affected by drug concentration.

To allow a comparison of the magnitude of directional transport among drugs examined in this study in the concentration equilibrium assay with LLC–MDR1 cells, the AUC in the apical chamber was calculated in percent above the initial concentration × time for all experiments (see Section 2 and Fig. 1 for details). As shown in Table 1, similar high AUCs were determined for digoxin and vinblastine, while 7–10 times lower AUCs were calculated for AEDs under the conditions of the equilibrium assay. Data shown in Table 1 are based on at least two (range 2–7) experiments per drug (each experiment performed in triplicate), so that the average AUCs may differ from the individual experiments shown in Figs. 1–4. Among AEDs, phenytoin showed the most pronounced directional transport, followed by phenobarbital, lamotrigine and levetiracetam, while carbamazepine was not actively transported under the conditions of the assay. Because transport of phenobarbital had been determined in both LLC–MDR1 and MDCKII–MDR1 cells (see above), the basolateral to apical transport of this AED could be compared by calculation of AUCs in both cell lines, resulting in almost identical values (189 ± 13.6 in LLC–MDR1 vs. 176 ± 17.6 in MDCKII–MDR1). Pretreatment of LLC–MDR1 cells with vincristine significantly enhanced transport of phenobarbital by 56% on average (Table 1). In addition to comparative data on drug transport, percent inhibition of drug transport by tariquidar or MK571 is shown in Table 1.

In many of the concentration equilibrium transport assays, baseline concentrations were below 100% on both sides (Figs. 2–4).

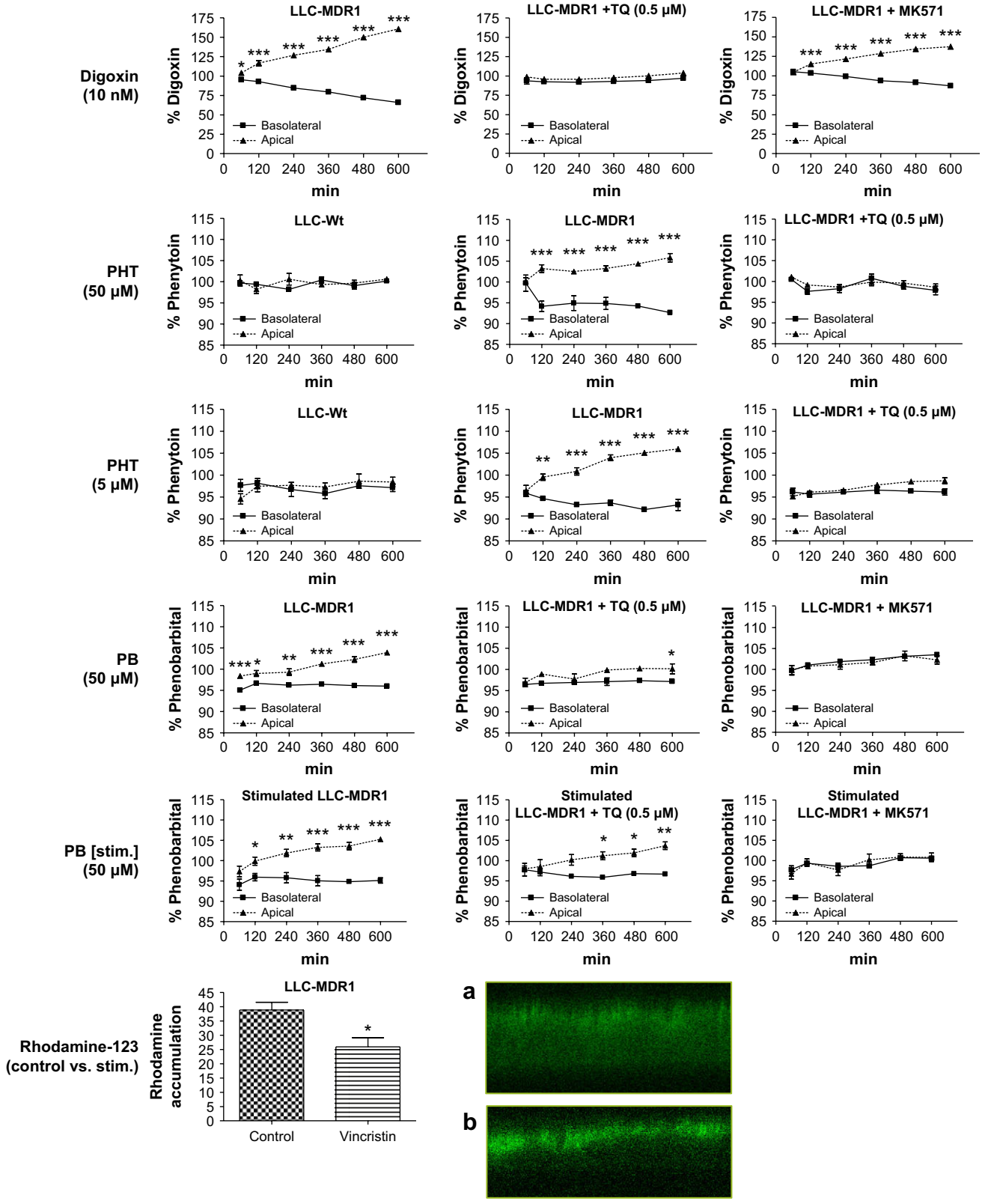

Fig. 4. Representative experiments with digoxin, phenytoin (PHT), and phenobarbital (PB) in LLC cells transfected with human *MDR1*, using the concentration equilibrium assay. Compared to the experiments shown in Fig. 2, study duration was increased from 6 to 10 h and more time points were added. For further details, see Fig. 2 legend. Since data in wildtype cells did not differ from those illustrated in Fig. 2, they are not shown for all experiments. For inhibition of basolateral to apical transport, either tariquidar (TQ; 0.5 μM) or MK571 (50 μM) was used. Phenobarbital was also tested in LLC cells that had been pretreated with vincristine (stim.) to enhance the apical expression and function of Pgp, which resulted in increased basolateral to apical transport of phenobarbital. In addition to testing phenobarbital in vincristine-pretreated cells, the effect of vincristine on Pgp function is also illustrated by rhodamine-123 accumulation in the lower bar diagram, showing significantly ($P < 0.05$) less rhodamine-123 uptake in the vincristine-pretreated LLC–MDR1 cells vs. non-pretreated cells (control). Tariquidar significantly enhanced uptake of rhodamine-123 in this experiment (not illustrated). Furthermore, enhanced apical membrane expression of Pgp was confirmed by confocal laser-scanning microscopy in LLC–MDR1 cells: the representative z-scans show apical membrane expression of Pgp in LLC–MDR1 control cells (a) and after pretreatment with vincristine (b).

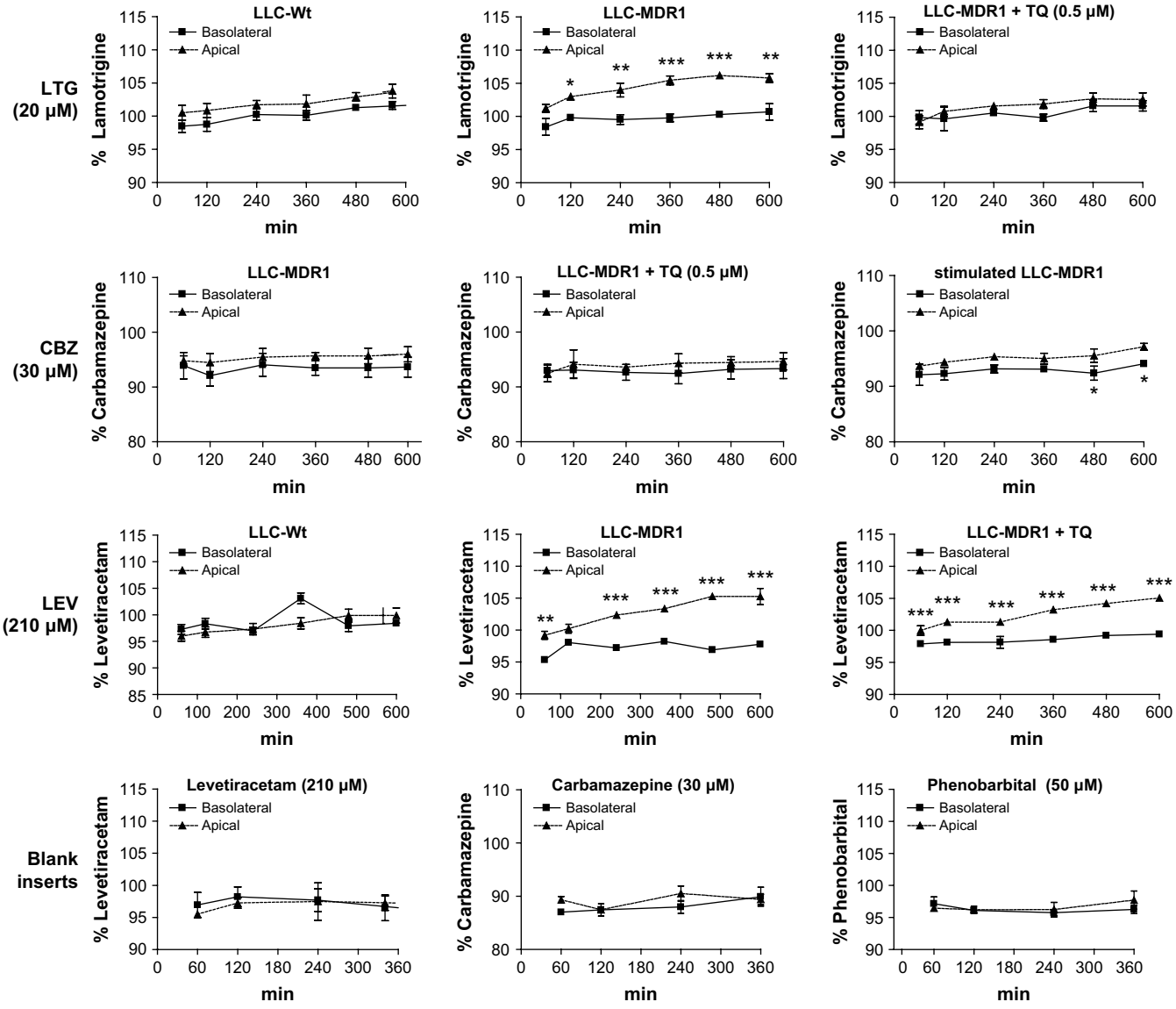

Fig. 5. Representative experiments with lamotrigine (LTG), carbamazepine (CBZ) and levetiracetam (LEV) in LLC cells transfected with human *MDR1*, using the concentration equilibrium assay (for further details, see Fig. 2 legend). Since no directional transport was observed in wildtype cells, data from wildtype cells are not illustrated for all drugs. For inhibition of Pgp-mediated basolateral to apical transport, tariquidar (TQ; 0.5 µM) was used. Carbamazepine was also tested in LLC cells that had been pretreated with vincristine (stimulated LLC–MDR1) to enhance the apical expression and function of Pgp (see also Fig. 2). In many of the transport assays, including those illustrated in Figs. 2–4, baseline concentrations of drugs were below 100% on both sides, which suggest adsorption issues. Therefore, all drug experiments were repeated with blank polyester membrane filter inserts. Three representative examples of such experiments are shown in the last row of the figure.

This is most obvious in Fig. 5, where concentrations of carbamazepine are no greater than 95% of the original concentration throughout the duration of the experiment. The most likely explanation for this finding is adsorption to components of the Transwell system. We therefore performed concentration equilibrium transport assays over 360 min with blank polyester membrane filter inserts for all drugs used in this study. Except digoxin, adsorption was observed for all drugs without any significant differences between basolateral and apical compartments (see representative examples in Fig. 5). Furthermore, the amount of adsorption did not change over the duration of the experiment, so that it can be excluded that this adsorption affected the transport studies with cell monolayers.

4. Discussion

Our recent study (Baltes et al., 2007a) indicating species differences in the Pgp-mediated transport of AEDs with significant transport of phenytoin and levetiracetam by mouse but not human Pgp in transfected LLC cells has cast serious doubt on the hypothesis that overexpression of multidrug transporters such as Pgp may mediate resistance to AEDs in patients with epilepsy (Löscher and Sills, 2007). The transporter hypothesis of AED-resistant epilepsy is fundamentally supported by four key observations: (1) Pgp (and MRPs such as MRP1 and MRP2) are overexpressed in epileptogenic brain regions of patients with AED-resistant epilepsy and in AED-resistant epileptic rats in models of temporal lobe epilepsy (Kwan and Brodie, 2005; Löscher and Potschka, 2005a,b); (2) in rodent models, the seizure-induced upregulation of Pgp is associated with a brain region-specific reduction in phenytoin levels (Rizzi et al., 2002; van Vliet et al., 2007) and such inverse relationship between the expression of Pgp and brain drug concentrations has also been reported for the primary active metabolite of oxcarbazepine in epilepsy patients (Marchi et al., 2005); (3) recent studies in rodents appear to suggest that most of the commonly used AEDs can be transported to some extent by Pgp and/or MRPs (Löscher and

Potschka, 2005a,b); and (4) inhibition of Pgp counteracts resistance to phenytoin and phenobarbital in rat models of epilepsy (Brandt et al., 2006; van Vliet et al., 2006). Thus, it is reasonable to conclude that Pgp plays a significant role in mediating resistance to AEDs in rodent models of temporal lobe epilepsy and that inhibition of Pgp can circumvent this mechanism, but whether this phenomenon extends to the human species remains unclear (Sills, 2007; Löscher and Sills, 2007). The present data are therefore important, because they are the first direct evidence that human Pgp transports several major AEDs.

It was clear from the beginning that AEDs cannot be high-affinity Pgp substrates such as digoxin, vinblastine or loperamide, because AEDs easily penetrate through the BBB at physiological expression of Pgp to reach their targets (Löscher and Potschka, 2005b). With some exceptions, this transcellular penetration of AEDs is governed by passive diffusion, because most AEDs are small, highly lipophilic molecules (Löscher and Frey, 1984; Löscher and Potschka, 2005b). Passive diffusion is defined by the movement of drug across a biological membrane in a manner solely driven by the concentration gradient, i.e., the drug moves from a region of greater concentration to a region of lesser concentration, e.g., from blood to brain. Thus, *in vitro* models used for identifying whether a drug is a Pgp substrate may fail to identify highly permeable compounds, when the model involves concentration gradient conditions, which is the case for all conventional *in vitro* assays used in this respect (FDA, 2006). This prompted us to use a modified transport assay for the present experiments, based on the fact that passive diffusion of a drug across a membrane is in equilibrium when drug concentration on both sides of the biological membrane is equal, thus allowing to identify if some form of carrier mediated transport is evident. To our knowledge, the concentration equilibrium transport assay (CETA) has been first described for measuring Pgp-mediated transport of PSC833 (valspodar) in LLC–MDR1 cells by Schinkel and Borst (Smith et al., 1998) and subsequently for measuring Pgp-mediated transport of highly permeable antibiotics in the Caco-2 model (Pachot et al., 2003).

The concentration equilibrium transport assay has a number of advantages. (1) Because drug concentrations are the same at both sides of the monolayer at the beginning of the transport experiment, the chance that directional transport is concealed by passive diffusion is reduced, thus enhancing the sensitivity of the assay to identify Pgp substrates. Thus, this assay is now used for new chemical entities by several pharmaceutical companies to avoid that Pgp substrates are missed by conventional, bi-directional transport assays. (2) The assay uses only ½ the number of transwells compared to the conventional bi-directional assay. (3) Another advantage when working with drug concentrations close to detection limits is that instead of analysing transport by looking for very small concentrations on the receiver side, one can look at higher concentrations with each HPLC measurement on both sides. As a consequence, equilibrium transport methods allow to measure changes at low initial concentrations based on detection methodology.

The key advantage of the concentration equilibrium transport assay is demonstrated by the direct comparison of conventional and modified assay for phenytoin and phenobarbital in the present study, in which significant transport by human Pgp was identified only by the concentration equilibrium transport assay. The latter assay also identified Pgp-mediated transport for lamotrigine and levetiracetam, but not carbamazepine. Thus, our data substantiate the recent suggestion of Robey et al. (2008) that AEDs may be Pgp substrates but that their low transport efficiency impedes detection of transport by conventional model systems.

Drugs that are transported by Pgp may inhibit transport of other Pgp substrates if they share the same drug-binding site on Pgp for which they can compete (Martin et al., 2000; Kimura et al., 2007; Yasuhisa et al., 2007). Weiss et al. (2003) demonstrated that phenytoin and lamotrigine at concentrations >100 μM significantly increased intracellular accumulation of the Pgp substrate bodipy-verapamil in LLC–MDR1 cells, indicating that these AEDs acted as competitive inhibitors, which can be explained by the present finding showing that both AEDs are weak substrates of human Pgp. However, Weiss et al. (2003) also reported that high concentrations (250 or 500 μM) of carbamazepine inhibited human Pgp, whereas we did not observe any specific transport of this AED in LLC–MDR1 cells. This may indicate that carbamazepine is a weak Pgp inhibitor that is not transported by Pgp. The findings of Weiss et al. (2003) were recently substantiated and extended by Hung et al. (2008), showing that phenytoin, carbamazepine, lamotrigine and phenobarbital (but not levetiracetam, valproic acid and gabapentin) inhibit transport of Pgp substrates in MDR1-transfected cells at high concentrations (100 or 250 μM) and that polymorphisms in MDR1 alter the inhibitory potency of AEDs. However, in a recent study with the Pgp substrate cyclosporin A in LLC–MDR1 cells, we did not determine any inhibitory effect of high concentrations of carbamazepine (224 μM) on Pgp (Baltes et al., 2007a). Lack of Pgp-inhibition by carbamazepine was also reported by Mahar Doan et al. (2002) in *MDR1*-transfected MDCKII cells and by Owen et al. (2001) in human lymphocytes. Thus, these findings seem to argue against any relevant inhibitory effect of carbamazepine on human Pgp. The fact that this AED has been shown to induce Pgp in human lymphocytes *in vitro* (Owen et al., 2006) and in the human intestinal tract *in vivo* (Giessmann et al., 2004; but see Magnusson et al., 2007) does not allow to conclude that carbamazepine directly interacts with Pgp, because induction of Pgp expression by carbamazepine and various other drugs is mediated via the nuclear pregnane X receptor (PXR; NR1I2), an important component of the body's adaptive defense mechanism against potentially toxic substances including xenobiotics (Xu et al., 2005; Löscher and Potschka, 2005a,b; Oscarson et al., 2006; Miller et al., 2008). Overall, current evidence suggests that resistance to carbamazepine is a result of brain target alterations (i.e., in voltage-dependent sodium channels) rather than overexpression of Pgp (Remy and Beck, 2006). In this respect it is important to note that target and transporter alterations may occur together in the same patient, resulting in resistance to a large variety of AEDs that act by different mechanisms (Schmidt and Löscher, 2005; Remy and Beck, 2006).

Substantiating transport of phenytoin and lamotrigine by human Pgp under the conditions of our study, the selective Pgp inhibitor, tariquidar, prevented any directional (basolateral to apical) transport of these AEDs, whereas tariquidar only partially inhibited transport of levetiracetam and phenobarbital. Inhibition of transport of phenobarbital by tariquidar was 54% on average, thus meeting the criterion of the FDA (2006) that reduction of transport of more than 50% by a known Pgp inhibitor allows the conclusion that the drug is a Pgp substrate. Similarly, average inhibition of transport of levetiracetam by tariquidar was >50%. Active transport of phenobarbital and levetiracetam could also be inhibited by MK571, indicating that MRPs are involved in transport of these AEDs. Induction of transporter expression and functionality by vincristine increased basolateral to apical transport of phenobarbital, which could be completely counteracted by MK571. Vincristine is transported by Pgp, MRP1, MRP2, and MRP3 and exposure to this chemotherapeutic drug induces the expression of these transporters in cell lines (Borst et al., 2000; Brügger et al., 2002; Huang et al., 2006). Yang and Liu (2008) have recently reported transport of phenobarbital in cultured rat brain microvascular endothelial cells, which could be partially blocked by both Pgp and MRP inhibitors, thus also suggesting that both Pgp and MRPs are involved in vectorial transport of phenobarbital in polarized cell lines. The apparent paradox in the present study that the preferential MRP inhibitor MK571 was more potent to inhibit

transport of phenobarbital and levetiracetam in LLC–MDR1 cells than the selective Pgp inhibitor tariquidar can most likely be explained by assuming that, at the concentration (50 μM) used, MK571 also partially inhibited Pgp, which is substantiated by the experiment with digoxin and MK571.

In addition to inhibiting Pgp, tariquidar inhibits breast cancer resistance protein (BCRP; ABCG2), which exhibits a similar tissue distribution as Pgp and transports various kinds of endogenous and exogenous compounds (Schinkel and Jonker, 2003). However, while tariquidar blocks Pgp at nanomolar concentrations, micromolar concentrations are needed to inhibit BCRP, indicating a much higher affinity for Pgp than for BCRP (Mistry et al., 2001; Robey et al., 2004). Thus, it is unlikely that inhibition of BCRP contributed to the effects of tariquidar at the inhibitor concentrations chosen in our experiments. Furthermore, LLC cells do not express any measurable BCRP (Takada et al., 2005), and significant transport of AEDs was only determined in MDR1-transfected LLC cells but not in LLC wildtype cells, further arguing against any role of BCRP in the present results.

Although the concentration equilibrium transport assay is increasingly being used by pharmaceutical companies, because it recognizes Pgp substrates likewise well for lowly and highly permeable compounds, only few studies that used this transport assay have been published as yet (Smith et al., 1998; Pachot et al., 2003). In the present study, we observed a difference between the conventional (bi-directional) and modified (equilibrium) assays that has not been reported previously. Thus, as generally known, in the bi-directional assay with Pgp-expressing polarized cells, a Pgp substrate is continuously transported from the basolateral to the apical compartment, starting immediately after onset of the experiment. In apparent contrast, in the concentration equilibrium assay no asymmetrical transport of Pgp substrates, such as digoxin or phenytoin, was observed over the first 60 min, but significant transport was only determined thereafter. We have no clear explanation for this observation. Of course, in the conventional, concentration gradient assay, both passive diffusion and active transport together will increase drug concentration in the apical chamber, whereas passive diffusion cannot increase drug concentration under equilibrium conditions in the apical chamber. Furthermore, adsorption to the components of the Transwell system as observed in the present study may delay onset of measurable active transport under equilibrium conditions. The interesting difference in onset of asymmetrical transport between the two assay conditions needs further evaluation. However, when using the concentration equilibrium assay with long study duration (6 h or more) as done in most of the present experiments, the initial delay in onset of active transport does not form any bias in identifying Pgp substrates.

Compared to prototype Pgp substrates such as digoxin or vinblastine, the Pgp-mediated transport determined for AEDs appeared weak. However, it has to be considered that even under the conditions of the modified transport assay used in this study, passive diffusion will affect the transport of highly permeable drugs. First, the directional transport from the basolateral to the apical compartment, as observed for several AEDs in the equilibrium assay, leads to a concentration gradient between the two compartments and thereby rediffusion from the apical to the basolateral chamber, thus reducing the amount of drug in the apical compartment. Such rediffusion is a likely explanation for the observation that AEDs such as phenytoin typically reached a plateau in the apical chamber once a concentration gradient was obtained by directional transport from the basolateral to the apical compartment (see for instance Fig. 2). As a consequence, directional transport by Pgp will be underestimated. However, as indicated by the experiment with a lower concentration (5 μM) of phenytoin, at least part of the plateau observed at a 10-fold higher concentration was based on saturation of transport, because no such plateau was observed at 5 μM. Saturation of asymmetrical transport would favor rediffusion from the apical to the basolateral chamber, so that transport should be preferably examined at low, non-saturating drug concentrations. Second, *in vitro* assays are performed over a limited period of time, usually 4–6 h, whereas under *in vivo* conditions, patients are treated with AEDs on a chronic basis, so that AED efflux by Pgp from brain to blood will occur more or less continuously. Third, *in vivo* endothelial cells are continuously exposed to shear stress generated by the flow of blood across their apical surface, whereas *in vitro* transport assays using the Transwell system are static models lacking shear stress that affects the transport of drugs across biological membranes *in vivo* (Santaguida et al., 2006). Thus, data from *in vitro* transport assays as used in the present study can be used as a proof-of-concept to determine whether a drug is a substrate of human Pgp, but only *in vivo* experiments can prove whether this transport affects therapeutic brain concentrations and thus the clinical response to this drug.

Janigro and coworkers have recently presented a new dynamic *in vitro* BBB model that recapitulates several of the functional and structural properties of the BBB, including shear stress (Santaguida et al., 2006; Cucullo et al., 2007). By using cocultures of human microvascular endothelial cells and astrocytes from normal or drug-resistant epileptic brain tissue, they found that the permeability of the *in vitro* BBB to phenytoin was 10-fold less when using cells from drug-resistant patients (Cucullo et al., 2007). A 3.5-fold increase in phenytoin permeability was observed in epileptic *in vitro* BBB pretreated with tariquidar, indicating that Pgp was involved in the reduced permeability of endothelial cells from epileptic patients. Because tariquidar had no effect on the permeability of phenytoin in normal endothelial cells that express physiological levels of Pgp, it appeared that measurable drug extrusion only occurred at overexpressed Pgp (Cucullo et al., 2007). This is substantiated by the present data with wildtype and MDR1-transfected LLC cells, showing significant directional transport of phenytoin only in the Pgp-overexpressing cells. Thus, the present model using *MDR1*-transfected kidney cells leads to similar conclusions than the model with human brain endothelial cells described by Cucullo et al. (2007), substantiating *MDR1*-transfected LLC or MDCK cells as a substitute for more labor-intensive *in vitro* or *in vivo* models of the BBB (Mahar Doan et al., 2002; Garberg et al., 2005; Bachmeier et al., 2006). Furthermore, our data suggest that, in addition to phenytoin, several other AEDs are transported by human Pgp, so that overexpression of Pgp as previously reported for epileptogenic tissue of patients with refractory epilepsy is likely to reduce AED concentrations in this tissue. The only method by which this likely possibility can be directly addressed *in vivo* is positron emission tomography (PET), using ^{11}C-labeled AEDs such as phenytoin (Baron et al., 1983; Langer et al., 2007). Such experiments are planned in patients with AED-resistant and responsive epilepsy.

In conclusion, by using a modified version of the widely used Transwell assay with *MDR1*-transfected LLC cells, the present experiments provide the first direct evidence that several AEDs are substrates of human Pgp. This assay can now be used to evaluate which other AEDs are transported by human Pgp. Furthermore, with the same assay, it is also possible to use *MRP1*- or *MRP2*-transfected LLC or MDCKII cells to identify which AEDs are transported by these efflux pumps, which are overexpressed in brain tissue of AED-resistant epilepsy patients (Kwan and Brodie, 2005). The present data with phenobarbital and levetiracetam suggest that both Pgp and MRPs act in concert to mediate basolateral to apical transport of some AEDs, but this has to be substantiated by cells that overexpress human MRPs. In addition to being transported by Pgp or MRPs, AEDs may induce such efflux transporters as recently demonstrated in human intestinal cells (Giessmann et al., 2004), human lymphocytes (Owen et al., 2006), human tumor cell lines (Schuetz et al., 1996; Eyal et al., 2006; Le Vee et al., 2006),

rat hepatocytes (Eyal et al., 2006) and rat astrocytes (Lü et al., 2004). We currently study whether exposure to AEDs increases the expression or functionality of Pgp in brain endothelial cells, which may restrict their penetration into the brain and add to the consequences of seizure-induced upregulation of Pgp and other drug efflux transporters at the BBB. However, one has to keep in mind that AEDs control seizures in about 70% of patients with epilepsy, so that any clinically relevant interactions between AEDs and Pgp are possibly restricted to AED-resistant patients. In this respect, it is important to note that polymorphisms in the human MDR1 gene significantly alter interactions between AEDs and Pgp (Hung et al., 2008), which should be dealt with in future studies on the transporter hypothesis of AED-resistant epilepsy.

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Chapter 3

The antiepileptic drug topiramate is a substrate for human P-glycoprotein

Chapter 3: The antiepileptic drug topiramate is a substrate for human P-glycoprotein

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3.1. Abstract

About one third of epileptic patients do not respond to common treatment with antiepileptic drugs, a condition termed pharmacoresistance that significantly impairs the quality of life of these patients. A plausible explanation to this phenomenon is the multidrug transporter hypothesis. It is based on the finding that several multidrug transporters (MDT), such as P-glycoprotein (Pgp) and multidrug resistance associated proteins (MRPs), are up-regulated at the luminal side of brain microvessels in pharmacoresistant epileptic patients, and in animal models of temporal lobe epilepsy. In the present study we used LLC-PK1 transfectants that over-express human Pgp in order to investigate whether topiramate is a substrate for this transporter. Using the concentration-equilibrium transport assay we found that topiramate is transported in transfectants, the transport is inhibited with tariquidar, and no effect was observed in parental cell lines. A comparison of data previously obtained and described in our laboratory with other antiepileptic drugs shows that the human Pgp-mediated transport of topiramate has the highest magnitude, followed by phenytoin, lamotrigine, phenobarbital and levetiracetam. In conclusion, our data demonstrates that topiramate is a substrate for human Pgp *in vitro*.

3.2. Introduction

In spite of the high availability of drugs with different pharmacokinetics to treat epilepsy, with 24 molecules approved in USA and Europe (Schmidt, 2009), about one third of epileptic patients are pharmacoresistant (Schmidt & Löscher, 2005). The mechanisms underlying this pharmacoresistance are only incompletely understood but may involve both pharmacodynamic and pharmacokinetic factors (Schmidt and Löscher, 2005). Alterations in several targets of antiepileptic drugs (AEDs), such as $GABA_A$ receptors, may affect drug-target relationship. On the other hand, alterations in the brain capillary endothelial cells with over-expression of MDT may lead to a diminished permeability of AEDs into the brain parenchyma (Schmidt & Löscher, 2005).

In a model of AED-resistant epilepsy in rats, i.e., phenytoin-resistant kindled rats, the resistance extended to topiramate and various other AEDs (Löscher, 2006), indicating that it probably occurs as a consequence of unspecific, pharmacokinetic factors rather than by specific drug-target alterations. Over-expression of MDT, especially Pgp, at the

blood-brain barrier (BBB) in the the epileptogenic focus region is a feature of phenytoin-resistant kindled rats (Potschka et al., 2004b), which would restrict the penetration of all drugs that are Pgp substrates into this region. This situation parallels the up-regulation of MDT observed in epileptogenic focus of human epileptic patients, and represents a plausible mechanism of multidrug resistance (Schmidt & Löscher, 2005). In this regard, it is important to investigate whether the AEDs are substrates for human MDT. By using a highly sensitive assay (concentration equilibrium transport assay [CETA]) we demonstrated recently that several major AEDs are substrates for human Pgp (Luna-Tortós et al., 2008). This prompted us to study whether topiramate is also transported by human Pgp. For this purpose, we used the CETA in polarized kidney cell lines (LLC-PK1) transfected with the gene encoding the human efflux transporter Pgp (LLC-MDR1). The present study shows for the first time that topiramate is transported by human Pgp.

3.3. Materials and methods

Cell lines and cell cultures

LLC-PK1 cells transfected with human *MDR1* (LLC-MDR1) and respective wildtype (Wt) LLC cells were kindly provided by Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands). After obtaining the cells, they were cultured as described in detail recently (Baltes et al., 2007a,b). LLC-PK1 cells and transfectants were cultured with Medium 199 (Gibco^{TM}/Invitrogen Corporation, Eggenstein, Germany) supplemented with 10% FCS and 1% penicillin-streptomycin. Cells were used within 10 passages or less after thawing from liquid nitrogen, and at a maximum of 13 passages after receiving them from Prof. Borst. Because transfected LLC cells may lose the transporter cDNA in the absence of a selection agent such as vincristine, they were regularly tested for vincristine resistance (640 nmol) before being used for transport experiments (for details see Baltes et al., 2007a).

Transcellular transport assays

Cells were seeded on transparent polyester membrane filters (Transwell-Clear®, 24 mm diameter, 0.4 µm pore size, Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.3 x 10^6 cells/cm^2 cultured for 1-2 days to confluence and used for transport assays between days 5 and 7 after confluence (for details see Baltes et al.,

2007a,b). Transport studies were performed with the filter inserts in Transwell® multiwell culture plates that allow studying drug transport between an apical and basolateral compartment. For the present experiments, 6-well plates were used. Before starting the transport experiments, the medium was replaced with Opti-MEM® (Gibco™/Invitrogen Corporation, Eggenstein, Germany) and the transwells were pre-incubated for one hour (with or without transport inhibitor, respectively; see below). This reduced serum medium was used without any additives according to the protocol of the laboratory that provided the cell lines (Prof. P. Borst) in order to minimize protein binding of the drugs. At the beginning of the experiment (t=0), the pre-incubation medium was replaced by fresh Opti-MEM® containing the drug in both chambers (see below). The volumes in the upper and lower compartment were 2000 µl and 2700 µl, respectively. For drug analysis, samples were taken at 60, 120, 240, and 360 min (in one experiment also 600 min). The transport assays including pre-incubation were performed at 37° C in a humidified incubator (5% CO_2) with shaking the transwells gently at 50 rpm. Monolayers were checked for integrity by measuring transepithelial electrical resistance (TEER) of the polarized cells before and after each transport experiment and by using [^{14}C]-mannitol (in separate wells) as described recently (Luna-Tortós et al., 2008). To check for functional Pgp or MRPs in the apical membrane of cell monolayers, transport of the reference substrate digoxin was tested in separate wells in each experiment with topiramate.

Each transport study was initiated by adding the drug to both (apical and basolateral) sides of the monolayer, so that initial drug concentration was the same in both compartments (concentration equilibrium transport assay, CETA). In experiments with transport inhibitors, the respective inhibitor was also added to both chambers. For drug analysis, aliquots were collected from both compartments over the course of an experiment (100 µl basolateral and 130 µl apical, in order to avoid influences by hydrostatic pressure). Each experiment was performed in triplicate and repeated at least once.

Drugs

Topiramate was kindly provided by Johnson & Johnson (Raritan, NJ, USA) and studied at a concentration of 10 µg/ml (30 µM), which is within the therapeutic plasma concentration range of this drug in patients with epilepsy (Rambeck et al., 2006). As a reference substrate for Pgp,

[^{3}H]-digoxin (40 Ci/mmol; PerkinElmer, Rodgau-Jügesheim, Germany) was diluted with unlabeled digoxin (Sigma-Aldrich; Taufkirchen, Germany) to give an activity of 0.05 µCi/ml and a final concentration of 10 nM in the assay. For selective inhibition of Pgp, tariquidar (kindly provided by Xenova, Slough, Berkshire, U.K.) was used at concentration of 0.5 µM.

Topiramate was dissolved in purified water and then diluted in the cell culture medium. Digoxin and tariquidar were dissolved in DMSO (< 0.1% DMSO in final solution).

Drug analysis

Topiramate was analyzed by liquid chromatography with a mass specific detector (LCMS) with an API-2000 triple quad MS (Applied Biosystems, Applera GmbH, Germany). Details are described elsewhere (Rambeck et al., 2006).

The radioactivity in samples from experiments with [^{3}H]digoxin and [^{14}C]mannitol was quantified using a scintillation counter.

Analysis of transport data

Results of the individual transport assays are presented for each chamber as the percentage of the initial drug concentration versus time. The statistical significance of differences between drug concentrations in the two chambers was calculated by two-way analysis of variance (ANOVA) for repeated measurements, followed by Bonferroni post- hoc tests. In order to compare the magnitude of transport, the area under drug concentration vs. time curves (AUC) in the apical chamber was calculated in percent above the initial concentration x time, by using the trapezoidal rule (for more details see Luna-Tortós et al., 2008 [chapter 2]).

3.4. Results

Transepithelial electrical resistance (TEER) and [^{14}C]mannitol controls.

The parallel tests for control of paracellular permeability showed TEER values of 180 ±43 Ω*cm^{2} in the monolayers used before experiment were started, and [^{14}C]mannitol permeabilities from basolateral to apical were under 12 nm/s, which were in accordance with previously described values for these cell lines in our laboratory (see Luna-Tortós et al., 2008 [chapter 2]).

Transport of reference compound (digoxin)

In MDR1 transfectants that express Pgp on the apical membrane, the concentration of the Pgp substrate digoxin rapidly increased in the apical chamber, while decreasing correspondingly in the basolateral chamber, as a result of basolateral to apical transport by Pgp (Fig. 3.1). This Pgp-mediated transport could be completely prevented by the Pgp inhibitor tariquidar. Some transport by endogenous Pgp, which could be inhibited by tariquidar, was determined in LLC-PK1 wildtype cells.

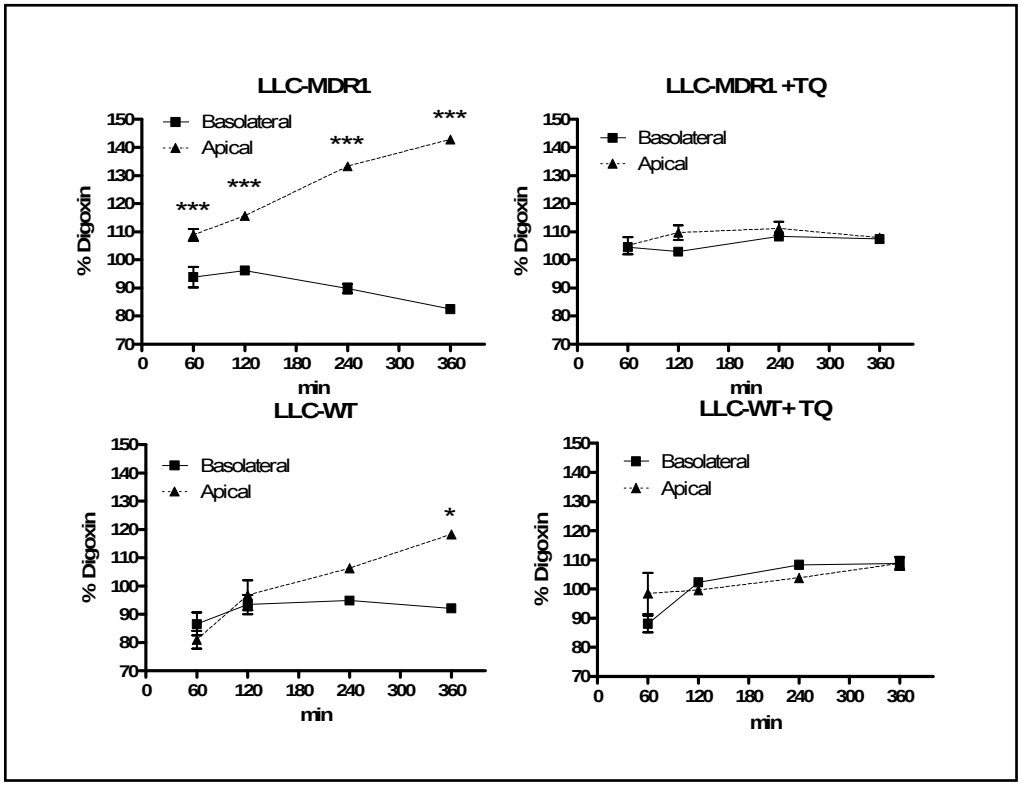

Figure 3.1 Transport of the reference compound digoxin. For transport experiments, drug was initially added in both compartments with or without inhibitor (concentration-equilibrium transport assay). Digoxin, a strong substrate of Pgp is rapidly enriched in the apical chamber (▲) of the human Pgp over-expressing cell lines (LLC-MDR1), while the concentration in the basolateral chamber (■) decreases. This transport was inhibited by tariquidar (0.5 μM). Less pronounced transport, also sensitive to tariquidar inhibition, was observed in the wildtype cell lines (LLC-WT), indicating active endogenous Pgp. Bars indicate SEM; when no bar is visible, standard errors were within the size of the symbols. Statistical significances are expressed with asterisks (*P<0.05; ***P<0.001).

Transport of topiramate by human Pgp in concentration equilibrium transport assay

In a first experiment with topiramate in LLC-MDR1 cells, a clear asymmetrical (basolateral to apical) transport was observed, indicating that this drug is a substrate for human Pgp (Fig. 3.2A). This was substantiated in a follow-up experiment (Fig. 3.2B), in which the transport of topiramate in LLC-MDR1 cells could be completely counteracted by tariquidar. Contrarily as for the case of digoxin, no transport was observed in the parental cell lines (Fig. 3.2B).

Regarding magnitude of transport, the AUC per hour in the apical chamber for the Pgp substrate digoxin (measured in our lab during last 2 years) showed a value of 1757 ± 281, while it was 446 ± 4.0 for topiramate in two experiments, meaning that the relative transport of topiramate in relation to digoxin equals 0.25 in LLC-MDR1 cells. Figure 3.3 shows a comparison of the relative AUCs for different AEDs we have investigated in our laboratory, including data we recently published (Luna-Tortos et al., 2008).

3.5. Discussion

Our results show that topiramate is a substrate for human Pgp, when investigated under concentration equilibrium transport assay using transfectants of the polarized porcine kidney cell line (LLC-PK1), which is widely used as an in vitro model for drug transport studies (Schinkel et al., 1996; Yamazaki et al., 2001). Transport of topiramate in LLC-MDR1 cells could be completely prevented by the selective Pgp inhibitor tariquidar, and no asymmetric transport was observed in parental cell lines, demonstrating the selectivity of the transport mediated by human Pgp in the transfected cells.

Sills et al. (2002) demonstrated that brain-serum concentrations of topiramate in *Mdr1a* knockout mice, which lack Pgp expression at the BBB, were two-fold higher than in wild-type animals, suggesting that Pgp mediates efflux of topiramate at BBB in this species (Sills et al., 2002). However, in view of species differences in substrate recognition by Pgp between mice and humans (Schinkel et al., 1996; Yamazaki et al., 2001; Baltes et al., 2007a), the study by Sills et al. (2002) did not allow to conclude that topiramate is also a substrate for human Pgp.

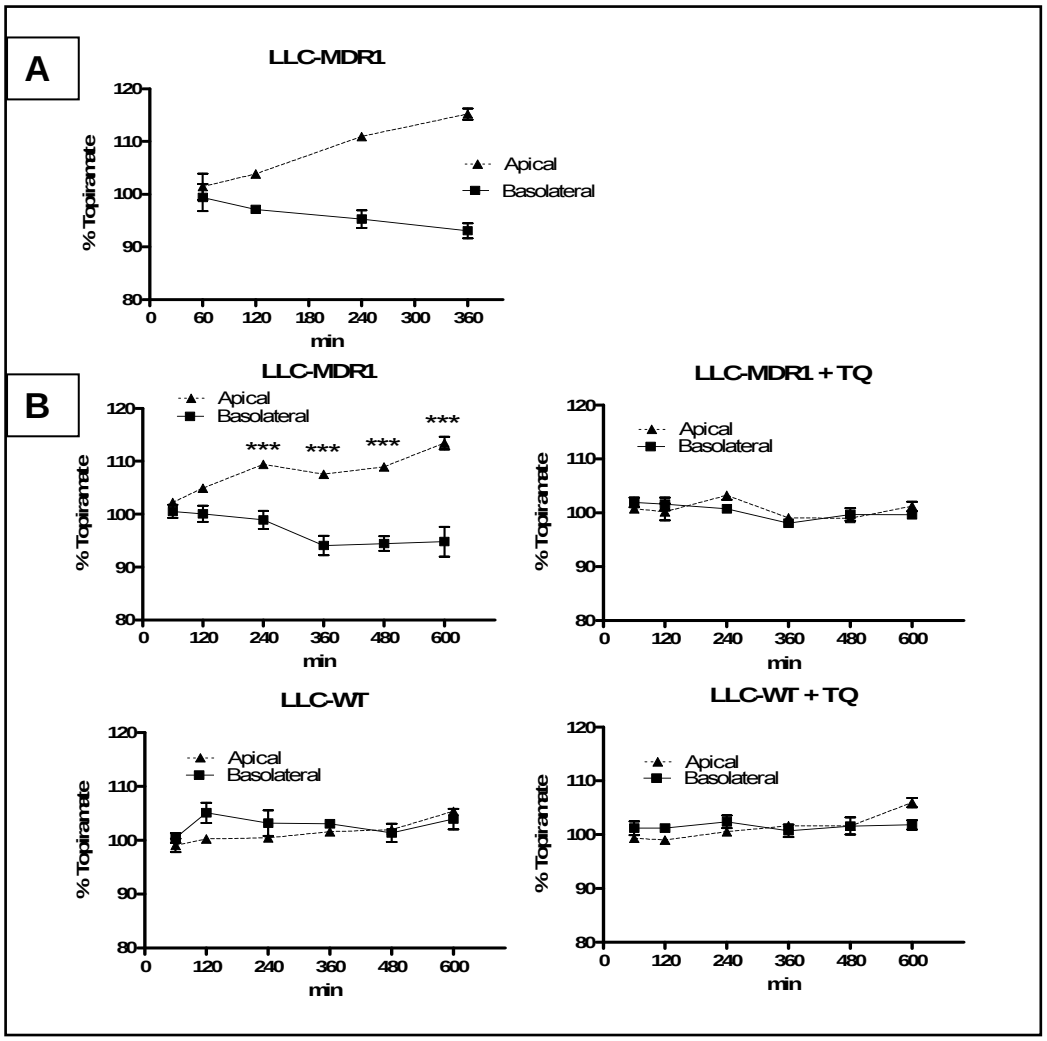

Figure 3.2 Transport of topiramate by human P-glycoprotein. For transport experiments, drug was initially added in both compartments with or without inhibitor (concentration-equilibrium transport assay). The antiepileptic drug topiramate is rapidly enriched in the apical chamber (▲) of the human Pgp over-expressing cell lines (LLC-MDR1), while the concentration in the basolateral chamber (■) decreases, in clear similar pattern as the Pgp substrate digoxin (see Fig. 3.1). The results of a pre-screening experiment are presented (**A**), where only two wells were used, and hence, no statistical analysis was performed. The experiment was repeated in triplicate for a longer time (n=3) and compared to wildtype cells (LLC-WT) and to monolayers incubated with tariquidar [0.5 μM] (+TQ) (**B**). Bars indicate SEM; when no bar is visible, standard errors were within the size of the symbols. Statistical significances are expressed with asterisks (***P<0.001).

Our data argue against the report of Crowe & Teoh (2006) who found no transport of topiramate mediated by human Pgp or MRPs. In their experiments a variety of AEDs were tested for their ability to be effluxed in monolayers of a Caco-2 sub-clone using bi-directional (apical to basolateral, and basolateral to apical) transport studies. No

evidence could be found to indicate Pgp-mediated efflux of topiramate in this immortalized line of heterogeneous human epithelial colorectal adenocarcinoma cells, but instead greater apical to basolateral transport was observed, which could not be affected by the MRP inhibitor MK571 (Crowe & Teoh, 2006). Caco-2 cells differ from the

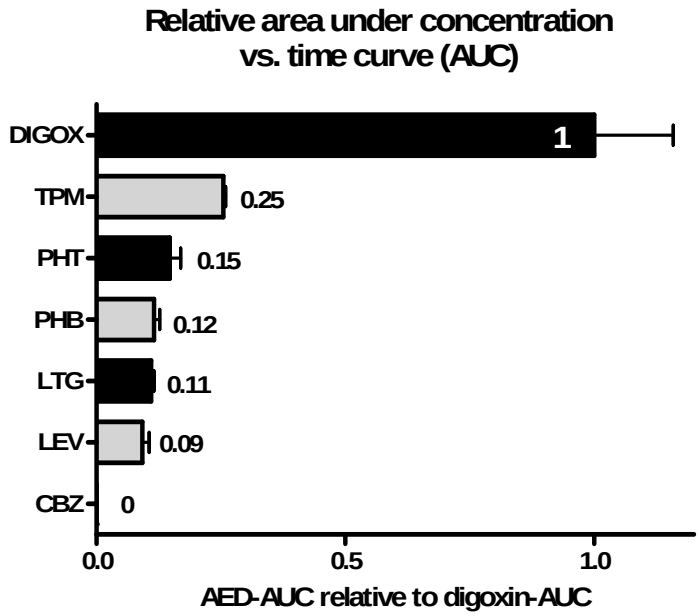

Figure 3.3 Relative area under concentration x time curves (AUC) for some human Pgp substrates. AUC in apical chamber obtained in CETA assay for several AEDs were normalized to digoxin AUC. Topiramate is the substrate showing the strongest transport among AEDs investigated in our lab by means of CETA in LLC-MDR1 cells. Data shows mean ± SEM.

LLC cells used in the present experiments by the expression of various influx transporters that could conceal active efflux by Pgp, what make Caco-2 cells a difficult model to dissect the participation of individual transporters (Wang et al., 2008). Furthermore, the conventional bi-directional transport assay with the Transwell® system used by Crowe and Teoh (2006), in which the drug is applied to either the apical or basolateral chamber for studying bi-directional transport, may fail to identify highly permeable compounds as Pgp substrates, because passive transcellular diffusion forms a bias in such assays by concealing active transport (Luna-Tortós et al., 2008). In the modified transport assay (CETA) used in the present experiments, the drug is initially added at identical concentration to both sides of the polarized, transporter-overexpressing cell monolayer, thus minimizing the influence of diffusion in response to concentration gradients (Luna-Tortós et al., 2008). Under these concentration

equilibrium conditions, a clear unidirectional (basolateral to apical) efflux of topiramate was determined in MDR1 transfectants in the present study, but it was not seen in the parental cell line.

In order to compare the magnitude of the Pgp-mediated transport of topiramate with that of the known Pgp substrate digoxin and other AEDs determined recently by us with the concentration equilibrium assay in LLC-MDR1 cells, the AUC in the apical chamber was calculated in percent above the initial concentration x time for all experiments. As shown in Fig. 3.3, topiramate exhibited the most pronounced directional transport among the AEDs that have been studied by us as yet. However, compared to the prototype Pgp substrate digoxin, the Pgp-mediated transport determined for AEDs appeared weak. However, a number of factors may lead to underestimation of drug transport in *in vitro* assays as used in the present study. First, it has to be considered that, even under the conditions of the modified transport assay used in this study, passive diffusion will affect the transport of highly permeable drugs such as most AEDs. The directional transport from the basolateral to the apical compartment, as observed for topiramate and several other AEDs in the equilibrium assay, leads to a concentration gradient between the two compartments and thereby rediffusion from the apical to the basolateral chamber, thus reducing the amount of drug in the apical compartment. As a consequence, directional transport by Pgp will be underestimated. In the case of digoxin, apart of the low concentrations used in these assays, it has been shown that increments in concentration gradient do not influence permeability of this compound in particular (Shirasaka et al., 2008), meaning that the substrate is more or less homogenously transported independently of the concentration. This seems not to be the case for AEDs, since lower concentrations in CETA lead to lower plateau on the apical transport of phenytoin (Luna et al., 2008). Second, *in vitro* assays are performed over a limited period of time, usually 4-6 h, whereas under *in vivo* conditions, patients are treated with AEDs on a chronic basis, so that AED efflux by Pgp from brain to blood will occur more or less continuously. Third, *in vivo* endothelial cells are continuously exposed to shear stress generated by the flow of blood across their apical surface, whereas *in vitro* transport assays using the Transwell system are static models lacking shear stress that affect the transport of drugs across biological membranes *in vivo* (Santaguida et al., 2006). Fourth, the physical barrier at the level of the BBB, mainly mediated by expression of TJ, forces most molecules to cross by the transcellular route instead of paracellular route at the BBB (Abbott et al., 2006). Epithelial cell lines used as

in vitro models are more “leaky” than the *in vivo* situation, meaning that paracellular re-diffusion of drugs in the CETA-model also influences the magnitude of transport seen in this system. Thus, data from *in vitro* transport assays as used in the present study can be used as a proof-of-concept to determine whether a drug is a substrate of human Pgp, but only *in vivo* experiments can prove if this transport affects therapeutic brain concentrations and thus the clinical response to this drug.

In conclusion, the present experiments provide the first direct evidence that topiramate is a substrate for human Pgp, when investigated in *MDR1*-transfected LLC cells and using a modified version of the widely used Transwell® system, i.e. the CETA, which is more adequate for investigating highly permeable compounds. Thus, it is plausible to hypothesize that up-regulation of Pgp at BBB, as found in patients with pharmacoresistant epilepsy, may restrict concentrations of topiramate at its brain targets and thereby contribute to resistance.

Chapter 4

Lack of active transport by human multidrug resistance associated proteins MRP1, MRP2 and MRP5 of several antiepileptic drugs in the MDCK II model

Chapter 4: Lack of active transport by human multidrug resistance associated proteins MRP1, MRP2 and MRP5 of several antiepileptic drugs in the MDCK II model

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4.1. Abstract

Resistance to antiepileptic drugs (AEDs) is a major concern for the management of epilepsy in about one third of the patients. One of the possible mechanisms of pharmacoresistance is the limitation of AED access to the seizure focus by over-expression of efflux transporters, including P-glycoprotein (Pgp) and multidrug resistance proteins (MRPs). But this mechanism would explain the phenomenon of pharmacoresistance only if the AEDs are substrates for these multidrug transporters (MDTs), thus it is important to investigate which AEDs are substrates for such MDT in humans. In the present study, we used the polarized canine kidney cell line MDCK II transfected with human MRP-genes (MRP1, MRP2 or MRP5) to evaluate transport of several major AEDs in the concentration-equilibrium transport assay (CETA). Known MRP substrates were used for comparison. The AEDs carbamazepine, lamotrigine, levetiracetam, phenobarbital, phenytoin, and topiramate were not transported by any of the MRPs investigated. Thus, the present data do not allow involving the former MRPs in the phenomenon of intractable in epilepsy.

4.2. Introduction

Epilepsy is a major central nervous system disorder which affects around 6 million people in Europe and 50 million people worldwide (Baulac & Pitkänen, 2009; WHO, 2005). The main therapy available for most of those patients is symptomatic instead of curative, with 24 molecules approved in USA and Europe (Schmidt, 2009). Unfortunately, around 30% to 40% of patients remain presenting epilepsy symptoms (i.e., seizures) in spite of receiving adequate antiepileptic drug therapy, and having appropriate plasma drug-levels (Schmidt & Löscher, 2005). Although the causes of this condition (named *pharmacoresistance*) are not known as yet, two main hypotheses have arisen. The *target hypothesis* proposes that seizure activity leads to changes in neuronal target molecules, what in turn affects the pharmacodynamic of the AEDs. *The multidrug transporter hypothesis* postulates that the overexpression of several multidrug transporters at the luminal side of blood-brain barrier (BBB) endothelium leads to an increased efflux of AEDs to the blood, what in turn reduces brain AED-concentrations, although their plasma levels remain unaffected (Löscher & Potschka, 2005a,b).

The multidrug transporters are part of a large transmembrane protein superfamily called ATP-binding cassette (ABC) transporters. Several members are expressed at the luminal side of the brain capillary endothelial cells of the BBB. Although the ABC transporter mostly investigated at time is *Pgp* (Pgp, or ABCB1), the *multidrug resistance associated protein* (MRP, or ABCC) subfamily is also represented at BBB, with several members such as MRP1 (ABCC1), MRP2 (ABCC2), MRP4 (ABCC4) and MRP5 (ABCC5) (Löscher & Potschka, 2005a,b). Similarly as Pgp, MRP1, MRP2 and MRP5 are up-regulated in epileptogenic brain tissue of pharmacoresistant epileptic patients (Kwan and Brodie, 2005).

While the meaning of this over-expression is not well understood, it has been shown that human Pgp is able to transport several AEDs (Luna-Tortos et al., 2008). Of particular relevance for understanding the possible role of the Pgp-overexpression was the demonstration that Pgp inhibition in non-responder epileptic rats can overcome the pharmacoresistance-condition, implying a central role for Pgp, but not a mere epiphenomenon, at least in animal models of temporal lobe epilepsy (TLE) (Brandt et al., 2006). On the other hand, there is *in vitro* evidence that species differences may be involved in the substrate specificity of ABC transporters (Baltes et al., 2007a). Thus, it is of particular relevance to study the transporter isotypes expressed in human tissue.

Since Pgp and MRPs are able to transport a wide range of drugs, and their spectrum can overlap, it is feasible to hypothesize that these transporters may act in conjunction to efflux AEDs from brain parenchyma, what would explain why a patient becomes resistant to several, chemically dissimilar drugs (Löscher & Potschka, 2005a,b).

With a view to explore the possible role of human MRPs in the phenomenon of pharmacoresistance in epilepsy, we used MDCK II cell lines which were stably transfected with the human genes encoding for MRP1 (MDCK-MRP1), MRP2 (MDCK-MRP2), and MRP5 (MDCK-MRP5) to assess the ability of these transporters to efflux several AEDs *in vitro*.

4.3. Materials and methods

Cell lines and cell cultures

MDCK type II cells transfected with human MRP1 (MDCK-MRP1), MRP2 (MDCK-MRP2) or MRP5 (MDCK-MRP2) and respective MDCKII wildtype cells were kindly provided by Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands).

After obtaining the cells, they were cultured as described in detail recently (Baltes et al., 2007a,b). Cells were used within 10 passages or less after thawing from liquid nitrogen, and at a maximum of 13 passages after receiving them from Prof. Borst.

Transcellular transport assays

Cells were seeded on transparent polyester membrane filters (Transwell-Clear®, 24 mm diameter, 0.4 µm pore size, Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.4 x 10^6 cells/cm^2, cultured for 1-2 days to confluence and used for transport assays between days 5 and 7 after confluence (for details see Baltes et al., 2007a,b). Transport studies were performed with the filter inserts in Transwell® multiwell culture plates that allow studying drug transport between an apical and basolateral compartment. For the present experiments, 6-well plates were used. Before starting the transport experiments, the medium was replaced with Opti-MEM® (Gibco™/Invitrogen Corporation, Eggenstein, Germany) and the transwells were pre-incubated for one hour (with or without transport inhibitor, respectively; see below). This reduced serum medium was used without any additives according to the protocol of the laboratory that provided the cell lines (Prof. P. Borst) in order to minimize protein binding of the drugs. At the beginning of the experiment (t=0), the pre-incubation medium was replaced by fresh Opti-MEM® containing the drug in both chambers (see below). The volumes in the upper and lower compartment were 2000 µl and 2700 µl, respectively. For drug analysis, samples were taken at 60, 120, 240, and 360 min (in some experiments also 480 and 600 min). The transport assays including pre-incubation were performed at 37° C in a humidified incubator (5% CO_2) with shaking the transwells gently at 50 rpm. Monolayers were checked for integrity by measuring transepithelial electrical resistance (TEER) of the polarized cells before and after each transport experiment and by using [^{14}C]-mannitol (0.1 µCi/ml, in separate wells) as described recently (Luna-Tortós et al., 2008). Monolayers used in these experiments showed Papp B-a for [^{14}C]mannitol of 5.3 ±1.5 nm/s and TEER of 70 ±12 Ω*cm^2. To check for functional MRPs in the apical membrane of cell monolayers, transport of the reference substrates (see below) was tested in separate wells in almost each experiment with AEDs.

Each transport study was initiated by adding the drug to both (apical and basolateral) sides of the monolayer, so that initial drug concentration was the same in both compartments. In experiments with transport inhibitors, the respective inhibitor was also

added to both chambers. For drug analysis, aliquots were collected from both compartments over the course of an experiment (100 µl basolateral and 130 µl apical, in order to avoid influences by hydrostatic pressure). Each experiment was performed in triplicate and, when indication of transport was seen, it was repeated once.

For selective inhibition of Pgp, tariquidar was used at concentrations of 0.5 µM. To avoid that expression of endogenous Pgp by MDCK cells interfered with MRP-mediated drug transport, tariquidar was added in all experiments with MRP-transfected MDCK cells, except in those performed with CMFDA.

Uptake experiments

For determining whether treatment reference compounds calcein-AM and CMFDA were transported by MRPs, uptake assays were performed essentially as described elsewhere (Decorti et al., 2001; Yasuda et al., 2002; Perrière et al., 2007), with cells cultured on 6-wells plates. Uptake of the fluorescent substrates was measured over 2 h (in the presence of the Pgp inhibitor tariquidar, for calcein-AM, and in the absence or presence of this inhibitor for CMFDA) and was calculated as absolute fluorescence in the cell lysate per mg protein. Experiments were performed in triplicate.

Drugs

Levetiracetam was kindly provided by UCB Pharma (Brussels, Belgium), phenytoin and carbamazepine by Desitin (Hamburg, Germany), lamotrigine by GlaxoSmithKline (Stevenage, Herts, U.K.), and tariquidar (XR9576) by Xenova Ltd. (Slough, Berkshire, U.K.). Phenobarbital (as sodium salt) was obtained from Serva (Heidelberg, Germany), and MK571 [*(E)*-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid, sodium salt] from Alexis Biochemicals (Axxora, Lörrach, Germany); [^{3}H]-vinblastine sulphate (9.8 Ci/mmol) and [^{14}C]-mannitol (61.0 mCi/ mmol), from Amersham (Buckinghamshire, U.K.), and cold vinblastine sulphate, from Sigma-Aldrich (Taufkirchen, Germany). Topiramate was kindly provided by Johnson & Johnson (Raritan, NJ, USA).

Calcein acetoxymethyl ester (calcein-AM) and chloromethylfluorescein diacetate (CMFDA, CellTracker Green) were obtained from Invitrogen Corporation (Eggenstein, Germany).

Tariquidar, Calcein-AM, and CMFDA were dissolved in DMSO (< 0.1% DMSO in final solution) and MK571 was dissolved in medium. Phenytoin, lamotrigine and

carbamazepine were dissolved in ethanol (≤ 0.4% ethanol in final solution). Phenobarbital and levetiracetam stock solutions were prepared in medium. Vinblastine sulphate and topiramate were dissolved in purified water. Freshly prepared stock solutions were dissolved in OptiMEM®

The choice of drug concentrations was based on therapeutic plasma concentrations of AEDs in epilepsy patients, previous transport studies with vinblastine, digoxin, tariquidar and MK571 (Smith et al., 1998; Baltes et al., 2007a,b) and FDA recommendations (FDA, 2006). The following concentrations were used: phenytoin, 50 µM; phenobarbital, 50 µM; levetiracetam, 220 µM; lamotrigine, 20 µM; carbamazepine, 30 µM; topiramate 30 µM. [^{3}H]-vinblastine sulphate (9.8 Ci/mmol) was diluted with unlabeled vinblastine sulphate to give an activity of 0.025 µCi/ml and a final concentration of 2 µM in the assay. Concentrations of calcein-AM (1 µM) and CMFDA (2.5 µM) were selected according to previous reports (Pratt et al., 2006; Nabekura et al., 2008). Tariquidar was used at 0.5 µM and MK571 at 50 µM. For paracellular permeability tests, [^{14}C]mannitol was added into the basolateal chamber at a concentration of 0.1 µCi/ml and measured from the apical chamber.

Drug analysis

Concentrations of AEDs were measured by high performance liquid chromatography (HPLC) with UV-detection as described earlier (Potschka and Löscher 2001; Potschka et al., 2001; Potschka et al., 2002; Potschka et al., 2004b). Limits of quantification in cell medium were as follows: carbamazepine, 25 ng/ml; lamotrigine, 25 ng/ml; levetiracetam, 400 ng/ml; phenytoin, 40 ng/ml; and phenobarbital, 25 ng/ml, respectively. Topiramate was analyzed by liquid chromatography with a mass specific detector (LCMS) with an API-2000 triple quad MS (Applied Biosystems, Applera GmbH, Germany). Details are described elsewhere (Rambeck et al., 2006). The radioactivity in samples from experiments with [^{3}H]vinblastine or [^{14}C]mannitol was quantified using a scintillation counter. Calcein and CMFDA-metabolite were quantified using a Fluoroscan II (LabSystems® Oy; Helsinki, Finland) with 485 nm excitation and 538 nm emission filters.

Statistics and calculations

Results of the individual transport assays are presented for each chamber as the percentage of the initial drug concentration versus time. Exceptions are the experiments

with calcein-AM, for which results are presented as absolute fluorescence of calcein per chamber. The statistical significance of differences between drug concentrations in the two chambers was calculated by two-way analysis of variance (ANOVA) for repeated measures, followed by Bonferroni post-tests. Drug transported was measured as area under concentration above initial concentration (in percent) x time curves for the case of vinblastine sulphate and antiepileptic drugs. For calcein and CMFDA-metabollite, the drug transported per chamber was calculated in absolute fluorescence per chamber (background fluorescence in medium with parental drug was subtracted from sample-values), and areas under absolute fluorescence x time curves were calculated for both chambers. In this case, a ratio between AUC basolateral and AUC apical was used to calculate relative differences between transfected and parental cell lines.

In the case of uptake assay, results are presented as absolute fluorescence normalized per protein content in mg.

4.4. Results

Transport of reference compounds

In order to assess functionality of the human transporters, the transport of corresponding reference compounds was evaluated by means of uptake assay and concentration equilibrium transport assay.

Figure 4.1 shows results for the MRP1 substrate calcein. The lipophilic calcein-AM diffuses into cells where it is cleaved by intracellular esterases, resulting in fluorescent calcein, which is actively extruded by MRP1 (Szakács et al., 1998; Dogan et al., 2004). Transport of calcein out of the cell mediated by MRP1 leads to lower accumulation of the compound, while increased accumulation can be mediated by the MRPs inhibitor MK571 (Fig. 4.1A). Since calcein can be transported also by Pgp, and the MDCK II cells express relatively high amounts of this endogenous transporter, the Pgp inhibitor tariquidar (0.5 µM) was used in all experiments. It can also be seen that uptake of calcein is affected by endogenous transporters, which can be blocked with MK571, since wildtype cells also transport calcein. Furthermore, the uptake assay does not allow differentiating whether other MRP transporters also transport calcein, since MRP2 and MRP5 transfectants have low calcein accumulation that can also be inhibited with MK571. Transport experiments with CETA method allow demonstrating that calcein is a substrate for MRP1 (Fig. 4.1B). This method permits the differentiation of basolateral

and apical transport. For the case of MRP1, which is expressed at the basolateral membrane of the cell, a preferentially basolateral transport should be observed. This occurs not only in MDCK-MRP1, but also in parental cell lines and in other transfectants (data not shown for the latter). However, basolateral vs. apical transport, measured by the ratio of the respective AUCs normalized per hour, were two fold higher in MDCK-MRP1 than in MDCK-Wt (see Table 4.1), while no difference between the MRP2 and MRP5 transfectants with the parental cells was found.

As expected, [^{3}H]vinblastine is a strong substrate for MRP2. Fig. 4.2 shows a clear basolateral to apical transport of this compound, corresponding to the apical expression of MRP2 in MDCK II transfectants. Transport could be inhibited with MK571 [50 µM] (>80% inhibition). On the other hand, only a slight, non-significant enrichment of [^{3}H]vinblastine in parental cells was observed, while no transport was determined in the MRP1 transfectants (Fig. 4.2).

Similarly to calcein-AM, CMFDA is a non-fluorescent, lipophilic compound that is hydrolyzed by intracellular esterases to a thiol-reactive, fluorescent, anionic intermediated (here referred as CMFDA-metabolite) that is impermeable and trapped within the cells, unless transported (Pratt et al., 2006). Figure 4.3A shows low accumulation of CMFDA-metabolite in MRP5 transfectants and parental cells that can be equally increased by the MRP inhibitor MK571 (50 µM), but not by Pgp inhibitor tariquidar (0.5 µM). Interestingly, although MK571-sensitive transport of CMFDA-metabolite can be demonstrated in parental cells, this inhibitor does not increase uptake in the MRP1 or MRP2 transfectants (tariquidar also does not, data not shown). Fig. 4.3B shows a clear basolateral transport of CMFDA metabolite in the MRP5 transfectants, but not in the parental cells, confirming the affinity of this transporter for this fluorescent metabolite. Because of lack of effect mediated by tariquidar in uptake assay, which indicates no role of endogenous Pgp for transport of CMFDA-metabolite, this inhibitor was not used during CETA-experiments. Corrected transport ratios are also shown in Table 4.1. Since no clear transport mediated by MRP1 or MRP2 transporters was observed in uptake assay (Fig. 4.3A), no experiment with CETA-method was performed with them.

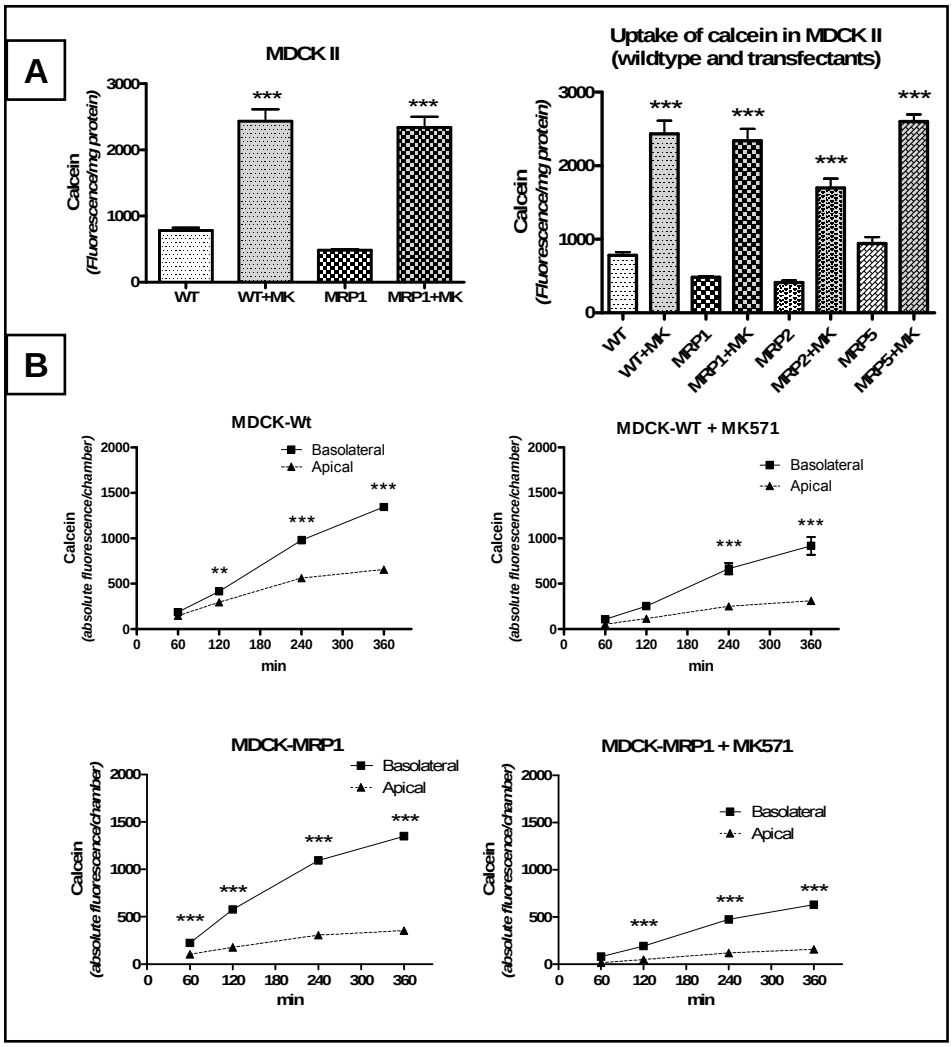

Figure 4.1 Calcein transport by human MRP1 measured in uptake and concentration-equilibrium transport assays (as described in Materials and Methods). Since calcein is transported by Pgp and MDCK II cells express this transporter, the Pgp inhibitor tariquidar (0.5 μM) was used during all experiments. Uptake assays in MDCK II parental cells and transfectants (A) show higher accumulation of drug in all cell lines with the MRPs inhibitor MK571 (50 μM). In transport experiments using CETA-method (B), only MRP1 transfectants show higher basolateral transport than the parental cell lines (here figures shown only for MRP1 transfectants and parental cells). Basolateral vs. apical transport measured by the ratio of the respective AUCs normalized per hour (see Materials and Methods for details) were two fold higher in MDCK-MRP1 than in MDCK-Wt (see table 4.1). Transport was not completely inhibited by MK571, but inhibition was higher than 50%. (■) basolateral chamber; (▲) apical chamber. Statistical analyses were performed with one-way ANOVA (A) or two-way ANOVA for repeated measurements (B) followed by Bonferroni post-hoc tests, with statistical differences expressed with asterisks (**P<0.01; ***P<0.001). (A): asterisks indicate difference for every cell line with and without inhibitor. Bars indicate SEM; when no bar is visible, errors are within the size of the symbols.

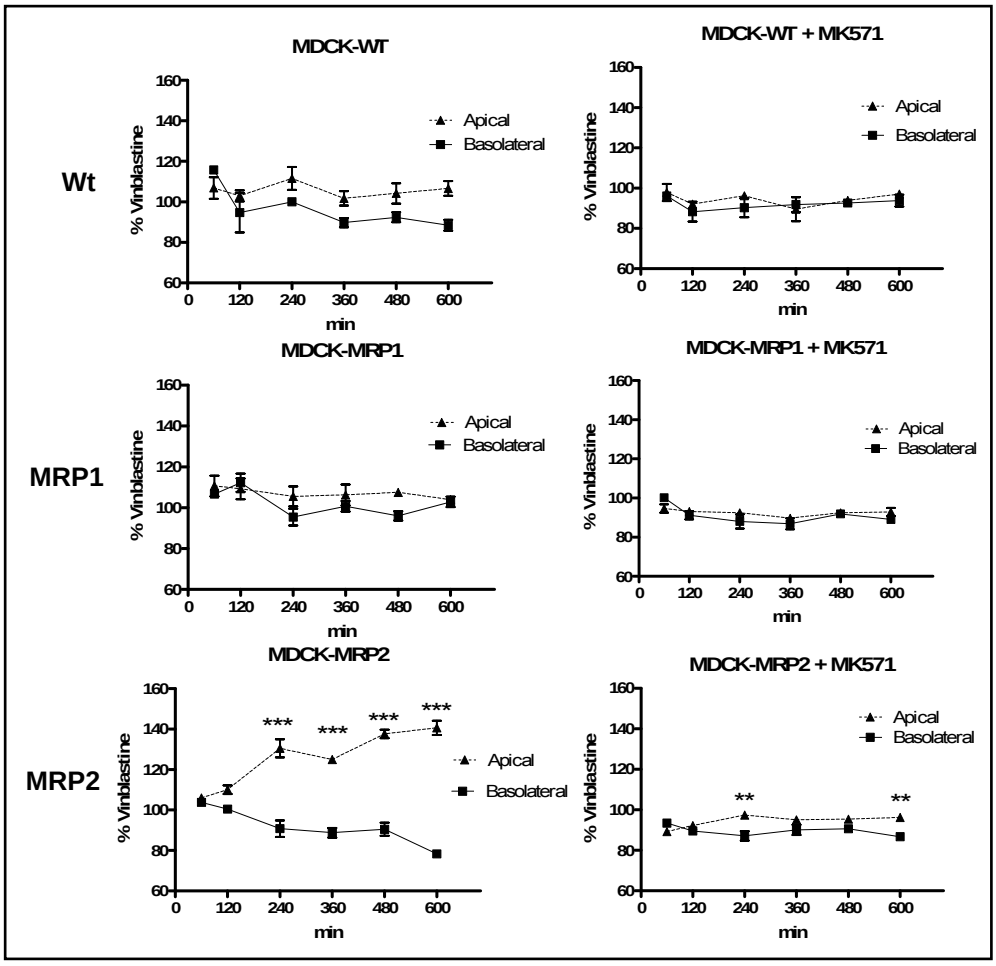

Figure 4.2 Transport of [^{3}H]vinblastine by human MRP2 in concentration equilibrium conditions. A clear enrichment in the apical chamber (▲) with corresponding decrease in basolateral chamber (■) can be seen in MDCK-MRP2 cells. Transport is mostly (>80%) inhibited by MK571 (50 μM). No significant transport was found in MRP1 transfectant or in parental cell lines. Statistical analyses were performed with two-way ANOVA repeated measurements, with Bonferroni post-hoc tests. Statistical differences are expressed with asterisks (**P<0.01; ***P<0.001).

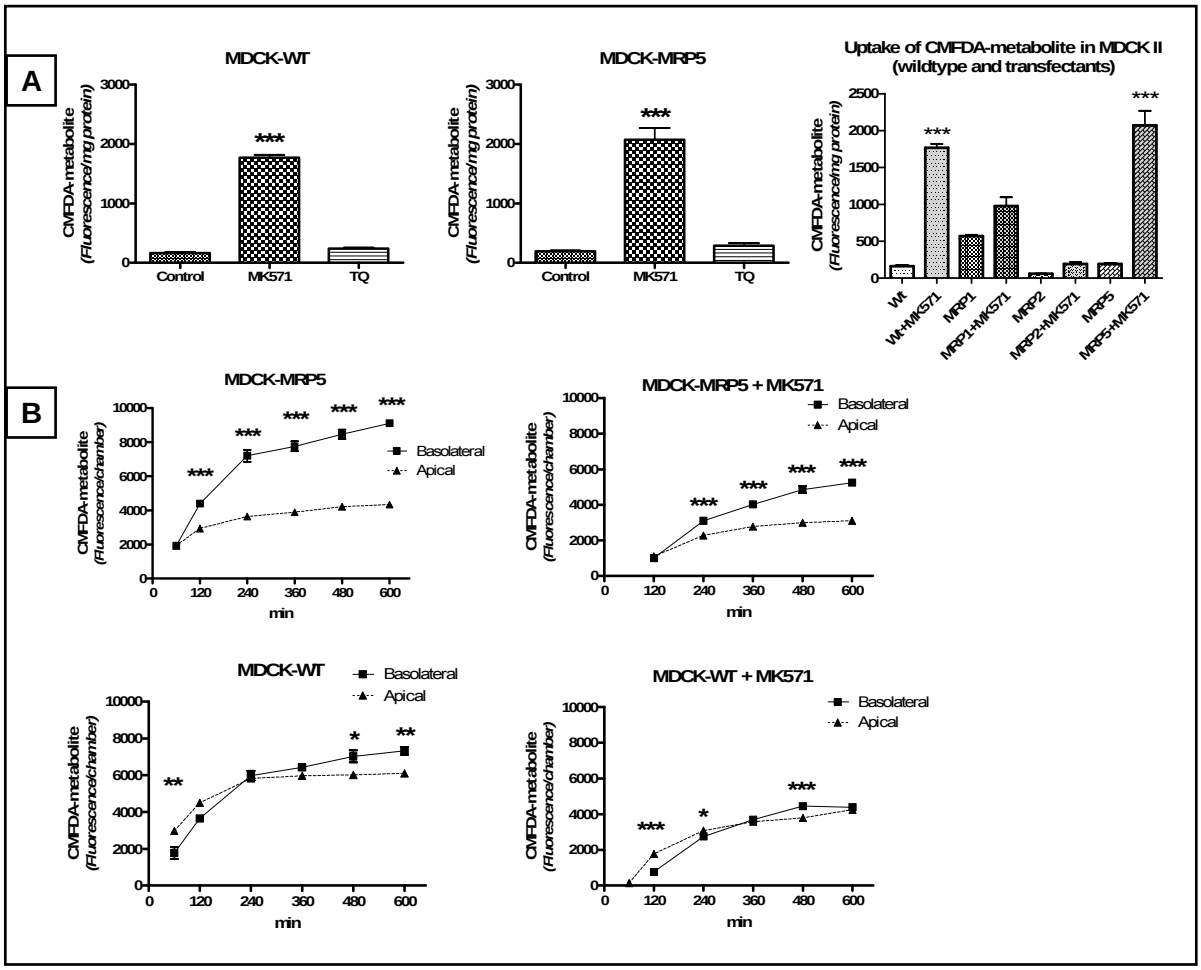

Figure 4.3 CMFDA-metabolite transport by human MRP5 measured in uptake and concentration-equilibrium transport assays (as described in Materials and Methods). Results of uptake assay (A) show no difference in transport of CMFDA-metabolite between parental cells (MDCK-WT) and MRP5 transfectants (MDCK-MRP5), since the transport in both is of similar magnitude and is equally inhibited by MK571 (50 μM). Endogenous Pgp does not seem to mediate transport, since no effect was seen with the Pgp inhibitor tariquidar (0.5 μM). In the human MRP1 and MRP2 transfectants (A) there is no demonstrable transport of CMFDA-metabolite. In a further investigation in CETA method (B) the transport in MRP5 transfectants is mainly basolateral (■), corresponding to the basolateral expression of this transporter in MDCK II transfectants, while there was only a small difference between apical (▲) and basolateral transport in parental cells. Transport in MRP5 was not completely inhibited by MK571, but basolateral AUC was reduced to a half. Statistical analysis were performed with one-way ANOVA (A) or two-way ANOVA for repeated measurements (B) followed by Bonferroni post-hoc tests, with statistical differences expressed as asterisks (*P<0.05; **P<0.01; ***P<0.001). (A): asterisks indicate difference for every cell line with and without inhibitor. Bars indicate SEM; when no bar is visible, errors are within the size of the symbols.

Table 4.1 Reference compounds for transporters MRP1, MRP2 and MRP5

Cell line and Transfectants	Calcein Bl/Ap ratio[1]	Vinblastine AUC/h (Ap)[2]	CMFDA metab. Bl/Ap ratio[1]
MDCK-Wt	1.8	388.6	1.0
MDCK-MRP1	3.9	122.1	n.d.
Corrected	**2.2**	0.3	n.d.
MDCK-MRP2	1.1	1151.2	n.d.
Corrected	0.6	**3.0**	n.d.
MDCK-MRP5	2.0	n.d.	2.1
Corrected	1.1	n.d.	**2.1**

n.d.: not determined. (1) For the basolateral transporters MRP1 and MRP5 their fluorescent substrates calcein and CMFDA are metabolized within the cell and transported to basolateral, but also to apical chambers, the basolateral vs. apical ratio (Bl/Ap) is shown. The corrected ratio for the fluorescent metabolites calcein and CMFDA-metabolite was calculated as the Bl/Ap ratio obtained in transfectants vs. the Bl/Ap ratio obtained in parental cells. (2) For the apical transporter MRP2 with the substrate [^{3}H]vinblastine, the area under drug concentration above initial concentration (in percent) x time curves (AUC) from the apical chamber was calculated (see Materials and Methods for more details). For [^{3}H]vinblastine sulphate, AUC obtained in transfectants was corrected by dividing by AUC from parental cells.

Transport experiments with antiepileptic drugs

Since several AEDs were shown to be transported by Pgp (Luna-Tortos et al., 2008), the inhibitor tariquidar (0.5 μM) was used in all experiments to prevent confounding effects mediated by this transporter. Contrarily to results obtained for the reference compounds, no transport of AEDs could be determined in the MDCK II model using human MRP1, MRP2 or MRP5 transfected cells, and the CETA-method. Figure 4.4A shows the results of first experiments, which were performed during 10 hours using MDCK-WT, MDCK-MRP1 and MDCK-MRP2, and which show lack of any clear transport of phenobarbital (50 μM) and levetiracetam (220 μM). Shorter experiments (6 hours) were assayed in MDCK-MRP5 cells; with equally negative results (Fig. 4.4B).

Given the former results, shorter (6 hours) experiments were carried out as screening tests for other AEDs using only transfected cells (Fig. 4.5). No transport was observed for lamotrigine (20 μM) or cabamazepine (30 μM) in any cell line, nor for phenytoin (50 μM) or topiramate (30 μM) in MDCK-MRP2 and MDCK-MRP5. Only for the cases of phenytoin and topiramate a slight, non-significant, basolateral enrichment was observed in the MDCK-MRP1 cells, corresponding to the basolateral expression of MRP1 in these cells. Hence, longer experiments were repeated for these two AEDs in MDCK-MRP1 cells, comparing to MDCK-WT and MDCK-MRP1 plus inhibitor (MK571 [50 μM]). As

shown in figure 6, no transport mediated by MRP1 could be demonstrated. The small, apical enrichment of drug in case of topiramate, seems to be mediated by endogenous transporters other than Pgp, since tariquidar was used in all experiments.

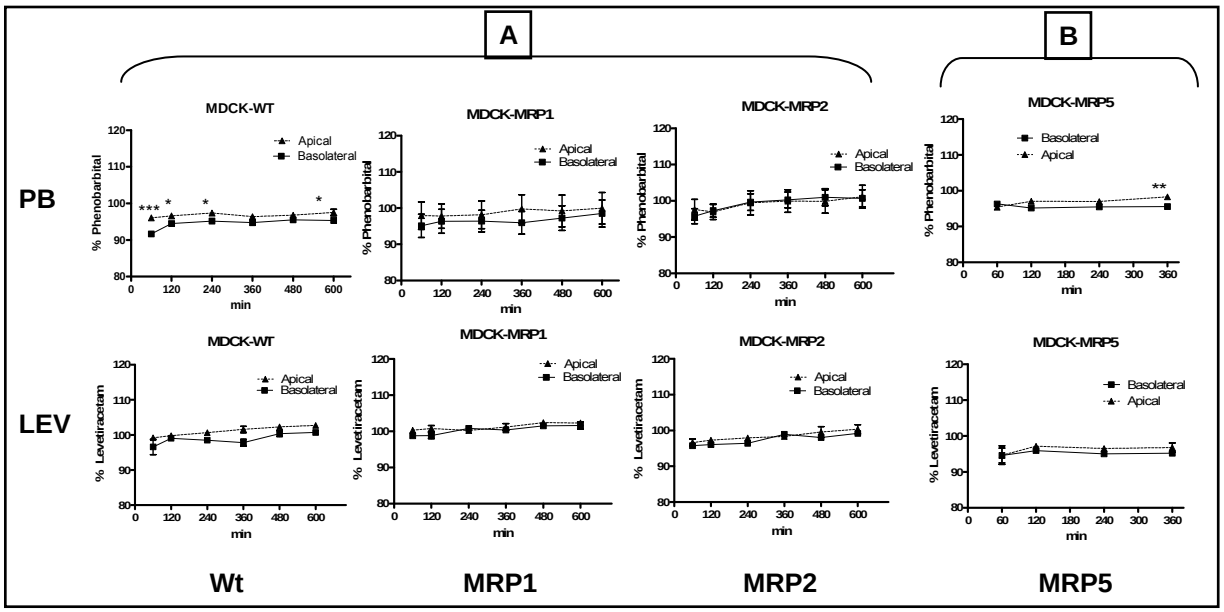

Figure 4.4 Transport experiments of antiepileptic drugs using CETA-method, where the drug is added in both chambers at same concentration (see Materials and Methods for more details). Experiments with phenobarbital (PB [50 μM]) and levetiracetam (LEV [220 μM]) during 10 hours (A) or 6 hours (B) show no transport of these AEDs mediated by human MRP1, MRP2 or MRP5. Although a small difference was seen in MRP5 transfectants for PB (B, above), it is not attributable to human MRP5, since this transporter is expressed at the basolateral membrane, but not at the apical one. The Pgp inhibitor tariquidar (0.5 μM) was used in all experiments. Wt: wildtype cells. For more details see legends of previous figures.

4.5. Discussion

Transport of reference compounds

Transport of calcein was corroborated in human MRP1 transfected MDCK II cells, coinciding with previous reports (Szakács et al., 1998; Yang et al., 2004). However, we found that uptake assay did not bring clear results regarding polarized transport of this compound, with similar results observed in MDCK-Wt. The polarized transport experiments using CETA-method allows differentiating between apical and basolateral transport. By means of this method, a higher basolateral transport was observed in MRP1 transfectants in relation to the parental cells. In this sense, these results show an advantage of tridimensional models over uptake assay.

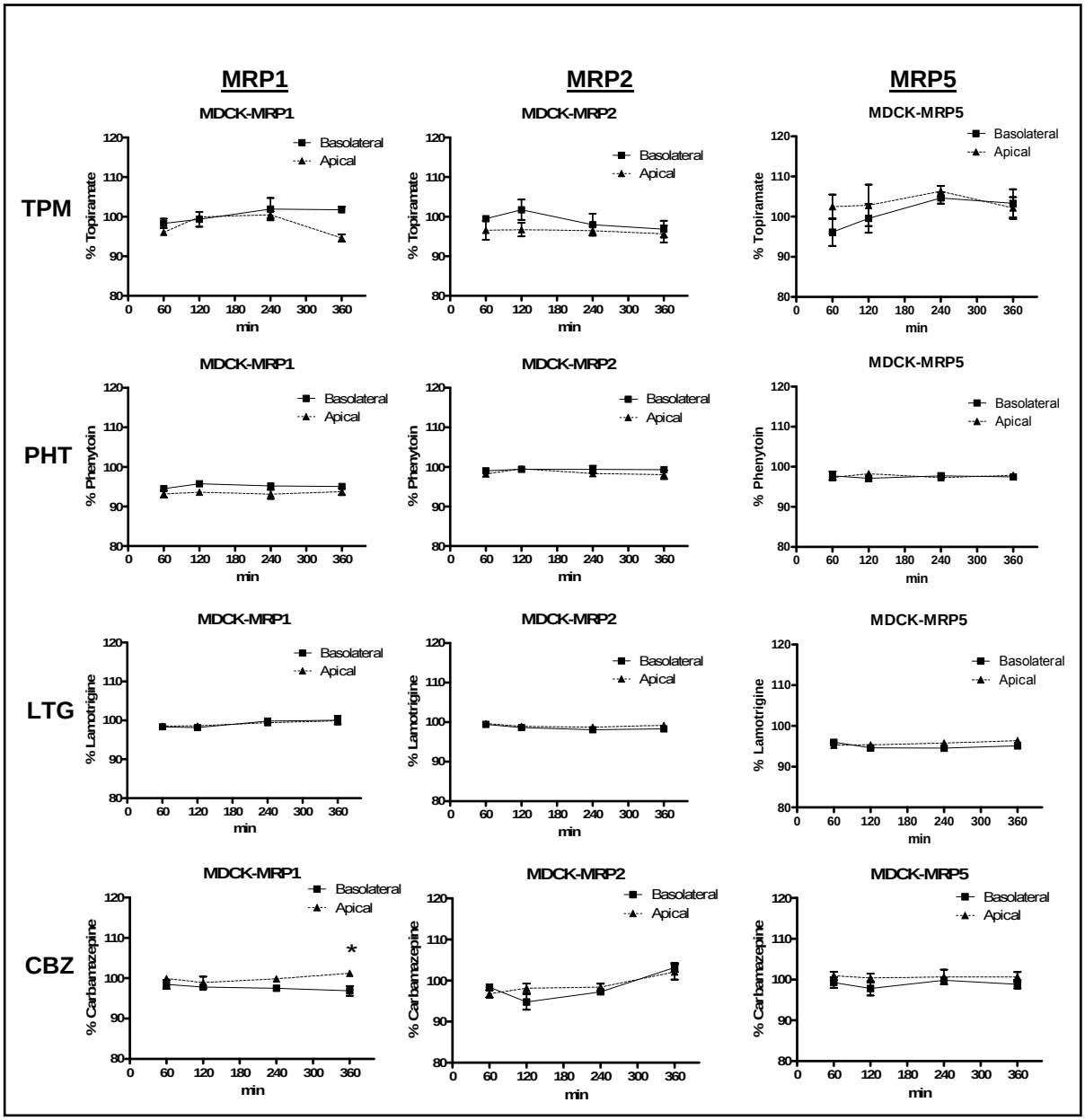

Figure 4.5 Transport experiments of antiepileptic drugs using CETA-method. Experiments with topiramate (TPM [29.5 µM]), phenytoin (PHT [50 µM]), lamotrigine (LTG [20 µM]), and carbamazepine (CBZ [30 µM]) during 6 hours show no transport of these AEDs mediated by human MRP1, MRP2 or MRP5. The Pgp inhibitor tariquidar (0.5 µM) was used in all experiments. For more details see legends of previous figures.

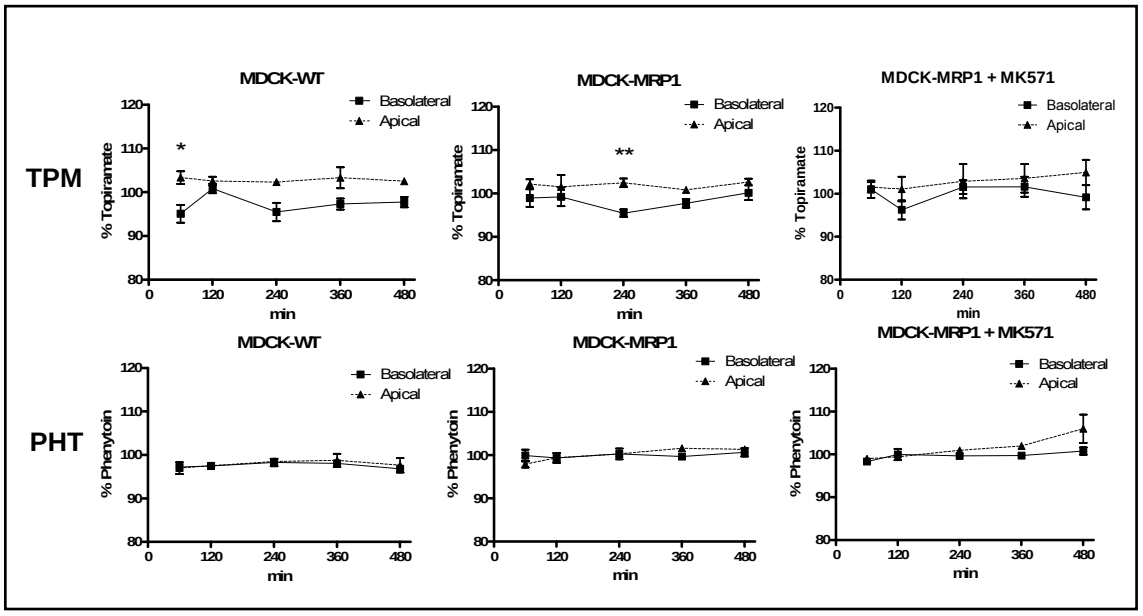

Figure 4.6 Transport experiments of topiramate (TPM) and phenytoin (PHT) using CETA-method. In fig. 4.5 a very slight, non significant difference was seen in the MRP1 transfectants favouring basolateral transport. Therefore assays were repeated for both AEDs in MDCK-MRP1 cells during 8 hours, comparing with the parental cells and MRPs inhibitor MK571. No transport of these AEDs that were attributable to human MRP1 was observed. The Pgp inhibitor tariquidar (0.5 μM) was used in all experiments. For more details see legends of previous figures.

Vinblastine is a common substrate for Pgp and MRP2 (Flanagan et al., 2002; Tang et al., 2002), and in our model with CETA method we previously demonstrated it is strongly transported in MDR1-transfected LLC-PK1 cells (Luna et al., 2008). Here we report about its transport in MDCK-MRP2 cells by using this concentration equilibrium transport assay, since a clear basolateral to apical transport takes place in this cell line, and it can be inhibited with MK571. Contrarily, we did not find transport of vinblastine in MDCK-MRP1 cells. Lack of transport of vinblastine mediated by MRP1 has been previously reported as well (Flanagan et al., 2002). Hence, our results argue against those of Yang et al. (2004). These authors found a high basolateral to apical ratio of vinblastine by using bidirectional transport assays in cells over-expressing human MRP1. Notwithstanding, they did not use Pgp inhibitor during transport experiments, what may have influenced the transport observed in those cells, since it is well know that Pgp mediates transport of vinblastine (Luna-Tortos et al., 2008). Furthermore, the cells we used express MRP1 at the basolateral membrane, while Yang et al. (2004) did not report on the polarized expression of the transporter. In case that those MDCK II transfected cells they used in their experiments expressed MRP1 at the basolateral membrane as well (what usually occurs in this epithelial cell line [Evers et al., 1996;

Flanagan et al., 2002; Shinkel & Jonker, 2003]), one would expect a very low b-A:A-b ratio (less than cero) because of a higher apical to basolateral transport (Flanagan et al., 2002). However, we can not discard that the lack of transport of vinblastine by MRP1 transfectants in our experiments may be due to high expression of apical, endogenous transporters such as MRP2 that conceal MRP1-mediated basolateral transport. Nevertheless, no clear transport of vinblastine was observed in the parental cell line.

Pratt et al. (2006) reported on the transport of CMFDA-metabolite mediated by MRP5. Here we corroborate that this fluorescent compound is transported by MRP5 in uptake assay and CETA, but probably not transported by Pgp, since tariquidar does not increase uptake in MDCK-Wt or MDCK-MRP5 cells. MK571 used at 50 μM does not completely inhibit polarized transport, and hence, the lower EC_{50} (16 μM) reported by Pratt et al. (2006) may be applicable for uptake assay, but not for polarized transport. It has also been reported that MRP5 is more resistant to MK571 inhibition than other MRPs (Dallas et al., 2006). Interestingly, low accumulation of CMFDA-metabolite was also observed in MDCK-MRP1 and MDCK-MRP2 cells, but it could not be inhibited by MK571. It probably indicates involvement of endogenous transporters other than MRPs.

Transport experiments with antiepileptic drugs

We recently reported on advantages of concentration equilibrium transport assay to assess transport of highly permeable AEDs by human Pgp (Luna-Tortos, et al., 2008). It was shown that this method can avoid problems created by diffusion gradient effects, which obviously occur when the conventional bidirectional method is used. This prompted us to employ stably transfected MDCK II cells that express human MRP transporters, in order to assess their ability to efflux several major AEDs.

We also previously reported on transport of levetiracetam and phenobarbital mediated by endogenous LLC-PK1 transporters, which was sensitive to inhibition by the MRP modulator MK-571 (Luna-Tortos et al., 2008). Since MRP1, MRP2 and MRP5 are over-expressed in epileptogenic tissue of pharmacoresistant patients (Kwan and Brodie, 2005), they are interesting candidates as pharmacoresistance mediators in epilepsy. According to the multidrug transporter hypothesis of pharmacoresistance in epilepsy, the over-expressed MRP transporters, which occur concomitantly with Pgp over-expression, may act in conjunction with the latter to extrude a variety of antiepileptic drugs from brain to blood (Löscher & Potschka, 2005a,b). However, we did not observe

any evidence of active transport of AEDs mediated by MRP1, MRP2 or MRP5 in the concentration equilibrium transport assay.

Although in a pre-screening experiment an apparently slight, non-significant enrichment of topiramate and phenytoin in the basolateral compartment was observed for MRP1 transfectants, which coincides with the basolateral expression of this transporter, no clear transport was found after repetition of the assay during a longer period.

Lack of transport of phenytoin, phenobarbital, carbamazepine, lamotrigine, and topiramate in Caco-2 cells was reported by Crowe & Teoh (2006). However, the use of such complex cell system that expresses several human transporters do not allow making final conclusions regarding involvement or exclusion of individual transporters (Wang et al., 2008).

Although species differences in substrate affinity have been reported for Pgp (Baltes et al., 2007a), no much information is available for the MRP subfamily, at least to our knowledge. Phenytoin was reported to be transported by rat Mrp2 *in vivo* (Potschka et al., 2003a). This difference with regard to our results may be due to several causes: 1) species differences in substrate affinity; 2) endogenous transporter expression in the MDCK II transfectants induces false negative results in the transport assay; and 3) MDCK II transfectants do not represent an approximate model for the *in vivo* situation. On the other hand, in another report Potschka et al. (2003b) did not find evidence of transport of lamotrigine or carbamazepine in MRP2 –deficient TR^- rats, although the anticonvulsant effect of the latter drug was increased in MRP2-deficient animals.

The MDCK II cells have been widely accepted as validated model for assessment of membrane permeation screening and for Pgp and MRP2 mediated transport (Irvine et al., 1999; Flanagan et al., 2002; Tang et al., 2002). However, it is also known that these cells have a high expression of endogenous transporters such as Pgp, MRP2, as well as various organic anion (OAT) and organic cation (OCT) transporters; but also, the endogenous-transporter expression can vary with transfection (Goh et al., 2002; Flanagan et al., 2002), what we also have observed in our laboratory (data not published). This is of particular relevance, since the spectrum of different ABC transporters can overlap, as part of their protective role against toxins (Löscher & Potschka, 2005a,b); meaning that endogenous transporters expressed on opposite side of the human MRP may be responsible of concealing active transport mediated by the latter.

In spite of their wide acceptance as a BBB-model, one caveat surges regarding this system: If an active transport were seen in the transfectants, but not in the wildtype cells (or in a lower rate than in the former), and the transport were successfully inhibited by a respective, specific modulator; the drug may be regarded as a substrate of the transporter in question. On the other hand, negative results may indicate that the drug is not transported, or that the transport is masked by endogenous transporters or by passive permeability. In other words, this model may be more reliable to discard false positives, but one can not discard false negative results. This point of view coincides with the observations reported by Feng et al. (2008) who investigated Pgp substrate specificity in MDCK-MDR1 and in Pgp-KO mice.

It would mean that the drug must be transported highly specifically by the exogenous transporter, and/or that endogenous transporters must be feasibly inhibited leaving a major participation by the exogenous one. An example for the first case is the transport of CMFDA-metabolite in MRP5 transfectants; and for the second case, the transport of calcein and vinblastine by MRP1 and MRP2, respectively, when Pgp is inhibited. Unfortunately, if the drug is transported by endogenous organic anion transporters and/or multidrug resistance associated proteins; no pharmacological inhibition is possible in order to dissect transport mediated by exogenous MRP transporters.

Of particular problem are the endogenous canine MRP transporters in MDCK cells, since it is not feasible to inhibit them pharmacologically, without affecting functionality of the exogenous human transporter. In the case of human MRP1 and MRP5 which are expressed on the basolateral membrane, an apically expressed endogenous transporter may conceal an eventual, active transport to the basolateral chamber.

One could be tempted to say that the more complex the system, the more representative of *in vivo* situation. But one important consideration here is that some models provide information whether a drug is transported by a given ABC transporter or not; while other models are more valid for translational purposes. The epithelial kidney cells can be used for assessment of transport in transfectants that over-express a given transporter, but the subcellular localization of their endogenous transporters differs in some instances from the subcellular localization of transporters at the BBB, and it is particularly remarkable for some MRPs. For example, MRP1 and MRP5 are expressed on the luminal membrane of the human BBB (Löscher & Potschka, 2005a,b), but they are expressed on the basolateral membrane of the MDCK II cells (Schinkel & Jonker, 2003).

Recently, Di et al. (2009) reported that the lipidic membrane composition of the MDCK II cells differs considerably from the BCECs from different species including humans. MDCK II cells have more fluidic membranes, while the cell membranes of BCECs are more rigid, and more hydrophobic. This finding correlates with higher permeability across the cell membrane of MDCK II. It also implies that MDCK II cells are less suitable than expected for carring out permeability studies involving highly permeable compounds (Di et al., 2009).

Hence, the ideal *in vitro* model for making more valid assumptions at the translational level should meet crucial characteristics of the BBB: 1) it must express adequate TJ and have very low paracellular permeability; 2) it must express transporters in the same subcellular localization as the *in vivo* BBB; and 3) it must possess a cell membrane that resembles more reliably the characteristic lipidic composition of the BBB-endothelium. A model that is more approximate to this theoretical prototype has been recently reported, where authors used human-derived endothelial cells, and built a tridimensional system in coculture with astrocytes to investigate phenytoin permeability across BBB (Cucullo et al., 2007). However, human-derived immortalized cell lines that can be used for large-scale screening of drugs in such a system are lacking so far.

In this sense, transfected MDCK II cells are adequate models to assess whether a drug is transported or not (albeit false negative results may occur), but not suitable to predict the magnitude of transport at the BBB level, especially because of the high paracellular permeability in this cell system that do not mimic the *in vivo* situation (Liu et al, 2008), what may lead to problems when investigating highly permeable compounds, such as AEDs.

In conclusion, the AEDs phenobarbital, phenytoin, carbamazepine, levetiracetam, lamotrigine and topiramate are not transported by human MRP1, MRP2 or MRP5 in transfected MDCK II cell lines. However, in virtue of the expression of endogenous transporters and the high drug-permeability in this system, negative results must be confirmed in other cell models with lower or no expression of endogenous ABC transporters. If confirmed, those results would mean that Pgp and MRP over-expression at the BBB in intractable epileptic patients do not act in conjunction to efflux the above mentioned AEDs, meaning that a major role is played by Pgp.

Chapter 5

Transport of valproic acid by endogenous transporters of LLC-PK1 and MDCK II kidney cells

Chapter 5: Transport of valproic acid by endogenous transporters of LLC-PK1 and MDCK II kidney cells

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5.1. Abstract

Valproic acid is a widely used antiepileptic drug (AED) with high efficacy for treatment of idiopatic partial and generalized epilepsies. Although able to reach brain parenchyma, it has been observed that valproate permeability through the blood-brain barrier is influenced by influx and efflux mechanisms. Pharmacological inhibition studies have pointed out that probable candidates for valproate efflux are members of the ATP-binding cassette transporters, subfamily C (ABCC, also called multidrug-resistance associated proteins [MRP]). Since up-regulation of several MRP transporters occur in the epileptogenic focus of pharmacoresistant patients, it has been hypothesized that they may play a role in the intractable epilepsy, by limiting AED concentration in target tissue. Hence, our aim was to investigate whether valproate is a substrate for several multidrug transporters, including P-glycoprotein (Pgp) and MRPs. To address this question, transport studies using porcine (LLC-PK1) and canine (MDCK II) kidney cells, and their transfectants that express human ABC transporters Pgp (LLC-MDR1), MRP1 (MDCK-MRP1), MRP2 (MDCK-MRP2), and MRP5 (MDCK-MRP5) were conducted in the concentration-equilibrium transport assay (CETA). No transport attributable to any of these transporters was observed, but an active transport to the apical chambers was recorded in LLC-PK1 cells (both parental and transfected), which was inhibited by the MRP modulator MK571. On the other hand, apical transport was also observed in MDCK II parental cells and their MRP5 transfectants that could not be inhibited by MK571, but was partially inhibited by probenecid. The present data show that endogenous transporters of these kidney cell lines are able to transport valproate, and that MRP transporters other than MRP1, MRP2 or MRP5 are probably involved in case of LLC-PK1 cells, but not in case of MDCK II cells.

5.2. Introduction

Valproate is a commonly used AED to treat new-onset idiopathic generalized and partial epilepsies. In spite of the availability of modern, very effective AEDs, such as lamotrigine and topiramate, valproate is still the drug of first choice for patients with idiopathic and symptomatic generalized epilepsies, because of its high efficacy (Schmidt, 2009). In a WHO report, valproate was included as first-line AED in the list of essential drugs by 86.7% of worldwide-participating countries (WHO, 2005). The mechanism of action of valproate is not completely understood, but it may involve the modulation of sodium and T-type calcium channels, while its effects on γ-aminobutyric

acid (GABA) system, e.g. increased GABA synthesis and turnover, are well recognized (Rogawski & Löscher, 2004).

In spite of its high efficacy for formerly mentioned epilepsies, valproate joins to the list of AEDs that fail to control seizures in about one third of patients (Schmidt, 2009), a condition termed pharmacoresistance. The two prevailing hypothesis to explain pharmacoresistance in epilepsy postulate, on the one hand, a pharmacodynamic model, where seizure-related (aquired) or innate changes in targets occur that affect drug-target relationship; and on the other hand, a pharmacodynamic model, with changes at the blood-brain barrier (BBB) affecting the brain concentrations of AEDs (Löscher & Potschka, 2005a,b; Schmidt & Löscher, 2009). Specifically, the latter hypothesis proposes that the over-expression of multidrug transporters (MDTs) at the BBB in pharmacoresistant patients leads to augmented extrusion of drugs from brain to blood. The MDTs belong to the ATPase –binding cassette (ABC) super-family of transporters that includes Pgp and the multidrug-resistance-associated proteins (MRP) subfamily, which are able to transport actively a wide range of chemically unrelated substrates against a concentration gradient. Those luminal MDTs of the BBB are one of the main defense components of the barrier and are assumed to be physiologically involved in the protection of the neural tissue from a wide range of xenobiotics (Löscher & Potschka, 2005a,b)

The MDT hypothesis is supported by several findings that showed that: a) an over-expression of several multidrug (efflux) transporters occurs at the BBB in epileptic patients and in animal models of epilepsy, especially Pgp, and several MRPs (MRP1, MRP2, MRP4 and MRP5) (Tishler et al., 1995; Sisodiya et al., 1999; Dombrowski et al., 2001; Aronica et al., 2003; Löscher & Potschka, 2005a; van Vliet et al., 2005; Volk & Löscher, 2005); b) over-expression of these transporters involves seizure-relevant brain structures (Seegers et al., 2002; Volk et al., 2004; van Vliet et al., 2007); and c) pharmacoresistance in animal models of epilepsy can be overcome by means of Pgp inhibition (Brandt et al., 2006, van Vliet et al, 2006).

However, one of the main open questions in this hypothesis is whether the human MDTs that are over-expressed in epilepsy can really transport AEDs. Our group has tried to address this question by using *in vitro* transport experiments with cells that express human MDTs. In this regard, we previously reported that valproate is not a substrate for Pgp, neither for murine nor for human isotypes, and is also not a substrate for MRP1 or MRP2 (Baltes et al., 2007b). On the other hand, given the fact that the bidirectional transport assay used for those experiments may overlook the transport of

highly permeable compounds, such as AEDs (Luna-Tortós et al., 2008), we decided to explore the transport of valproate in human MDR1 transfected LLC-PK1 cells, and human-MRP (-1, -2, and -5) transfected MDCK II cells by means of concentration equilibrium transport assay.

5.3. Materials and methods

Cell lines and cell cultures

LLC-PK1 cells transfected with human *MDR1* (LLC-MDR1) and respective wildtype (Wt) LLC cells as well as MDCK type II cells transfected with human MRP1 (MDCK-MRP1), MRP2 (MDCK-MRP2) or MRP5 (MDCK-MRP2) and respective MDCKII wildtype cells were kindly provided by Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands). After obtaining the cells, they were cultured as described in detail recently (Baltes et al., 2007a,b). Cells were used within 10 passages or less after thawing from liquid nitrogen, and at a maximum of 13 passages after receiving them from Prof. Borst. Because transfected LLC cells may lose the transporter cDNA in the absence of a selection agent such as vincristine, they were regularly tested for vincristine resistance (640 nmol) before being used for transport experiments (for details see Baltes et al., 2007a).

Transcellular transport assays

Cells were seeded on transparent polyester membrane filters (Transwell-Clear®, 24 mm diameter, 0.4 µm pore size, Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.3 x 10^6 cells/cm^2 (LLC) or 0.4 x 10^6 cells/cm^2 (MDCK), cultured for 1-2 days to confluence and used for transport assays between days 5 and 7 after confluence (for details see Baltes et al., 2007a,b). Transport studies were performed with the filter inserts in Transwell® multiwell culture plates that allow studying drug transport between an apical and basolateral compartment. For the present experiments, 6-well plates were used. Before starting the transport experiments, the medium was replaced with Opti-MEM® (Gibco™/Invitrogen Corporation, Eggenstein, Germany) and the transwells were pre-incubated for one hour (with or without transport inhibitor, respectively; see below). This reduced serum medium was used without any additives according to the protocol of the laboratory that provided the cell lines (Prof. P. Borst) in order to minimize protein binding of the drugs. At the beginning of the experiment (t=0), the pre-incubation medium was replaced by fresh Opti-MEM® containing the drug in

both chambers (see below). The volumes in the upper and lower compartment were 2000 µl and 2700 µl, respectively. For drug analysis, samples were taken at 60, 120, 240, and 360 min (in some experiments also 480 and 600 min). The transport assays including pre-incubation were performed at 37° C in a humidified incubator (5% CO_2) with shaking the transwells gently at 50 rpm. Monolayers were checked for integrity by measuring transepithelial electrical resistance (TEER) of the polarized cells before and after each transport experiment and by using [^{14}C]-mannitol (in separate wells) as described recently (Luna-Tortós et al., 2008). Values of [^{14}C]-mannitol apparent permeability (Papp) in direction basolateral to apical, and TEER were, respectively, 3.0 ±1.2 nm/s and 85.5 ±15 Ω*cm^2 for MDCK II cells (both wildtype and transfectants), and 6.8 ±2.6 nm/s and 409.7 ±197 for LLC-PK1 cells (both wildtype and transfectants). To check for functional multidrug transporters in the apical membrane of cell monolayers, transport of the reference substrates digoxin (for Pgp), calcein-AM (for MRP1), vinblastine (for MRP2) and CMFDA (for MRP5) were tested in separate wells in each experiment with valproate.

Each transport study was initiated by adding the drug to both (apical and basolateral) sides of the monolayer, so that initial drug concentration was the same in both compartments. In experiments with transport inhibitors, the respective inhibitor was also added to both chambers. For drug analysis, aliquots were collected from both compartments over the course of an experiment (100 µl basolateral and 130 µl apical, in order to avoid influences by hydrostatic pressure). Each experiment was performed in triplicate and, except for MRP1 and MRP2 transfected cells, repeated at least once.

Drugs

Valproate (used as its sodium salt) was diluted from a commercial aqueous solution (Orfiril; Desitin, Hamgburg, Germany). Tariquidar (XR9576) was kindly provided by Xenova Ltd. (Slough, Berkshire, U.K.). MK571 [*(E)*-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid, sodium salt] was obtained from Alexis Biochemicals (Axxora, Lörrach, Germany), and [^{3}H]-vinblastine sulphate (9.8 Ci/mmol) and [^{14}C]-mannitol (61.0 mCi/ mmol) from Amersham (Buckinghamshire, U.K.). Calcein acetoxymethyl ester (calcein-AM) and chloromethylfluorescein diacetate (CMFDA, CellTracker Green®) were obtained from Invitrogen Corporation (Eggenstein, Germany). Cold vinblastine sulphate and probenecid were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Tariquidar, Calcein-AM, and CMFDA were dissolved in DMSO (≤ 0.1% DMSO in final

solution) and MK571 was dissolved in medium. Valproate commercial solution was dissolved directly in medium. Vinblastine sulphate was dissolved in purified water. Probenecid was diluted in ethanol (≤ 0.1 % ethanol in final solution). Freshly prepared stock solutions were dissolved in OptiMEM®.

The choice of drug concentrations was based on therapeutic plasma concentrations of valproate in epilepsy patients, and previous transport studies with vinblastine, digoxin, tariquidar, MK571, and probenecid (Baltes et al., 2007b; Luna-Tortos et al., 2008; Schmidt, 2009). The following concentrations were used: Valproic acid (415 µM); [^{3}H]-vinblastine sulphate (9.8 Ci/mmol) was diluted with unlabeled vinblastine sulphate to give an activity of 0.025 µCi/ml and a final concentration of 2 µM in the assay. Concentrations of calcein-AM (1 µM) and CMFDA (2.5 µM) were selected according to previous reports (Pratt et al., 2006; Nabekura et al., 2008). For paracellular permeability tests, [^{14}C]mannitol was added into the basolateal chamber at a concentration of 0.1 µCi/ml. Tariquidar, MK571 and probenecid were used at concentrations of 0.5 µM, 50 µM and 100 µM, respectively.

Drug analysis

Concentrations of valproate were measured by high performance liquid chromatography (HPLC) with UV-detection as described earlier (Baltes et al., 2007b). The limit of quantification in cell medium was 6.25 µg/ml. The radioactivity in samples from experiments with [^{3}H]vinblastine or [^{14}C]mannitol was quantified using a scintillation counter. Calcein and CMFDA were quantified using a Fluoroscan II (LabSystems® Oy; Helsinki, Finland) with 485 nm excitation and 538 nm emission filters.

Statistics and calculations

Results of the individual transport assays are presented for each chamber as the percentage of the initial drug concentration versus time. Exceptions are the experiments with calcein-AM and CMFDA, for which results are presented as absolute fluorescence of calcein and CMFDA-metabolite per chamber, respectively. The statistical significance of differences between drug concentrations in the two chambers was calculated by two-way analysis of variance (ANOVA) for repeated measures, followed by Bonferroni post-tests. Drug transported was measured as area under concentration above initial concentration (in percent) x time curves for the case of vinblastine sulphate and valproate (see Luna-Tortos et al., 2008, for more details). For calcein and CMFDA-

metabollite, the drug transported per chamber was calculated in absolute fluorescence per chamber (background fluorescence in medium with parental drug was subtracted from sample-values).

5.4. Results

Reference compounds

As shown in figure 5.1, parallel tests with reference compounds that were performed during valproate transport experiments demonstrate functionality of human transporters in the respective cell lines. In this case, only a few wells (usually one or two) were used to test transporter functionality, since their affinity for such compounds were previously standardized (see chapter 4 for more details). Hence, no statistical analyses were carried out for controls.

Transport of valproate in LLC-PK1 cells (wildtype and transfectants)

Results of valproate transport in LLC-PK1 cells are resumed in figure 5.2 (only results from representative experiments are included). First experiments showed a strong transport in direction basolateral to apical, in both parental and transfected cell lines, that could not be inhibited with the Pgp modulator tariquidar. The lack of effect of Pgp inhibitor and the comparable magnitude of transport in both cell lines discard the involvement of human Pgp in the transport of valproate. The assay was repeated, but additionally comparing to the MRP inhibitor MK571. Once again, transport could not be inhibited by tariquidar, but was abolished by MK571 in both cell lines, suggesting a probable role of endogenous MRP transporters. Another repetition of experiments, this time adding tariquidar plus MK571 and tariquidar plus probenecid in parallel, confirmed that cells with only tariquidar can still transport valproate, but the concomitant addition of MK571 or probenecid could inhibit this effect by more than 50% (Fig. 5.2B, and Table 5.1).

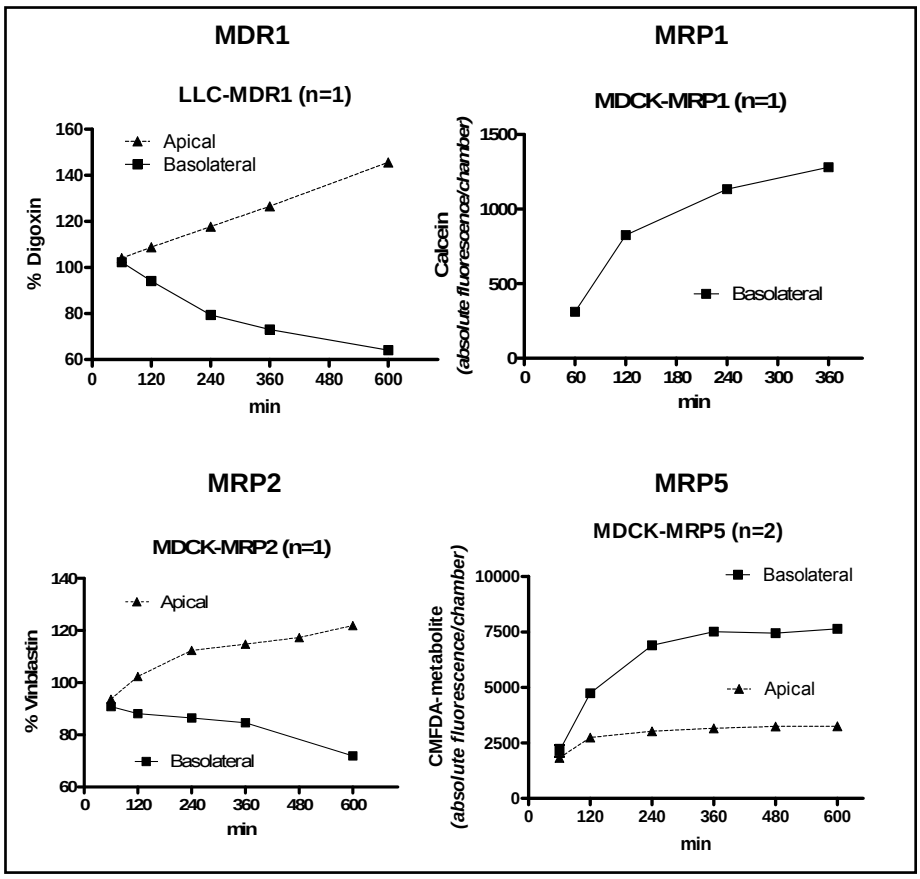

Figure 5.1 Representative examples for transport of reference compounds in concentration equilibrium conditions, as described in Materials and Methods. To demonstrate functionality of human transporters in the transfectants, transport assays with reference compounds were performed in parallel to valproate experiments. Transport of digoxin and vinblastine by human Pgp (MDR1) and human MRP2, respectively (graphs on the left); occur in direction basolateral (■) to apical (▲), since drug concentrations are clearly enriched in the apical compartment. Transport of calcein and CMFDA (intermediate, fluorescent products) are transported mainly to the basolateral compartment (graphs on the right) by MRP1 and MRP5, respectively. In the case of calcein (right, above), no fluorescence was detected in the apical compartment in the experiment shown here (background fluorescence in medium was subtracted from fluorescence in samples). Usually only one or two wells were used as controls ("*n*" indicated in brackets) in parallel to the experiments with valproate.

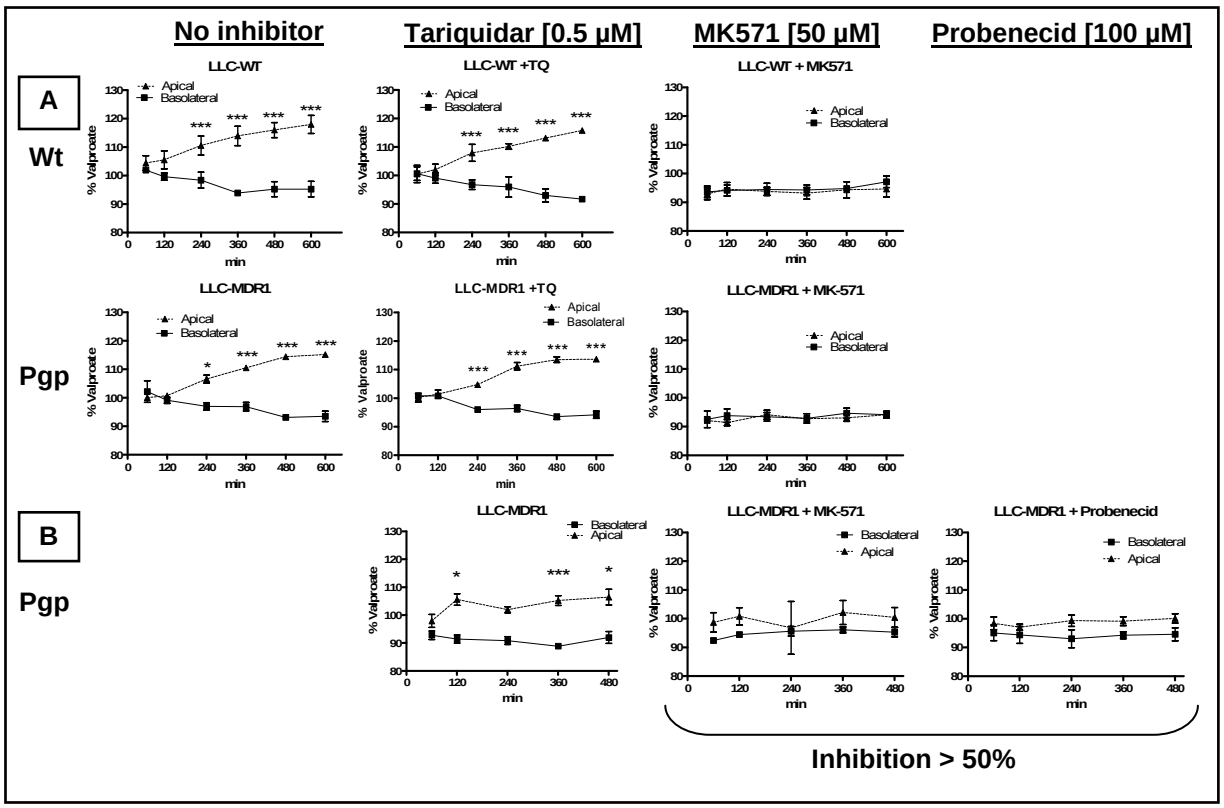

Figure 5.2 Transport of valproate in LLC-PK1 cells in the concentration equilibrium transport assay (CETA). (A) Representative results of experiments with MDR1 transfectants and parental (Wt) cell lines, where a clear basolateral (■) to apical (▲) transport occurs, that can be inhibited with MRP inhibitor MK571, but not with tariquidar, a Pgp blocker. (B) Results of experiments in which tariquidar was added alone or concomitantly with MK571 or probenecid. In this case, transport was inhibited by MK571 and by probenecid. Statistical analyses were performed with two-way ANOVA for repeated measurements followed by Bonferroni post-hoc tests, with statistical differences expressed with asterisks (*P<0.05; **P<0.01; ***P<0.001). Bars indicate SEM; when no bar is visible, errors are within the size of the symbols.

Transport of valproate in MDCK II cells (wildtype and transfectants)

Given that valproate transport in LLC-PK1 cells is efficiently inhibited by MK571 and, thus, probably mediated by endogenous MRPs, we wanted to explore the role of human MRPs in the transport of this compound. For this purpose we utilized MDCK II cells that were transfected for expression of respective human MRP1, MRP2 and MRP5, and compared them to the parental cell line. Surprisingly, no MRP transporter of the investigated ones was clearly implicated in the transport of this AED (Fig. 5.3). valproate concentrations were slightly, but significantly enriched in the apical chamber of parental cell lines and MRP5 transfectants. However, no implication of human MRP5 could be demonstrated, since this transporter is expressed at the basolateral membrane, and MK571 did not influence the observed basolateral to apical transport. More interesting is

the fact that, although an evident involvement of endogenous transporters, no transport was observed in MRP1 or MRP2 transfectants, meaning that such an endogenous transporter is differentially expressed among these MDCK II sub-clones. Transport experiment was repeated for MRP5 transfectants, comparing cells incubated with tariquidar plus MK571, and cells with tariquidar plus probenecid (Table 5.1), where a transport to the apical chamber was evident even in presence of MK571 and probenecid, but the latter inhibited the transport of valproate by 78% (figure not shown).

Table 5.1 Apical area under concentration x time curves for valproic acid

Cell line	AUC/h in apical chamber					
	No inhibitor	TQ	MK	Inhibition (%)	PBC[a]	Inhibition (%)
LLC-WT	301.5	489.9	0.0	100	Nd	nd
LLC-MDR1	283.0	321.1	21.2	93	119.7	58
MDCK-WT	117.0	nd	132	0	Nd	nd
MDCK-MRP1	0.0	nd	0.0	--	Nd	nd
MDCK-MRP2	0.0	nd	79.3	0	Nd	nd
MDCK-MRP5	155.8	nd	156.4[b]	0	33.8	78

TQ: tariquidar; MK: MK571; PBC: probenecid; AUC: area under concentration (in percent) x time curve; nd: not determined. Results are the mean of at least two experiments, except for all experiments with probenecid, and experiments with MDCK-MRP1, and MDCK-MRP2, where one experiment was performed in triplicate.

(a) In experiments with probenecid, the inhibitor tariquidar was also present.

(b) In one of the experiments, tariquidar was also present.

5.5. Discussion

Pharmaco-physical and pharmacokinetic characteristics of valproate suggest that it is transported into, but also out from brain tissue by active transporters and/or carriers. First, although lipophilicity of valproate at isoelectrical point (logP) is relatively high (calculated logP= 2.54, experimental logP= 2.7 [http://www.drugbank.ca]), at physiological pH valproate is, instead, poorly lipophilic (Löscher & Esenwein, 1978) with a calculated logD (pH 7.4) that varies from -0.02 to 0.16 (Liu et al., 2004; http://www.chemspider.com). Second, although this unfavourable characteristic, valproate rapidly permeates into the brain after intravenous injection in rats, mice and dogs (Löscher & Esenwein, 1978; Löscher & Nau, 1983), and its pharmacological effects in neural tissue demonstrate its permeation into the brain. These previous characteristics point out there is an uptake system influencing valproate permeation at the BBB. Third, although able to reach brain parenchyma, valproate has low brain-to-

plasma concentrations in humans, rats and dogs which are usually below cero (Vajda et al., 1981; Löscher & Nau, 1983; Wieser, 1991). Fourth, there is strong evidence that valproate-efflux occurs at the BBB and that the efflux clearance is higher than the influx clearance (Cornford & Oldendorf, 1986; Kakee et al., 2002). Fifth, brain and cerebrospinal concentrations of valproate are increased by systemic administration of probenecid (Frey & Löscher, 1978; Scism et al., 2000).

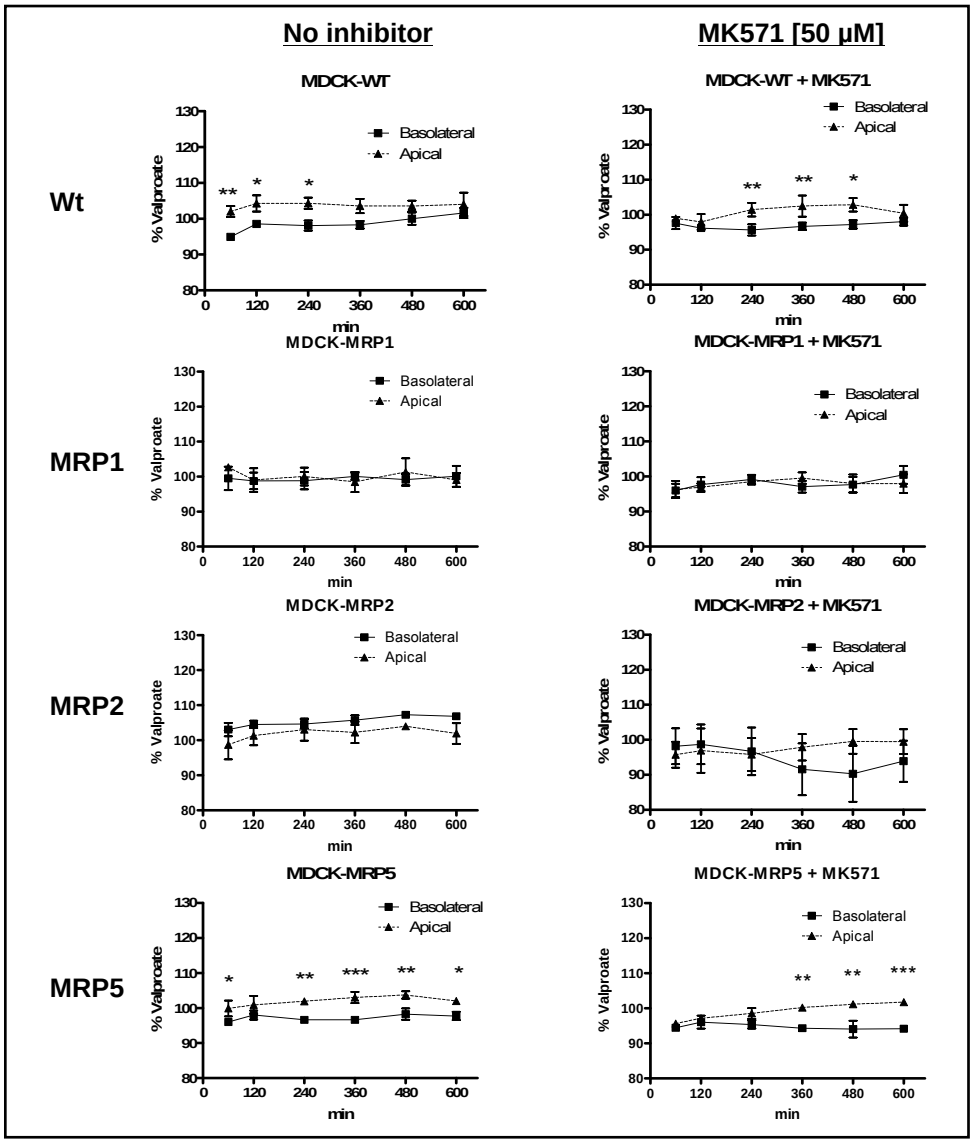

Figure 5.3 Transport of valproate by endogenous transporters of MDCK II cells in concentration-equilibrium transport assay, as described in Materials and Methods. Small apical transport of valproate was observed in parental cells (Wt) and MRP5 transfectants, but not in MRP1 or MRP2 transfectants. The use of MRPs inhibitor MK571 did not influence the basolateral-to-apical transport. For more details see legends of previous figures.

Influx carriers of valproate are presumably located at luminal and abluminal phases of BBB. Medium-chain fatty acids can compete with valproate uptake in the rat brain-

perfusion model, and the uptake process measured in rat brain capillaries can be inhibited with probenecid (Naora & Shen, 1995; Adkinson & Shen, 1996). Fischer et al. (2008) also described the inhibition of valproate-uptake in the rat brain capillary immortalized cell line RBE4, and in Caco-2 cells, but it was also inhibited by several other compounds. The authors suggested that the monocarboxylate transporter 1 (MCT1) is the possible transporter mediating valproate influx in these cell lines (Fischer et al., 2008). On the other hand, little is known about the efflux transporters involved in valproate brain-extrusion, and whether they belong to the group of known up-regulated transporters in pharmacoresistant epilepsy.

The clear transport of valproate we report here in the LLC-PK1 cells is an active process, since the drug is extruded to the apical compartment, creating a concentration difference between both chambers. Furthermore it is evident that the apical transporter involved in the process is not Pgp, since no difference between Pgp-over-expressing cells and the parental cell lines exist, and the Pgp inhibitor tariquidar can not influence the transport. MK571, a commonly used MRP inhibitor for investigation of drug transport mediated by these proteins (Dallas et al., 2006), completely inhibited valproate transport. On the other hand, probenecid, a drug that can inhibit MRPs less potently than MK571 but also inhibits several organic anion transporters (Dallas et al., 2006; Borst et al., 2007), showed a 58% inhibition in LLC-MDR1 cells. Gibbs et al (2004) reported that uptake of valproate in bovine brain endothelial cells is susceptible to inhibition with probenecid and indomethacine, and that this inhibition has a biphasic behaviour, with lower concentrations inhibiting efflux transporters, and higher concentrations inhibiting influx transporters. Notwithstanding, the concentrations of probenecid they utilized were far higher than the ones we used here (from 0.2 to 2.0 mM in their case), and as previously mentioned, it also inhibits organic anion transporters. Similarly, Ogawa et al. (2006) studied the transport of valproate in inside-out vesicles of erythrocytes, and found that it can not only inhibit the transport of 2,4-dinitrophenyl-S-glutathione, a substrate for MRPs, but also is directly transported in this model, and the transport is susceptible to inhibition with indomethacine, taurocholic acid and prostaglandin E1, which are known MRP inhibitors (Ogawa et al., 2006). Other study performed in rabbits using microdialysis, shows that probenecid can increase valproate concentrations in brain tissue, although brain-plasma partition coefficient was just slightly changed (Scism et al., 2000). However, those studies used MRP inhibitors that also can affect other transporters. In our study, we used the rather specific MRP inhibitor MK571, what in turn reflects more directly the role of this transporter subfamily

in active efflux of valproate.

Functional analyses have shown that LLC-PK1 has low, but functional expression of breast cancer related protein (BCRP or ABCG2 [Imai et al., 2003]). BCRP inhibition requires a higher concentration of tariquidar than the one we used here, to be achieved effectively (Mistry et al., 2001; Robey et al., 2004; Kühnle et al., 2009). However, it is not known that MK571 inhibits BCRP. In fact, it is widely used for pharmacological inhibition in order to dissect BCRP or Pgp functionality from MRPs functionality (van der Kolk et al., 2002; Schrickx et al., 2007). Hence, a role for this protein in transport of valproate is not very likely. The experiments in LLC-PK1 altogether demonstrate that there is an active transporter with moderate affinity for valproate, and that it probably belongs to the MRP subfamily of ABC-transporters, since it is clearly susceptible to inhibition with MK571 and probenecid.

In our laboratory we have observed a relatively low expression of endogenous transporters in LLC-PK1 cells (data not published); therefore it is the cell line of choice for transport experiments with human Pgp. On the other hand, other authors have shown that LLC-PK1 cells can express MRP1 and MRP2 (measured by mRNA expression [Goh et al., 2002]). However, no transport of valproate in MRP1 or MRP2 over-expressing MDCK II cells was evident. In fact, no transport at all was seen in these cell lines that could suggest its affinity for valproate, what is in accordance with a previous report (Baltes et al, 2007b).

Since MDCK-MRP2 cells express human MRP2 on the apical membrane, and the transport of valproate in parental cells is directed to apical chamber, one would expect a higher transport to the apical chamber if human MRP2 were involved, but this did not occur.

Human MRP1 and MRP5 are expressed on the basolateral membrane of MDCK II transfectants. Since the slight transport observed in the parental cells is basolateral to apical, it may occur that endogenous transporters conceal any apical to basolateral transport of human MRPs. However, if it were the case one would expect an apical transport in MRP1 transfectants similar to parental cells, after adition of MK571, but this did not occur in MDCK-MRP1.

Our results in MDCK-MRP1 and MDCK-MRP2 suggest that the endogenous transporters influencing valproate efflux in parental cells are down-regulated in these transfectants. It is a common feature of MDCK II that expression of exogenous transporters after transfection leads to up or down-regulation of endogenous ones (Flanagan et al., 2002). For instance, we have observed a higher expression of

endogenous Pgp in MDCK-MRP2 and MDCK-MRP5 than in parental cell lines, as measured by means of Western blot (data not published). Taken together, the results obtained from MDCK II cells, both parental and transfectants, do not allow suggesting an involvement of specific MRP transporters in the transport of valproate. Contrarily to what occurred in LLC-PK1 cells, slight transport in MDCK II parental cells could not be inhibited with MK571.

In conclusion, our data support that valproate is a substrate for endogenous (pig) MRP transporters expressed in LLC-PK1 immortalized cells, but endogenous (canine) transporters of MDCK II cells other than MRPs are involved in the transport of valproate. No evidence of valproate transport mediated by human MRP1, MRP2 or MRP5 was found.

Given the charactetistics of valproate at physiological pH (i.e., low lipophilicity) it is probably suitable to investigate its transport in the vesicle-uptake model. The latter is perhaps not so suitable for other AEDs, with high lipophilicity at physiological pH, and very fast permeability. It would be also interesting to investigate which other apical MRP transporters are expressed in the LLC-PK1 cells, and to screen valproate transport in cells where the respective MRP transporters were selectively silenced.

Chapter 6

Assessment of several types of blood-brain barrier endothelial cells for studies of Pgp-mediated drug transport of highly permeable compounds

Chapter 6: Assessment of several types of blood-brain barrier endothelial cells for studies of Pgp-mediated drug transport of highly permeable compounds

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6.1. Abstract

Multidrug transporters that are expressed at the luminal side of the blood-brain barrier (BBB) endothelium, such as Pgp (Pgp), limit the brain disposition of a wide range of drugs. On the other hand, it has been demonstrated that certain central nervous system (CNS)-active drugs are also substrates for Pgp, meaning that the drugs can still overcome the influence of this transporter in virtue of their high passive permeability. According to the multidrug transporter hypothesis of pharmacoresistance in epilepsy, the over-expression of Pgp and other transporters at the BBB may reduce antiepileptic drug (AED) concentration in the brain-target tissue. It has been shown that Pgp can transport several AEDs, such as phenytoin, but it is not known whether and in which extent its expression in endothelial cells can influence drug efflux. Hence, it was our aim to standardize cell models based on brain capillary endothelial cells (BCECs) from rat and swine for testing the drug transport of the AED phenytoin. For this purpose, uptake experiments in immortalized rat BCECs (GPNT and RBE4) and a transport experiment with primary porcine BCECs were performed. The preliminary results of this work show that GPNT cells that over-express Pgp had lower phenytoin accumulation than cells with lower Pgp expression. Furthermore, RBE4 cells transfected with human MDR1 (Pgp encoding gene) had also lower phenytoin accumulation than the non-transfected cells. Finally, our preliminary results with primary cultures show high variability in growth and tightness of monolayers among preparation-charges, especially in case of rat BCECs. One experiment with phenytoin showed that this drug is transported in this cell system, and that the cells express Pgp. Conclusion: The present preliminary results show that uptake experiments in immortalized brain endothelial cells using AEDs may constitute a feasible model for evaluation of drug transport. However, further experiments with Pgp inhibitors are required to fully standardize the model. Primary porcine endothelial cells also may be useful as transport model, but optimization of the method, the manipulation of transfected cells, and the determination of the best time for the assay are required for the standardization of this model.

6.2. Introduction

Drug permeation into the brain is influenced by several mechanisms including passive permeability and ATP binding cassette (ABC) transporters, among the most important (Mahar Doan et al., 2002; Pardridge et al., 2003). Most CNS active drugs are

characterized by small molecular sizes, usually less than 500 Da (Pardridge, 2003); and high passive permeabilities that allow them crossing the BBB (Mahar Doan et al., 2002). On the other hand, ABC transporters located on the luminal membrane of the BBB endothelium, such as Pgp, extrude actively a wide range of chemically dissimilar compounds and, hence, limit drug access into brain tissue. Because of their efflux ability, many potentially active CNS drugs do not gain access into the brain and are consequently inactive when administered systemically, e.g. the opioid loperamide. However, several reports evidence that some CNS active molecules are also substrates for Pgp, e.g., risperidone, and that they are still able to reach the brain parenchyma in virtue of their high passive permeability (Feng et al., 2008).

The multidrug transporter hypothesis of pharmacoresistance in epilepsy proposes that the observed over-expression of ABC transporters, and particularly Pgp, at the BBB in pharmacoresistant patients and in animal models of epilepsy, can lead to an increased efflux of AEDs into the blood, with consequent lower concentrations in the target tissue (Löscher & Potschka 2005a,b; Schmidt & Löscher, 2009). It has been reported that the localized transporter over-expression is functional and mediates pharmacoresistance to phenobarbital and phenytoin in animal models of epilepsy (Brandt et al., 2006; van Vliet et al., 2006). Both drugs are highly permeable compounds with good CNS pharmacokinetics. Thus, it seems that at least in animal models of epilepsy the over-expression of efflux transporters affects significantly the brain permeation of a drug that otherwise is able to reach high enough brain concentrations. However, such a direct evidence of altered drug pharmacokinetics in intractable epileptic patients is lacking so far

We recently reported on the ability of the human Pgp to transport several AEDs in epithelial kidney LLC-MDR1 cells, which are used as an *in vitro* model for investigation of substrate specificity (Luna-Tortós et al., 2008). This finding supports the multidrug transporter hypothesis of intractable epilepsy, since it represents direct evidence that several AEDs are substrates for human Pgp. Notwithstanding, it is not known whether differences in Pgp expression in BBB endothelial cells affect drug efflux to a significant extent. Garberg et al. (2005) reported on remarkable differences in drug transport by Pgp between the epithelial cell line MDCK II and several brain endothelial cells (primary and immortalized) from different species. Hence, our aim in the present work was to establish BBB-endothelium-based cell models that allow us investigating Pgp-mediated drug efflux of highly permeable substrates. For this purpose we used the AED phenytoin, a highly permeable, human- and mouse-Pgp substrate (Baltes et al., 2007a;

Cucullo et al., 2007; Luna et al., 2008) as positive control for testing the transport in immortalized (GPNT and RBE4), and primary (porcine and rat) brain capillary endothelial cells. Here we show preliminary results obtained from uptake and concentration-equilibrium transport assays.

6.3. Materials and methods

Chemicals and drugs

Alpha-MEM, Opti-MEM®, DMEM, DMEM-F12, Ham's F10, Medium 199, trypsin-EDTA, HEPES, HBSS and penicillin/streptomycin were obtained from GIBCO® (Invitrogen Corporation, Eggenstein, Germany), basic Fibroblast Growth Factor (b-FGF) from Invitrogen (Carlsbad, CA, USA); EBM-2 from Cambrex (Lonza Verviers SPRL, Aachen, Germany); and fetal calf serum and percoll from Linaris GmbH (Wertheim-Bettingen, Germany). Fibronectin was obtained from Biochrom AG (Berlin, Germany). Collagen type I, Complete® Protease Inhibitor Cocktail, Dispase II neutral protease and collagenase-dispase were obtained from Roche Diagnostics (Mannheim, Germany). Monoclonal mouse-anti-Pgp antibody (C219) was obtained from Signet™ Laboratories (Dedham, MA, USA). Polyclonal rabbit-anti-GFAP (glial fibrillary acidic protein), and secondary anti-mouse IgG-HRP and anti-rabbit IgG-HRP antibodies were purchased from Dako Cytomatics (Glostrup, Denmark). Polyclonal rabbit-anti-GLUT1 was obtained from Chemicon (Hofheim, Germany). Secondary Cy2-conjugated or biotin-conjugated-streptavidin antibodies were obtained from Jackson Immunoresearch (Jackson Immunoresearch Laboratories Inc., USA). Other reagents for Western blot were from Carl Roth (Karlsruhe, Germany), unless otherwise specified. Phenytoin was provided by Desitin (Hamburg, Germany), and [14C]-mannitol from Amersham (Buckinghamshire, U.K.). Puromycin-dihydrochloride, hydrocortisone, cAMP, collagen type IV from human placenta, lucifer yellow, 4',6-Diamino-2-phenylindole dihydrochloride (DAPI), trypan blue, and monoclonal mouse-anti-vimentin-Cy3 and rabbit-anti-actin antibodies were obtained from Sigma–Aldrich (Taufkirchenn, Germany). RO 20-1724 was obtained from Tocris/Biotrend (Cologne, Germany), and plasma-derived bovine serum (BPDS) was obtained from First Link Ltd. (U.K.). Stock solutions of phenytoin were prepared in ethanol (0.4% final ethanol concentration in medium), and lucifer yellow was dissolved in medium.

Cell culture

GPNT and RBE4 cells

The GPNT and RBE4 cell lines were kindly provided by Prof. Françoise Roux (INSERM U26, Hôpital Fernand Widal, Paris, France). Both cell lines were cultured on collagen type I-coated 100 mm Petri-dishes and maintained (according to a protocol provided by F. Roux) in alpha- MEM/Ham's F-10 (1:1 vol/vol) medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 ng/ml basic b-FGF in 5% CO_2, 95% air-humidity at 37°C. RBE4 cells were also supplemented with 2 mM L-glutamine. GPNT cells were supplemented with 5 mg/ml puromycin (only first two passages, then without puromycin).

Endothelial cell preparation and cultivation

a. Rat brain capillary endothelial cells

Rat brain capillary endothelial cells (rBCECs) were prepared according to Perrière et al. (2005). Two to three weeks old Sprague Dawley rats from own breeding were decapitated after CO_2 anaesthesia. Heads were disinfected in 70% ethanol solution, and transported in PBS on ice. Dissection of brains was performed under sterile conditions. Brains were removed from skull and placed on PBS supplemented with 1% penicillin-streptomycin on ice. The midbrain, meninges and larger vessels were removed by dissection and by rolling the brains on autoclaved, dry gel-blotting paper. Brain cortices were washed with PBS and supernatant was removed. The tissue was chopped with scalpels and resuspended in 15 ml enzyme solution per 5/6 brains (DMEM/F12 containing 270 U/ml collagenase II, 2 mg/ml dispase II, 5 ng/ml DNAse, and 1% penicillin-streptomycin). The suspension was incubated during 1.5 hours at 37ºC with gentle agitation. First digestion was stopped with 20% BSA in DMEM/F12 and the suspension was centrifuged (1,000 g, 15 min, 4ºC). Separated myelin and detritus were decanted. The microvessel-containing fraction (on the bottom of the tube) was resuspended in enzyme solution (5 ml for 5/6 brains), and incubated for second digestion during 1 hour at 37ºC with gentle agitation. The suspension was filtered through a 10 µm pore nylon mesh. The remaining capillaries were gently washed with DMEM/F12 and seeded onto 35 mm collagen IV-coated Petri dishes. Cells were maintained in selection-medium during 48 hours (EBM-2 medium supplemented with 20% BPDS, 1% penicillin-streptomycin, 10 µM HEPES, 2 mM glutamine, 0.5 µg/ml hydrocortisone, and 4 µg/ml puromycin). Puromycin allows for selection of Pgp-expressing endothelial cells. Thereafter, medium was changed to growth-medium

consisting of the same components, but without puromycin and supplemented with 2 ng/ml bFGF. Monolayers reaching 80 to 90% confluences were harvested by trypsinization and cells were seeded on collagen IV-fibronectin-coated inserts of 12-well Transwell plates (Transwell-Clear®, Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.1 to 0.2 x 10^6 cells/cm^2, and maintained with differentiation-medium consisting of the same basal medium without puromycin, but supplemented with 1 ng/ml bFGF.

b. Porcine brain capillary endothelial cells

Porcine brain capillary endothelial cells (pBCECs) were prepared as described elsewhere (Audus & Borchardt, 1986; Gutmann et al., 1999). Two 30 kg-weight pigs (Institut für Tierzucht Mariensee, Neustadt, Germany) were anaesthetized with azaperone (2 mg/Kg) and pentobarbital (80 mg/Kg) and euthanized with pentobarbital. Brains were removed from skull and transported in artificial cerebrospinal fluid NaCl 118 mM, KCl 3 mM, Na_3PO_4 x 12 H_2O 0.7 mM, $NaHCO_3$ 18 mM, urea 2 mM, D-glucose 12 mM, $MgCl_2$ x 6 H_2O 0.8 mM, $CaCl_2$ x 2 H_2O 1.4 mM, pH 7.4) on ice. Further steps were performed at the clean bench. Brains were cut sagitally into halves, and meninges and larger vessels were carefully removed by dissection. Brain hemispheres were washed on ice cold artificial cerebrospinal fluid and further removal of vessels and meninges was carried out (this was repeated until visibly complete removal of meninges and vessels). Thereafter, the grey matter was removed with a sterile scalpel, collected onto a cell culture dish placed on ice, and mechanically dissociated with scalpels. The homogenate was suspended in 90 ml enzyme solution (5 mg/ml dispase dissolved in preparation medium consisting of Medium 199 supplemented with 0.8 mM L-glutamine, 1% penicillin-streptomycin, 100 µg/ml gentamycin and 10 mM HEPES, pH 7.4) and incubated for 2 hours at 37ºC with gentle agitation. 30 minutes after beginning of incubation, pH was checked again and calibrated to 7.4. After first digestion, suspension was centrifuged (1,000 g, 10 min, 4ºC), supernatant was discarded and pellets were resuspended in preparation medium containing 15% dextran followed by centrifugation (5,000 g, 10 min, 4ºC). The supernatant was carefully removed and the microvessel-containing pellets were resuspended and collected in 5 ml of preparation medium. Following centrifugation (1,000 g, 5 min, 4ºC), tissue was resuspended in 7 ml collagenase/dispase solution (1 mg/ml dissolved in preparation medium, warmed up till 37ºC just before use) and incubated for 1.5 h to 2 h at 37ºC. Thereafter, cell suspension was filtered through a 150 µm-pore nylon mesh adding cold preparation medium

supplemented with 10% horse serum (HS) for washing; then, centrifuged (130 g, 10 min, 4ºC), resuspended in 2 ml preparation medium with 10% HS, and 1 ml of the suspension was carefully placed on the top of a percoll-gradient solution consisting of high (1.07 g/ml) and low (1.03 g/ml) density percoll-phases (phenol-free HBSS 1x, 0.8 mM L-glutamine, 1% penicillin-streptomycin, 10 mM HEPES, percoll 1.13 g/ml and phenol-containing HBSS 10x); and centrifuged (1,250 g, 10 min, 4ºC). The fraction between both phases containing the microvessels was carefully collected with a Pasteur pipette and resuspended in 20 ml preparation medium with 10% HS and centrifuged (130 g, 10 min, 4ºC). Cells were resuspended, filtered (150 µm-pore nylon mesh), and centrifuged again. After discarding the supernatant, the cells were resuspended in culture medium (similar composition as preparation medium, but without gentamycin and supplemented with 20% HS), seeded on collagen type IV-coated 75-T flasks at a density of 7 x 10^6 cells per flask, and cultivated during 48 hours in presence of 4 µg/ml puromycin. Thereafter, cells were rinsed with HBSS and maintained in medium without puromycin. When 90% confluence was reached, cells were harvested with trypsin-EDTA and seeded on collagen IV-fibronectin-coated inserts (see below) or cryo-preserved in culture medium with HS (20 % final concentration) and DMSO (10% final concentration).

Astrocyte preparation and cultivation

Astrocytes were prepared from cerebral cortices of 1 day old Wistar rats (Charles River, Sulzfeld, Germany). A mechanic method and an enzymatic method were used separately (5 animals per group). In both cases, after decapitation the heads were briefly disinfected in 70% ethanol, and transferred to a sterile clean-bench, where brains were aseptically removed, and placed on sterile culture dishes containing ice-cold PBS. The midbrain, meninges and large vessels were removed by dissection, and brains were split into two groups for different dissociation methods. The mechanical method was performed as described elsewhere (Vantelon et al., 2007). Cerebral cortices were chopped with scalpels, then Dulbecco's modified Eagle medium (DMEM) containing 1% penicillin-streptomycin was added, and tissue was further dissociated with a Pasteur pipette for 2 min. The suspension was filtered through a 70 µm pore size nylon mesh. Cells were counted with trypan blue and seeded at a density of 1X10^6 living cells/ml on 75-T flasks (2 flasks per 5 brains were obtained) and maintained with DMEM supplemented with 1% penicillin-streptomycin and 10% foetal calve serum (FCS).

An enzymatic dissociation method has also been employed as described elsewhere (Zhang et al., 2006). Cerebral cortices were chopped with scalpels and 10 ml dissociation mixture consisting of DMEM, 0.4% trypsin-EDTA, 20 µM HEPES and 100 µg/ml DNAse were added per 5 brains. Tissue was further mixed with 5 ml pipette, and resuspended cells were incubated during 30 min at 37ºC with gentle agitation. Digested tissue was further dissociated with a pipette and centrifuged (400 g, 5 min). The pellet was resuspended with 15 ml astrocytes-medium (DMEM supplemented with 10% FCS and 1% penicillin-streptomycin). The cell suspension was seeded onto a 75-T flask, and medium was changed on next day.

Every 3 days, cells were shaken during 2 hours at 200 rpm. to dislodge microglia, and medium was replaced. When cells reached about 90% confluence (on the 4th culture day for enzymatic method, and the 10th culture day for mechanic method), they were shaken at 200 rpm overnight, split at 1:5 ratio, and seeded on the bottom of Transwells®, or cover slips for further experiments, or on 75-T flasks for collection of astrocytes-conditioned medium. The intermediate filament glial fibrillary acidic protein (GFAP) was used as specific marker for astroglial cell differentiation (Reuss et al., 2003).

Effect of bFGF, hydrocortisone and endothelial cell medium supplements on astrocytes

No co-culture with rBCECs was performed, but with pBCECs. Since the latter need different culture medium, growth of astrocytes was tested under conditions of pBCEC culture. For this purpose, cells from passage 1 obtained by both preparation methods and fed with astrocyte medium were compared for morphology and growth with their counterparts that were fed with pBCEC medium (see above for medium composition). No differences between both media were observed.

On the other hand, cells were tested for the effect of several factors that are usually added to BCECs. In this case, cells were seeded on cover slips and cultured with astrocyte medium either without further supplements (controls) or with 500 ng/ml hycrocortisone, 1 ng/ml bFGF, or both. Cells were stained on the 5th day after beginning of treatment for determination of glial fibrilary acidic protein (GFAP) and vimentin expressions by means of immunocytochemistry (see below).

GPNT cell selection

For GPNT selection, cells were split several passages without puromycin, in order to obtain cells with low Pgp expression. Monolayers of cells growing on collagen-coated 6

well plates were divided into two groups from the day of confluence. One of them was further supplemented with 5 mg/ml puromycin in order to stimulate the Pgp expression, while the other group was cultured without puromycin until day of the uptake assay or collection for Western blots.

RBE4 and pBCEC cell transfection

For cell transfection, RBE4 growing on collagen-coated 100 mm Petri-dish and pBCEC on collagen type IV-fibronectin-coated 24 mm diameter inserts (Transwell-Clear®) were transfected with human MDR1 as they reached 80% to 90% confluence. EGFP(green-fluorescent protein)-MDR1 cDNA (kindly provided by Prof. Borst, The Netherlands Cancer Institute, Amsterdam, Netherlands) was diluted in OptiMEM® and Fugene® HD transfection reagent (Roche Applied Science) and added to the cells according to manufaturer's instruction. The pBCEC were transfected directly on the assay plate, 1 µg cDNA per 24 mm diameter insert (from 6-well Transwell-Clear® plates) was used, and cells were incubated for about 24 hours with the transfection complex. For 90% confluent RBE4 cells growing on 100 mm Petridish, 6 µg cDNA were added and cells were incubated overnight. On the next day, RBE4 cells were split and seeded at high density (0.1 $x10^6$ cells/cm^2) onto 6 well plates, so that monolayers were 90% confluent within 24 hours. After incubation with transfection complex, medium was routinely changed. In both cases, viability of cells exposed to transfection complex did not appear to be affected, when their growth and confluence were compared with the non-transfected cells. No further selection was used after cell transfection to avoid induction of endogenous transporters. After functional experiments, samples of cells on plates/inserts were washed with PBS and directly observed under a microscope for expression of green fluorescent protein (GFP) before preparing the cells for immunofluorescence.

Western Blotting

Cells grown in parallel to uptake assay (GPNT, RBE4) or used for transport assays (pBCEC) were washed with ice-cold PBS and lysed with protein lysis buffer (25 mM Tris, 50 mM NaCl, 0.5% Na-deoxycholate, 0.5% Triton X-100, 1x Complete® Protease Inhibitor Cocktail, pH=8.0), which was directly applied on wells/inserts on ice, following 30 min incubation on ice and centrifugation for 15 min at 16,000 g to separate DNA. Aliquots were immediately frozen until analysis by Western blotting. For analysis of Pgp expression, 50 µg total cell proteins were separated on 7.5% polyacrylamide gel (SDS-

PAGE) and transferred onto PVDF membrane. Pgp was probed using the C219 (dilution 1:200) / antimouse IgG-HRP (1:1000) antibodies. Actin (loading control) was probed with anti-actin (1:5000) / anti-rabbit IgG-HRP (1:10000). Bands were visualized by ECL Super-signal Pico assay (Pierce Biotechnology, Rockford, IL, USA) and X-ray film (Pierce Biotechnology). Signal on film was analyzed by densitometry using ScionImage 4.0 software (Scion Corp., Frederick, MD, USA).

Immunofluorescence

For immunofluorescence, cells on cover slips or inserts were washed three times with PBS, and fixed with ice cold acetone-methanol (1:1 vol/vol) during 10 min followed by permeabilization with 1% Triton X-100 in PBS for 20 min at room temperature. Cells were incubated 2 hours at room temperature or overnight at 4ºC with blocking buffer containing 3% bovine serum albumin in 1% Triton X-100 solution in PBS. For protein expression, the respective primary antibodies were dissolved in blocking solution (1:20 dilution for all, except for Cy3-conjugated anti-vimentin, 1:100) for 1 to 2 hours at room temperature, followed by incubation with the respective secondary antibodies (1:50), with several washing steps with blocking solution in between. Finally, cells were washed once with PBS containing 0.5 µg/ml DAPI; once with PBS, and finally were mounted on glass slides with Dako Fluorescent Mounting Medium (Dako Cytomatics, Glostrup, Denmark). Cells were examined using an Axioskop microscope (Zeiss, Germany).

Measurement of TEER and paracellular permeability

Rat and porcine BCEC were seeded on collagen IV-fibronectin-coated polyester membrane filters (Transwell-Clear®, 12 mm diameter or 12-well Transwells, 0.4 µm pore size, Corning Costar Corporation, MA, USA) at a density of 0.1 cells/cm^2, either after first splitting (rBCEC and pBCEC) or after thawing from liquid nitrogen (pBCEC). For one experiment, pBCEC were also seeded on 24 mm diameter inserts (6-well Transwells) directly after thawing from liquid nitrogen. Cells were cultured till confluence, and TEER measurements were periodically performed using an EVOM resistance meter (WPI Inc., Sarasota, FL), connected to a STX-2 chopstick electrode (for 12-well Transwell plates) or to an Endohm™ measurement chamber (for 6-wells Transwell plates). Measurements were carried out at room temperature in the respective culture medium.

For paracellular permeability, the marker was applied to the apical chamber of the Transwell®-System and samples were taken periodically from the contralatteral

chamber. Polar drugs that diffuse exclusively through the paracellular route were used: Lucifer yellow (100 µM), a fluorescent marker, or [^{14}C]-mannitol (0.1 µCi/ml), a radioactive marker. Volumes of medium were: 1,500 ml basolateral and 500 µl apical for 12-wells Transwells; and 2,700 µl basolateral and 2,000 µl apical for 6-wells Transwells. Volume of samples from basolateral chambers were 50 µl for 12-wells Transwells, and 100 µl for 6-wells Transwells. Appropriate volumes from the apical chambers were removed in order to avoid influences by hydrostatic pressure. Values of empty inserts were subtracted from values of the monolayers.

For two experiments with rBCEC, monolayers were treated with Ro 20-1724 (17.5 µM) and 8-(4-chlorophenylthio)-cAMP (250 µM) 24 hours before recording TEER and permeability values, in order to improve TJ formation (Rubin et al., 1991).

Transport experiment with astrocyte-pBCEC coculture on Transwell®-System

For one experiment with porcine BCECs, a co-culture with astrocytes was performed. pBCECs were thawed from liquid nitrogen and seeded at a density of 0.05 x 10^6 cells/cm^2. When they reached 80 to 90% confluence, the monolayer-containing inserts were transferred to a separate 6-well plate, and cells from one plate were transfected with EGFP-MDR1 cDNA as described above, leaving one plate as parental cells. Then, astrocytes were cultured on the bottom of the Transwells whose inserts were removed. After confluence and transfection, the monolayer-containing inserts were placed back into the respective Transwell-plates, where the astrocytes were growing. It was performed this way to avoid transfection of astrocytes. Since the latter cells were not mature, astrocyte-conditioned medium from 10 days-confluent flasks was mixed with the endothelial medium (1:1 vol/vol). TEER values were recorded daily. At day seven of confluence, transport of phenytoin (50 µM) was performed in concentration equilibrium conditions as previously described (Luna-Tortos et al., 2008). Briefly, the cells were pre-incubated in OptiMEM® containing phenytoin (50 µM) during 30 min to allow equilibration with the medium and to reduce probable influence of drug uptake by astrocytes during the transport experiment. Then, transport experiment was carried out by applying 2,000 µl (apical chamber) and 2,700 µl (basolateral chamber) of OptiMEM® containing 50 µM of phenytoin. Samples were removed from both chambers after 30, 60, 180 and 240 min. Thereafter, medium was changed and [^{14}C]-mannitol test was performed as described above. Samples were removed after 30 and 60 min.

Phenytoin uptake assay

GPNT and RBE4 cells were seeded on collagen-coated 6 well plates and experiments were performed when they reached 6 to 7 days post-confluence. On the day of experiment, cells were washed once with PBS, and pre-incubated in serum-free OptiMEM® for 30 min to allow for equilibration with this low serum medium. Thereafter, cells were incubated with phenytoin [50 µM] for 1 hour (37ºC, 5% CO_2, 95% humidity). Following incubation with the substrate and medium-samples removal, cells were placed on ice, washed once with ice cold PBS, lysed with 100 µl 1% triton X-100 in purified water, the DNA was separated by centrifugation (20,000 g for 15 min), and the supernatant was immediately frozen until analysis by HPLC. Protein concentration in cell lysates was measured by Lowry protein assay from samples before freezing. Amount of accumulated phenytoin in cells (in nmol) was normalized to amount of protein in the sample.

Drug analysis

The concentration of phenytoin in cells and medium was quantified by high performance liquid chromatography (HPLC) with UV-detection as described earlier (Potschka and Löscher 2001; Potschka et al., 2001; Potschka et al., 2002; Potschka et al., 2004a). The radioactivity in samples from experiments with [^{14}C]mannitol was quantified using a scintillation counter. Lucifer yellow was quantified using a Fluoroscan II (LabSystems® Oy; Helsinki, Finland) with 485 nm excitation and 538 nm emission filters

Data analysis and statistics

Data were statistically analyzed by unpaired t-test or two-way ANOVA using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Tests used were two-sided and a $p<0.05$ was considered significant.

6.4. Results

Primary brain endothelial cells and astrocytes

A total of 9 preparations were performed for rBCECs, 1 for pBCECs, and 1 for rat astrocytes. The methods for preparation of endothelial cells and astrocytes yielded cultures with relatively high purity. Primary endothelial cells showed their characteristic spindle morphology in culture (Figure 6.1). Porcine BCEC could be successfully

cultured after keeping them 2 years in liquid nitrogen, and adding endothelial cell growth factor (10 ng/ml) in the medium after thawing. Both primary cell lines typically lost their morphology after second passage; but in some preparations of rBCEC, the morphology was changed after the first passage, indicating cell dedifferentiation. Immunocytochemistry of glucose transporter 1 (GLUT-1; as marker for endothelial cells [Farrell & Pardridge, 1991; Dobrogowska & Vorbrodt, 1999]) showed between 90 and 100% positive pBCECs. The porcine primary cultures also expressed the ABC transporter Pgp. No immunostaining for GLUT-1 was performed in rBCECs.

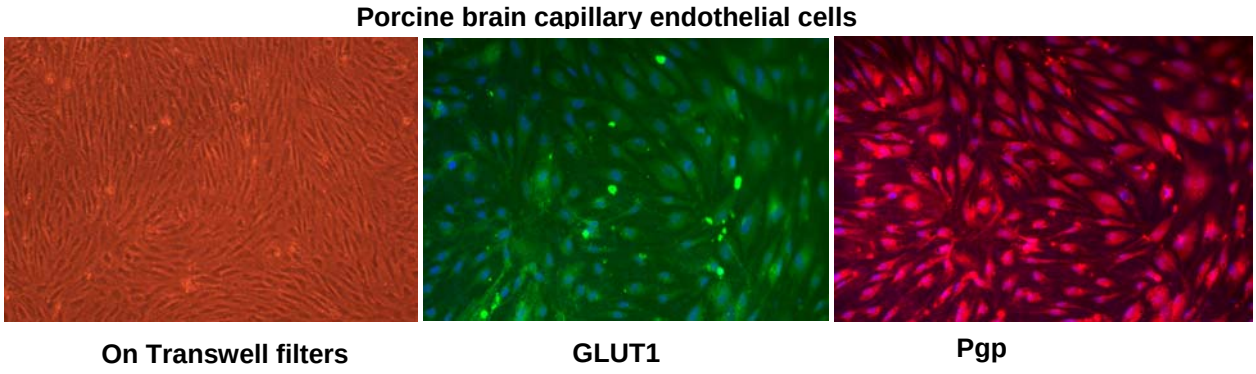

Figure 6.1 Primary porcine brain capillary endothelial cells. Cells show their typical spindle morphology in culture (left; 100x magnification), and express glucose transporter 1 (GLUT1), which is a marker for endothelial cells (middle of the panel; 400x magnification). Pgp (right; 400x magnification) is also expressed in this culture. Cells were immunostained after transport experiment of phenytoin. GLUT1: green; Pgp: red; DAPI: blue.

GFAP was expressed in 73.2% ±7.1 astrocytes cultured in maintenance-medium (Fig. 6.2B). However, expression of vimentin, a marker for all mesenchymal cells that is also expressed in immature astrocytes (Lin & Goldman, 2009), increased after treatment with bFGF. Hence, this factor was not added in medium of co-cultured pBCEC and astrocytes. On the other hand, addition of pBCEC-medium to astrocytes did not alter their morphology or their expression of GFAP (Fig. 6.2B). Addition of 500 ng/ml hydrocortisone for 5 days did not change the expression of GFAP and vimentin (Fig. 6.2D), as compared with the controls.

Table 6.1 summarizes the results for TEER values and paracellular permeability recorded in rat and porcine BCEC monolayers cultured on 12-well Transwell-plates. From the nine rBCEC preparations, only 3 could be used for TEER measurements, since other monolayers did not reach confluence before or after seeding on Transwells. For both primary cell lines, TEER values were usually below 100 $\Omega*cm^2$, although the

permeability for paracellular markers was below 10 x 10^{-6} cm/s. The addition of Rho 20-1724 and cAMP to rBCEC mono-cultures improved the electrical resistance, but did not change the permeability to mannitol.

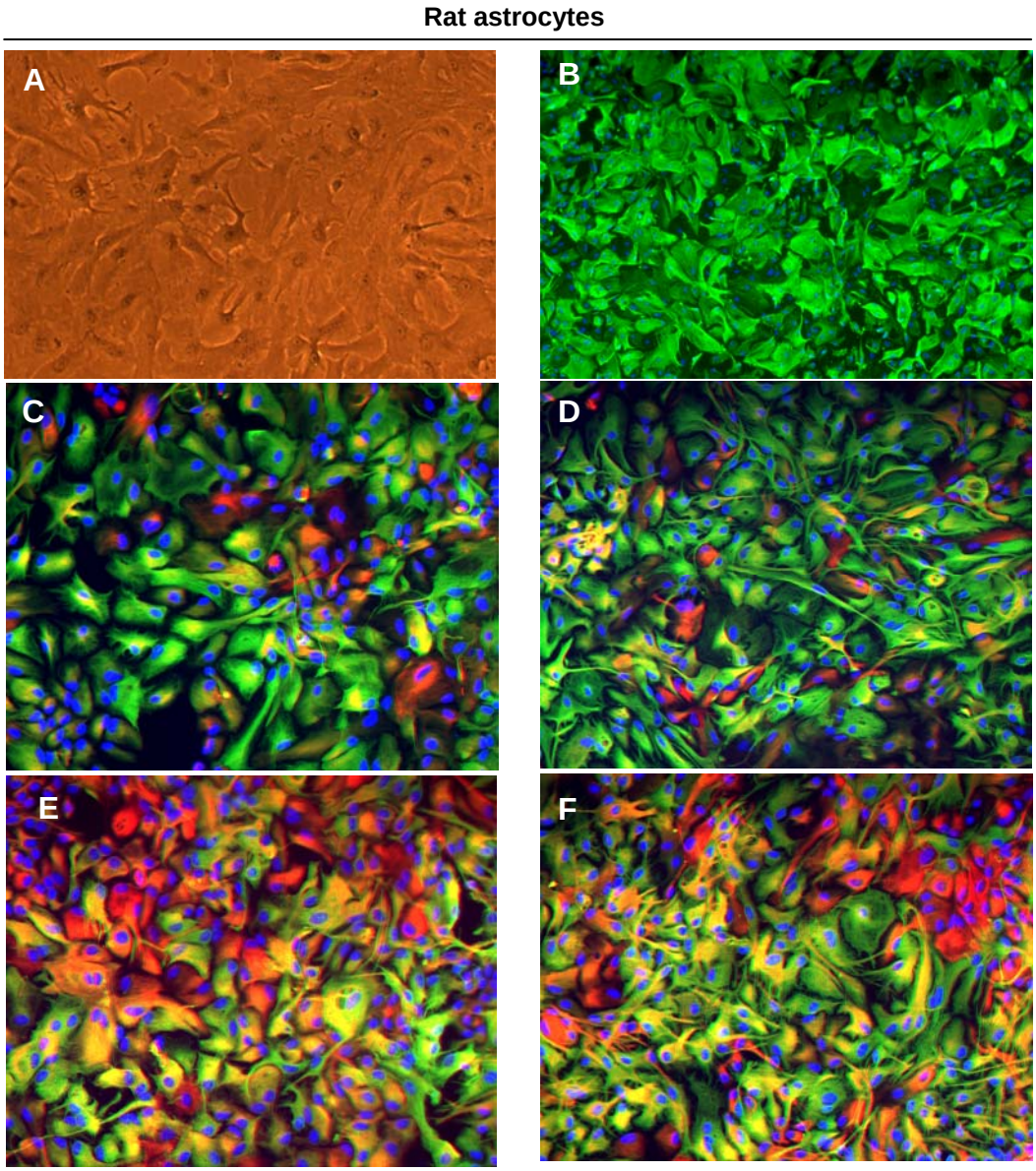

Figure 6.2 Primary cultures of astrocytes. A: Characteristic morphology of astrocytes in culture. B: Astrocytes cultured in pBCEC-medium also express GFAP (green) and maintain their morphology. These cells were immunostained after transport experiments with phenytoin; i.e., when used for coculture with endothelial cells. C: Cells cultured in astrocyte medium, used as controls for GFAP and vimentin expression. D: cells exposed during 5 days to hydrocortisone alone (500 ng/ml) show similar GFAP and vimentin expression as the controls. The addition of bFGF (1 ng/ml), either alone (E) or concomitantly with hydrocortisone (F) increased the expression of vimentin, and they also reached confluence two days earlier than controls or cells treated with hydrocortisone alone. GFAP: green; vimentin: red, DAPI: blue. Magnification: 200x.

The co-culture of pBCEC and astrocytes, and the additional supplementation with astrocyte-conditioned-medium did not improve TEER values in pBCEC (Fig. 6.3). Similarly, [^{14}C]-mannitol permeability was relatively high (16 x 10^{-6} cm/s for the non-transfected cells, and 12 x 10^{-6} cm/s for the transfected ones).

Table 6.1 Transendothelial electrical resistance and paracellular permeability of rat brain endothelial cells

Cells	TEER (Ω*cm^2)		[^{14}C]-mannitol (x 10^{-6} cm/s)		Lucifer yellow (x 10^{-6} cm/s)
	No Tx	Tx	No Tx	Tx	No Tx
rBCEC	65 ±19	70 ±12	3.0 ±0.5	4.4 ±0.9	--
	66 ±2	113 ±7	--	--	--
	65 ±10	--	--	--	7.3 ±3
pBCEC	51 ±19	--	--	--	7.1 ±1

Cells were cultured without astrocytes (monocultures) on 12-wells Transwell-plates as described in materials and methods. No Tx: no treatment; Tx: treatment. Treatment was performed with Ro 20-1724 (17.5 µM) and 8-(4-chlorophenylthio)-cAMP (250 µM) 24 hours before recording TEER and permeability values, in order to improve TJ formation.

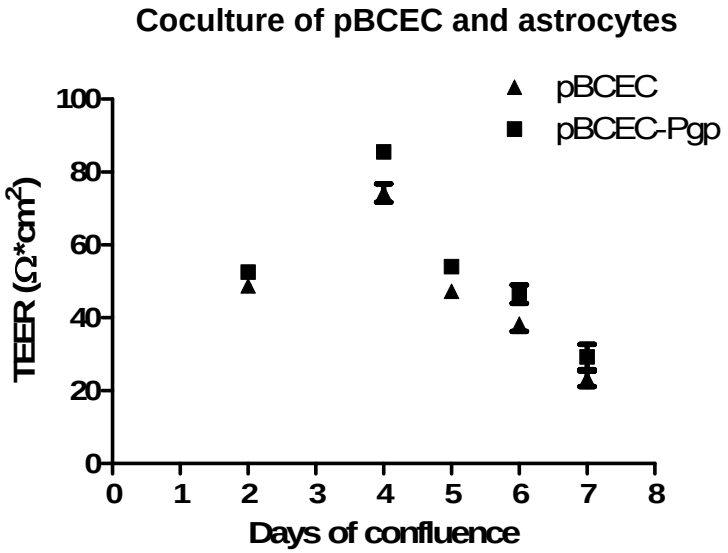

Figure 6.3 Transendothelial electrical resistances of Pgp-transfected and non-transfected pBCEC monolayers in coculture with astrocytes. Cells were transfected on different plate, and maintained separated till day 4 of confluence. At day 4, monolayers were transferred to astrocytes-containing wells (which were just confluent), and cultured with 1:1 vol/vol endothelial-medium/astrocyte-conditioned-medium (the latter from 10 day confluent cultures). At day 6, cells were supplemented with hydrocortisone (110 nM). Transport of phenytoin and [^{14}C]-mannitol permeability determination were performed at day 7.

Pgp expression in primary endothelial cells

As shown in figures 6.1 and 6.5, primary porcine endothelial cells express Pgp, as corroborated by immunocytochemistry. The transfection of primary cells with human MDR1 was successfully corroborated by fluorescence microscopy for GFP expression (Fig. 6.5), although the background fluorescence did not allow for exact positive-cells counting. The relative high Pgp expression in parental cells does not allow for differentiation of Pgp expression between transfected and non-transfected cells by immunocytochemistry technique.

Uptake of phenytoin in GPNT and RBE4 cells

In order to assess the feasibility of uptake experiments for the highly permeable AED and Pgp-substrate phenytoin, puromycin-selected and non-selected GPNT cells were used. A total of six repetitions per group were employed in order to increase power for statistical analysis. As shown in figure 6.4, the induction of endogenous Pgp by means of puromycin selection significantly reduced the intracellular accumulation of phenytoin, indicating rat Pgp-mediated transport. Hence, a similar assay was performed with RBE4 cells, but comparing with transfected cells. Since RBE4 cells previously showed to express low amounts of Pgp in our laboratory conditions, the cells were transfected with human MDR1 (encoding for Pgp) to allow for assessment of human Pgp-mediated drug transport. Figure 6.4 shows that, similarly as Pgp over-expressing GPNT cells, the uptake of phenytoin in transfected RBE4 cells was significantly reduced, indicating functional expression of human Pgp. Fluorescent microscopy showed expression of the fluorescent GFP protein. Unfortunately, a highly fluorescent background in the parental cells did not allow for exact positive-cell counts.

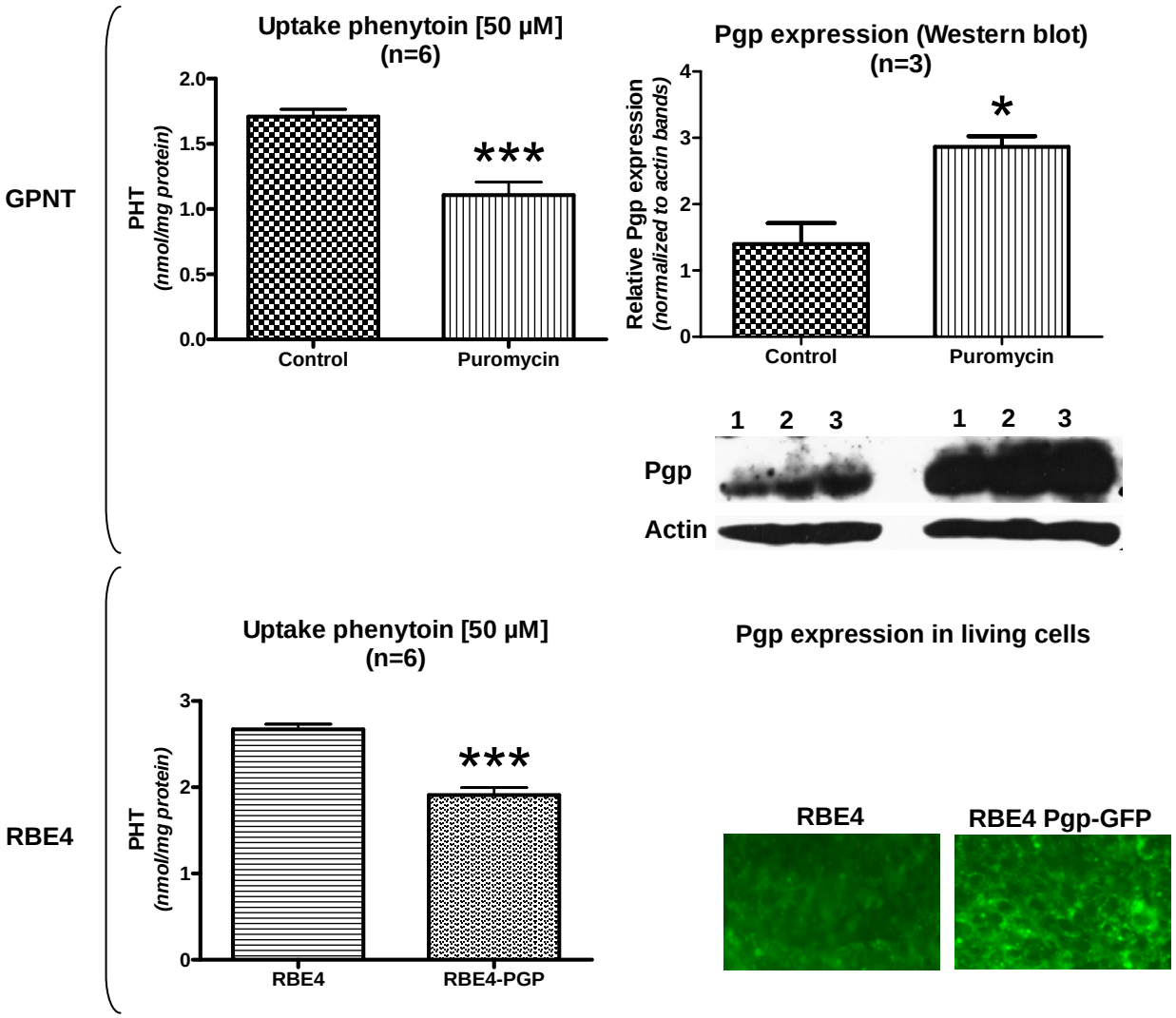

Figure 6.4 Functional uptake assays with the Pgp substrate phenytoin. Cells were incubated with medium containing phenytoin (50 µM) during 1 hour and phenytoin accumulation was measured in cell lysates. GPNT cells that were selected with puromycin express significantly more Pgp, as corroborated by Western blot (right upper panel), and accumulate less phenytoin. Similarly, RBE4 cells transfected with human MDR1 gene show reduced intracellular accumulation of phenytoin. Transfected cells were positive for GFP expression (lower right panel). Unfortunately, Western blot of parallel samples could not be performed in RBE4 cells, since they deattached from the culture plate 3 days before the assay was performed (possibly due to bad collagen-coating). Bars indicate SEM. Statistical analysis was performed with t-Test for unpaired samples. Asterisks show level of significance (*P<0.05; ***P<0.001).

Transport of phenytoin in pBCEC cells

Transport experiments were performed in pBCEC at day 7 of confluence. The figure 6.5 shows that phenytoin was slightly but significantly enriched in the apical chamber of non-transfected pBCEC. Unfortunately, most monolayers were not longer confluent on

the day of experiment, and only three from the non-transfected cells and one from the Pgp-transfected cells could be used for the experiment. Hence, no statistical analysis was possible in case of Pgp transfected cells. The apical-to-basolateral Papp for [^{14}C]-mannitol after phenytoin experiment was 16 x 10^{-6} cm/s for the non-transfected cells, and 12 x 10^{-6} cm/s for the transfected ones. Microscopical examination and immunostaining of cells after experiment show expression of GFP in transfected cells, and a relatively similar Pgp expression in parental and transfected cells, as compared by immunocytochemistry (Fig. 6.5). Areas under concentration above initial concentration (in percent) x time curves normalized per hour were 84.2 and 162.2 in parental cells and transfectants, respectively.

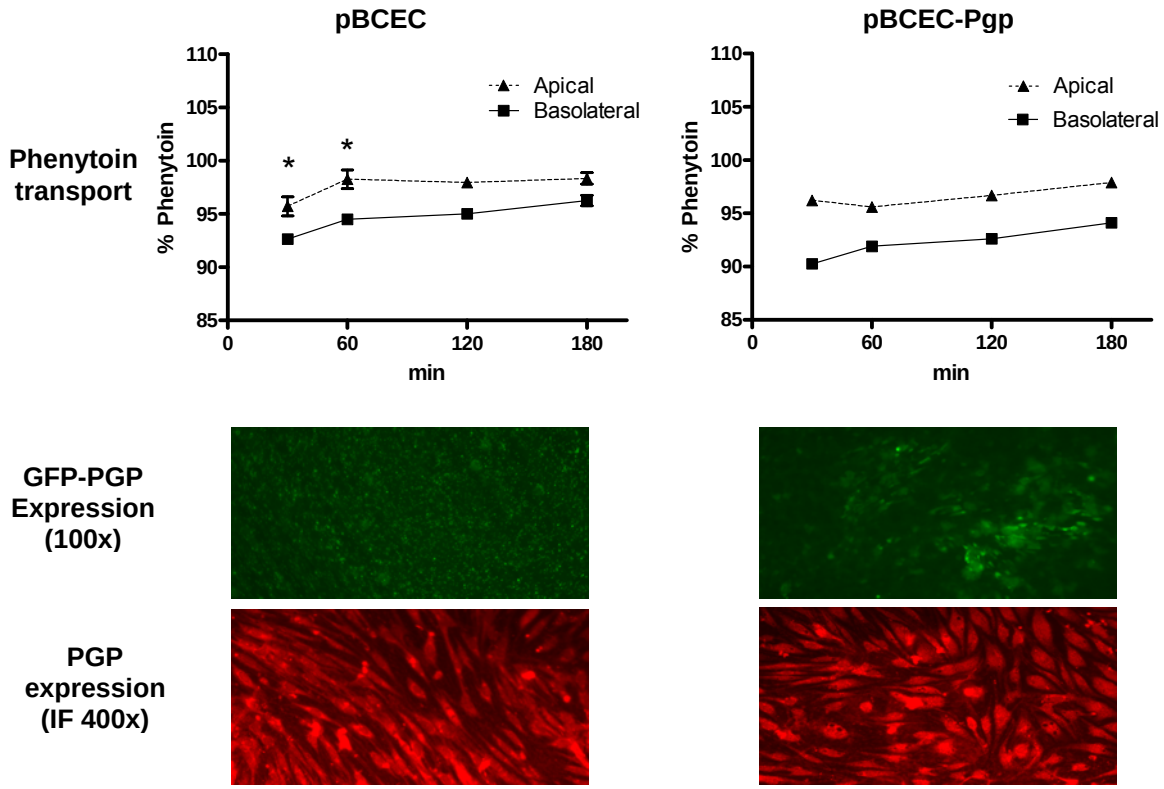

Figure 6.5 Transport experiment and Pgp expression in primary porcine brain capillary endothelial cells (pBCEC) comparing with transfected cells (pBCEC-Pgp). For transport experiments with phenytoin [50 µM] the drug was incubated in both chambers and samples were removed at specified time intervals. For parental cells, 3 wells were used, and statistical analysis was performed with two-way ANOVA for repeated measurements followed by Bonferroni post-hoc tests, with asterisks representing level of significance (*P<0.05). For transfected cells only one well could be used, hence no statistical analysis was possible. Fluorescent GFP-PGP expression was also detected in the transfected cells after transport experiment. Immunocytochemistry for Pgp performed with the cells after transport experiment

demonstrates relatively high expression in both parental (endogenous Pgp) and transfected (endogenous and human Pgp) pBCEC.

6.5. Discussion

Primary brain endothelial cell cultures in the Transwell®-System have been a subject of intensive research in view of the search for better *in vitro* models of the BBB that allow for reliable investigation of CNS drug permeation, and the role of multidrug transporters at the BBB (Garberg et al., 2005; Perrière et al., 2005; Zhang et al., 2006). However, the high variability among preparations and laboratories is known to be one of the most frequent challenges, with considerable variations in TEER values and paracellular permeability (Zhang et al., 2006). Yet it is widely accepted that these tridimensional models, either in monoculture or in co-culture with astrocytes, are far from simulating the tightness of the *in vivo* BBB (Liu et al., 2008).

The methods described in the literature for preparation of primary endothelial cells and astrocytes (Gutmann et al., 1999; Perrière et al., 2005; Zhang et al., 2006; Vantelon et al., 2007) yield high purity cultures. Our results in mono-cultures of rBCEC indicate similar paracellular permeability values to those reported by Perriére et al. (2005), although the recorded resistance was quiet low, and did not increase more than 113 $\Omega*cm^2$ after treatment with RO 20-1724. High variability occurred among preparations and in some cases the cells could even not be used for experiments, since they did not reach confluence. One of the possible explanations for the early stop of growing observed in rBCECs may be the addition of hydrocortisone to the medium during all the cultivation period. It is known that hydrocortisone induces endothelial cell differentiation (Förster et al., 2005), and the continuous exposition to this agent probably induces the cells not to grow further and, thus, some charges never reached appropriate confluence.

Both astrocyte-preparation methods also yielded similar results. Our experiments demonstrate that astrocytes can be co-cultivated with pBCEC using the same medium as the latter, but bFGF should probably be avoided in the medium. It is known that this growth factor induces astrocytes proliferation and reduces the expression of GFAP, increasing the expression of vimentin, and, contrarily, corticosteroids induce the expression of GFAP (Gomes et al., 1999; Avola et al., 2004). This implicates that maturation of astrocytes is affected by bFGF, what in turn could affect the astrocyte-mediated induction of endothelial cells diferenciation.

In the case of pBCEC, the cells showed better growth rates, and are feasible to be kept under freezing conditions. They also showed TEER values in monoculture (Fig. 6.3, until day 4) that are similar to those found in the literature (Zhang et al., 2006). Passive permeability to mannitol was considerably lower than previously obtained by Zhang et al. (2006), who reported values of 94 x 10^{-6} cm/s. Unfortunately, while the inserts with confluent monolayers were handled to place them onto the wells with astrocytes (at day 4 of confluence), the membranes were slightly attached to the bottom of the well where the transfection was carried out. It is probable that mechanical disruption of the monolayers occurred during this step, what would explain the decay in TEER after transferring the pBCECs for co-culture with astrocytes.

Surprisingly, even under conditions of relatively high paracellular permeability (if compared with models such as LLC-PK1 and MDCK II), a clear enrichment of phenytoin in the apical chamber occurred, indicating polarized transport. However, given the technical problems that appeared during this experiment, optimization of the model regarding handling for transfection and co-cultivation with astrocytes, as well as the determination of the best moment for transport experiments should be performed. In the present conditions, it is not possible to conclude that phenytoin is transported by human Pgp in this model with transfected pBCECs.

We performed uptake experiments with the immortalized GPNT and RBE4 cells, since it is known that these cell lines do not reach appropriate confluence for transport experiments in the Transwell system (Roux & Couraud, 2005). The uptake model was first validated in GPNT cells, since the over-expression of Pgp can be easily induced by supplementation with puromycin (Roux & Couraud, 2005), and phenytoin is a substrate for mouse Pgp (Baltes et al., 2007a). Using an appropriate number of samples (in this case, six samples per group) this test showed enough power to detect significant differences between cells with lower and higher Pgp expression. But, since our main goal is to investigate whether highly permeable AEDs are substrates of human Pgp using endothelial cells derived from BBB, we transfected RBE4 cells for expression of human Pgp. RBE4 transfectants also showed similar patterns of phenytoin uptake than the GPNT selected cells, with about 0.75 nmol phenytoin per mg protein less in both cells than in their corresponding parental or non-selected counterparts. Notwithstanding, these experiments require to be repeated and the effect of Pgp inhibitors has to be evaluated, in order to fully standardize this model for assessment of drug substrate specificity.

Conclusions: The present results show that uptake experiments in immortalized brain endothelial cells with AEDs promise to be a feasible model for evaluation of drug transport. However, further experiments with Pgp inhibitors are required to completely standardize the model. Primary porcine endothelial cells may be also useful as transport model, but optimization of the method, the manipulation of transfected cells, and the best time for the assay are required for the standardization of this model.

Chapter 7

Discussion

Chapter 7: Discussion

7.1. General considerations

Since the main therapy option available for epileptic patients is the pharmacological treatment based on antiepileptic drugs (AED [Schmidt, 2009]), it is a major concern that about one third of the patients are or become resistant (non-responsive) to this kind of therapy. In those cases, the surgical resection of the epileptogenic focus constitutes an alternative therapy, but unfortunately it is not applicable for all resistant cases, either because of high costs (what constitutes a problem in developing countries), or because the patient is not a suitable candidate for surgical resection. Hence, understanding the subjacent mechanisms of pharmacoresistance in epilepsy will allow developing suitable therapies for the treatment of such undesirable condition.

The two prevailing hypotheses about intractable epilepsy focus on two different mechanisms, but both end up affecting the drug-target relationship. In the first case, the "target hypothesis" attributes the condition to alterations in brain targets, postulating, thus, a pharmacodynamic approach. In the second case, the "multidrug transporter (MDT) hypothesis" proposes that the augmented expression of efflux carriers at the blood-brain barrier (BBB) limits the drugs to gain access to the target tissue; this is a pharmacodynamic approach (Schmidt & Löscher, 2005; Schmidt & Löscher, 2009). However, a common feature of intractable epilepsy is that the resistance extends to several AEDs, either in human patients or in animal models of temporal lobe epilepsy (Schmidt, 2009; Löscher, 2006). This points out that an unspecific mechanism underlies the condition. The fact that multidrug transporters pertaining to the ATP-binding cassette (ABC) superfamily of transporters, and specially P-glycoprotein (Pgp), can efflux a wide range of chemically dissimilar drugs; that several members are expressed at the luminal side of the brain capillary endothelium (the main component of the barrier phenotype of the BBB-structure); and that several of them are over-expressed in epileptogenic focus; make the MDT hypothesis a plausible explanation for intractable epilepsy (Löscher & Potschka, 2005a,b; Schmidt & Löscher, 2009).

It is known that MDTs mediate pharmacoresistance to phenytoin and phenobarbital in epileptic, nonresponder rats, since they are over-expressed in the BBB of those animals, these AEDs are *in vitro* substrates of the MDT Pgp expressed in rats (Abcb1a, or Mdr1a [Baltes et al., 2007a]), and the resistant condition can be overcome by means

of Pgp inhibition (Brandt et al., 2006; van Vliet et al., 2006). In this regard, it seems that Pgp over-expression in brain tissue of non-responder epileptic rats is far more than a mere epiphenomenon. But little is known about substrate specificity of human Pgp for AEDs and, consequently, about the possible role of Pgp over-expression in brain parenchyma of epileptic patients. Hence, it was our aim to investigate the *in vitro* substrate specificity of several ABC transporters for the AEDs phenobarbital, phenytoin, cabamazepine and valproic acid (with a widespread use worldwide [WHO, 2005]), and the modern drugs topiramate, levetiracetam, and lamotrigine.

7.2. Transport of antiepileptic drugs by human Pgp

Several characteristics of Pgp make this protein a feasible candidate for pharmacoresistance in central nervous system (CNS) pathologies. Its gene is the mostly expressed among all the ABC transporters of the BBB investigated so far, there is a universal luminal expression at the BBB-endothelium in mammals, it is able to transport a wide range of chemical compounds most of them lipophilic, and it is over-expressed in the epileptogenic focus of pharmacoresistant patients (Dean et al., 2001; Fricker & Miller, 2004; Schinkel & Jonker, 2003; Löscher & Potschka, 2005a,b; Warren et al., 2009). Furthermore, previous reports of our group showed that, unlike mouse Pgp, human Pgp may not transport AEDs *in vitro* (Baltes et al., 2007a), what constituted a challenge for us, when considering it as starting point.

In the first series of experiments using LLC-PK1 cells transfected with human MDR1 gene (LLC-MDR1 transfectants) for assaying the transport of phenytoin by means of the conventionally used bidirectional transport method, no transport of this AED was observed. One of the main questions at that point was whether the high permeability of the AEDs in conjunction with the non-sufficient TJ (TJ) formation in the already available models (not only LLC-PK1, but also MDCK II cells and endothelial primary cultures) make it difficult to differentiate passive diffusion, caused by diffusion gradient in the system, from active transport of –possibly– weak substrates. This prompted us to modify the assay setup, so that we avoided creating a diffusion gradient, at least at time zero of the experiment. As a consequence, and after discussion with scientists working in the pharmaceutical industry, we decided to employ the concentration-equilibrium transport assay (CETA) for evaluation of AED transport in LLC-PK1 parental cells and transfectants.

First experiments with the positive controls digoxin and vinblastine sulphate

(Flanagan et al., 2002; Taipalensuu et al., 2004) showed a strong affinity of human Pgp for these substrates, thus allowing us validating this model, so that following experiments were carried out with several major AEDs. The results derived from them allow us classifying all tested AEDs, except carbamazepine, as human Pgp substrates. From highest to lowest magnitude of transport the order is: topiramate > phenytoin > phenobarbital > lamotrigine > levetiracetam. Criteria for classification as human Pgp substrates are: 1) they are transported actively in human MDR1 transfectants; 2) transport can be completely or partially (but by more than 50%) inhibited with the Pgp modulator tariquidar used at nanomolar concentrations; and 3) no transport was detected in parental cells. To our knowledge, this is the first direct evidence that human Pgp can actually transport *in vitro* several major AEDs at a significant extent.

Negative results for several AEDs have been reported previously. First, Crowe and Teoh (2006) assessed the transport of phenobarbital, lamotrigine, carbamazepine, phenytoin and topiramate, among others, in a sub-clone of Caco-2 cells with high Pgp expression, and by means of Transwell®-System with the bidirectional transport assay. None of the former AEDs were shown to be transported by human Pgp. Second, Baltes et al. (2007a) and Feng et al. (2008) found no transport of levetiracetam, phenytoin or carbamazepine using the bidirectional transport assay with LLC-MDR1 and MDCK-MDR1 transfectants, and of lamotrigine, phenytoin or carbamazepine using the bidirectional transport assay and the calcein inhibition assay with MDCK-MDR1 transfectants. Regarding the Caco-2 model, it is a quite predictable model for intestinal drug absorption, but some authors have questioned its utility for studies of substrate specificity, especially because of the high expression of endogenous transporters (Wang et al., 2008), and its high paracellular permeability (Liu et al., 2008). As an example, Crowe and Teoh (2006) reported that phenytoin showed a higher permeability from apical to basolateral side of the monolayer, meaning that an uptake-flux occurred in the system, what seems to recapitulate the good intestinal absorption of phenytoin *in vivo* (Pade & Stavchansky, 1998), but not necessarily the brain/plasma concentration of this drug in human beings, which has been shown to be around 1 (Houghton et al., 1975). On the other hand, the MDCK-MDR1 and the LLC-MDR1 transfectants are quite good models for assessing substrate specificity of human Pgp (Feng et al., 2008), but this seems to be more the case for low permeability compounds than for highly permeable ones (Polli et al., 2001). AEDs are *per se* highly permeable compounds because of their low molecular weight and their good enough –but not too high– lipophilicity. These characteristics do not coincide with the common descriptors of the

classical Pgp substrates (Didziapetris et al., 2003). The relatively high paracellular permeability of the LLC-PK1 and MDCK II models are less than optimal, when compared with the BBB (Liu et al., 2008), what in conjunction with the high permeability of AEDs, and the diffusion gradient created in the bidirectional transport assay may conceal a potential transport mediated by Pgp. In contrast, the CETA can ameliorate this problem and allow observing Pgp-mediated transport, when diffusion gradients are avoided at the start of the experiment (Luna-Tortos et al., 2008), meaning that this method permits studying the transport of highly permeable compounds still in cell systems with relatively high paracellular permeability. Finally, the calcein inhibition assay is based on the ability of the investigated drug for inhibiting the efflux of calcein from the cells to the extra cellular fluid (Polli et al., 2001; Feng et al., 2008). However, a lack of interaction in this assay does not necessarily mean the drug is not a substrate of Pgp. There is not necessary correlation between Pgp substrates and Pgp inhibitors, for example, Polli et al. (2001) classified several well known Pgp substrates such as puromycin, vincristine and dexamethasone as “transported substrates”. Drugs in this category classically showed a high transport ratio in MDCK-MDR1 monolayers, but failed in the ATPase activation and calcein-inhibition assays. Furthermore, calcein-inhibition assay tends to produce more false negatives and false positives than the bidirectional transport assay in MDCK-MDR1 cells (Feng et al., 2008). Because of this, the negative results related to AED interaction with human Pgp obtained in inhibitory assays (Weiss et al., 2003) do not exclude that Pgp may transport these drugs. Finally, the consistent negative results for carbamazepine in CETA, bidirectional transport assay and calcein-inhibition assay (Mahar Doan et al., 2002; Baltes et al., 2007; Feng et al, 2008; Luna-Tortos et al., 2008) strongly suggest that this AED is neither a substrate nor a consistent inhibitor of Pgp.

An open question is whether the cell type is relevant for assessing Pgp-mediated drug transport. Garberg et al. (2005) found a high transport ratio (5.7) for [^{14}C]-phenytoin using human primary brain endothelial cells, but not in the MDCK-MDR1 cells, which showed a transport ratio (non-corrected) of 1.5. On the other hand, the dynamic, three-dimensional model utilized by Cucullo et al. (2007) (where brain endothelial cells from human beings are cultured in the lumen of capillary structures, with astrocytes grown on the abluminal side, and the continuous flux of the media through capillaries replies the physiological shear stress) recapitulates more reliably the BBB *in vitro* (Liu et al., 2008). Under these conditions, there was an efflux of phenytoin in Pgp over-expressing cells that could be diminished with tariquidar, indicating that this

drug is a substrate of human Pgp (Cucullo et al., 2007).

Although further development and validation of the endothelial cell models are needed, our preliminary results indicate that uptake assay in immortalized BCECs, and, possibly, transport experiments with the CETA method may be suitable models for studying transport of highly permeable compounds.

It seems that the high permeability of a compound and the high paracellular permeability of the *in vitro* systems influence the ability of the assay to find Pgp substrates. An example of this is the calcium-channel blocker verapamil, which is a non-CNS drug. This is a highly permeable compound that is known to be substrate and inhibitor for Pgp (Löscher & Potschka, 2005b), but the MDCK-MDR1 model fails to identify it as substrate, possibly because of the high permeability of the compound, and the high paracellular permeability of this model (Polli et al., 2001). But *in vivo* studies in rats using positron emission tomography consistently showed that tariquidar can dramatically enhance the permeation of ^{11}C-verapamil into the brain (Bankstahl et al., 2008b), meaning that verapamil is a strong substrate for Pgp *in vivo*.

Given contradictory results regarding Pgp substrate specificity that have been obtained in different models, it is a common agreement that better models, i.e. models with better formation of TJ, are required in order to assess more reliably the role of Pgp transport at the BBB (Garberg et al., 2005; Liu et al., 2008).

Central nervous system pharmacokinetics and the influence of Pgp-mediated transport of antiepileptic drugs

Mahar Doan et al. (2002) described that a common characteristic that differentiates CNS drugs from non-CNS drugs is the high passive permeability of the former, with most drugs having apparent permeability values (Papp) in the MDCK-MDR1 model that are higher than 150 nm/s. The authors also described that some of the 43 CNS drugs they investigated could be classified as Pgp substrates, but still, the high permeability of the compounds allows them reaching enough concentrations in the brain to be active, and they conclude that this high permeability and a Pgp mediated efflux ratio in MDCK-MDR1 cells (basolateral to apical / apical to basolateral) lower than 2.5 (which means they are not good Pgp substrates) are adequate parameters for the prediction of CNS permeation (Mahar Doan et al., 2002). Data from our laboratory indicate that AEDs investigated at time in the last years, that we reported to be substrates for human Pgp only in CETA, have significantly higher apical-to-basolateral Papp values in LLC-Wt cells, than the group of the well known Pgp substrates digoxin, vinblastine sulphate and

cyclosporine A (Fig. 7.1); yet the Papp values of AEDs are much lower in this model than those reported to be obtained from MDCK-MDR1 cells (>200 nm/s for carbamazepine, lamotrigine and phenytoin [Feng et al., 2008]).

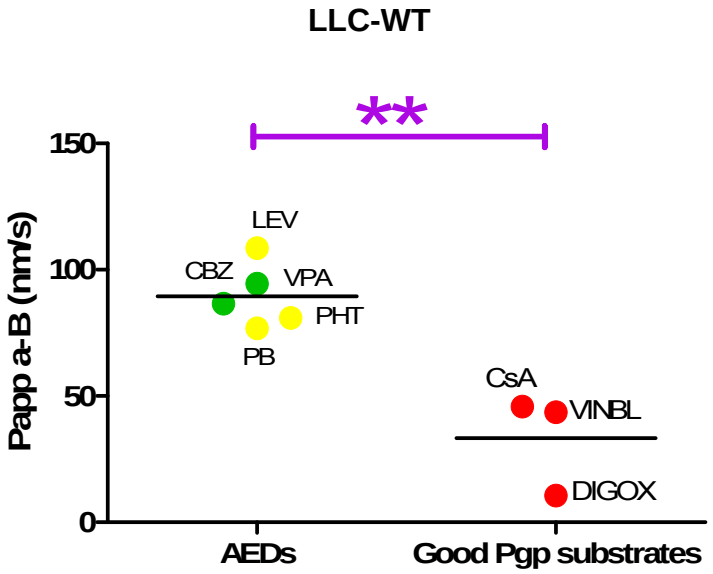

Figure 7.1 Apical-to-basolateral apparent permeabilites (Papp) measured in LLC-PK1 parental cells (LLC-WT) for several antiepileptic drugs and good Pgp substrates. Red points represent good Pgp substrates, yellow points, the CETA-possitive Pgp substrates; and green points represent non-substrates. Each point represents the average obtained from several experiments for each drug. Bars indicate mean of the groups. Statistical comparison between both groups was performed with unpaired t-Test (**P<0.01). CBZ: carbamazepine; CsA: cyclosporine A; DIGOX: digoxin; LEV: levetiracetam; PB: phenobarbital; PHT: phenytoin; VINBL: vinblastine; VPA: valproic acid.

Predicting the bioavailability of a compound in the brain has been a challenge in the last years. Many efforts have been made in order to find common pharmacokinetic descriptors for drugs that permeate well into the CNS (for instance: Kaznessis et al., 2001; Mahar Doan et al., 2002; Liu et al., 2004; Garberg et al., 2005; Zhang, 2006); but in fact it is not obvious which parameters should be used to define good brain penetration (Liu et al., 2008), and it is possibly due to different mechanisms mediating or limiting brain penetration such as carrier-mediated influx, passive permeation, and transporter-mediated efflux (Garberg et al., 2005; Abbott et al., 2006).

On the other hand, for cellular barriers other than the BBB the permeability of a drug shows a direct relationship with its oil-water partition coefficient (logP), but in case of the BBB it seems that this relationship is not continuous for all drugs, meaning that many

highly lipophilic compounds are excluded from the brain parenchyma compartment (Fricker & Miller, 2004; Garberg et al., 2005; Neuwelt et al., 2008). Most of these drugs are substrates of MDTs, and particularly Pgp. It is well accepted that one of the most common characteristics of Pgp substrates is that they are amphipathic (Schinkel & Jonker, 2003).

A general denominator among the substances that permeate the BBB by passive diffusion is their very low molecular weight. The molecular weights of virtually all CNS drugs used in clinical practice today are under 500 Da (Pardridge, 2003), and this includes AEDs. In general, it seems that two main parameters are common denominators of CNS drugs: lipophilicity and very low molecular weight; and both are probably important characteristics influencing Pgp functionality at the BBB as well, since both contribute together to the typical high passive permeability to the CNS drugs.

The widely accepted mechanistic model for the Pgp translocation of its substrates postulates that the drug enters a high-affinity site into the transporter from the cytoplasmic leaflet of the membrane, and upon the binding and/or hydrolysis of ATP, the affinity of the drug binding pocket decreases causing the release of the drug into the outer leaflet or the aqueous environment (Seeger & van Veen, 2009). This model explains, at least partially, why most Pgp substrates are lipophilic compounds, since the drug has to partition in the lipidic microenvironment of the cell membrane in order to gain access into the high affinity pocket of the transporter. Highly lipophilic compounds will preferentially partition into the cell membrane, what in turn facilitates drug extrusion into the outer leaflet, while the increment of drug concentration in the extracellular fluid will be favoured by diffusion gradient from the membrane to the aqueous phase. On the other hand, as previously suggested, this model implies that the efflux by Pgp will only result in clear distribution asymmetry if the rate of active transport for the drug is of considerable magnitude in relation to the passive diffusion rate; but in case the latter is relatively high, the pump activity will be overwhelmed by the passive diffusion component (Eytan et al., 1996; Schinker & Jonker, 2003). This situation may change when the transporter is over-expressed because of higher molecular collision.

A last consideration on Pgp function relates to its lipidic microenvironment. It has been observed that Pgp can transport lipids, Pgp over-expression leads to changes in the lipid composition of the cell membrane, and the adequate function of Pgp depends on its lipid microenvironment (Ferté, 2000; Orlowski et al., 2006). Lipophilic drugs that are able to permeate through cell membranes have to partition firstly into the lipidic microenvironment of the outer membrane leaflet, secondly in the hydrophobic core of

the bilayer, and thirdly in the lipidic inner leaflet. On the other hand, the cell membrane is asymmetric in regard to its inner and outer lipidic composition, and it has been shown that different drugs have different affinities for specific lipid species that constitute the bilayer (Ferté, 2000). Furthermore, whereas Pgp substrates tend to have a lower flip-flop (i.e., crossing from outer to inner bilayer leaflets), the Pgp inhibitors tend to diffuse much more quickly (Eytan et al., 1996). These observations also support the mechanistic model of Pgp function described above, since slower diffusion of a drug would relate to more chances to collide with the transporter's active site, theoretically localized in the inner leaflet (Ferté et al., 2000; Schinkel & Jonker, 2003; Seeger & van Veen, 2009).

A recent work demonstrated that the differences in lipid composition observed between brain endothelial cells and MDCK cells, with the former having more rigid membranes, highly impact the passive permeability of a drug and lead to a poor correlation of drug permeability between the mouse *in situ* brain perfusion model and the MDCK-MDR1 model (Di et al., 2009). Such a factor for drug permeation would explain why other authors have found similar apical-to-basolateral permeabilities for the highly permeable drug, and Pgp-substrate, verapamil in primary human brain endothelial cells and in MDCK-MDR1 cells (160 and 155 nm/s, respectively), even when the former had showed a 66-fold higher permeability for the paracellular marker sucrose (Garberg et al., 2005). However, it is not known whether Pgp-overexpression-mediated changes in lipid composition may alter the permeability pattern of the highly permeable AEDs, and whether this situation may further impact the drug efflux in case of Pgp over-expression in brain endothelial cells. If so, the significance of localized Pgp over-expression on AEDs pharmacokinetics may be higher than expected.

Predictability for the in vivo conditions

In vitro transport mediated by Pgp does not necessarily reflect the situation *in vivo* (Feng et al., 2008). Thus, the fact that human Pgp transports several major AEDs supports the multidrug transporter hypothesis of pharmacoresistance in epilepsy, but does not allow predicting what the impact of the *in vivo* transport on the AED pharmacokinetics may be. Notwithstanding, the results available in animal models of intractable epilepsy may bring some light regarding this issue. The AED phenytoin was classified as weak substrate for mouse Pgp, according to data obtained in the bidirectional transport assay using LLC-PK1 cells that over-express mouse Pgp (Mdr1a [Baltes et al., 2007a]). *In vivo* experiments using knockout mice (Mdr1a/1b [–/–, –/–])

compared to wildtype mice showed a small, although significant difference in the ratio knockout vs. wildtype of the brain-to-plasma concentrations, pointing out that the drug is possibly transported weakly by Pgp (Doran et al., 2005); but, because of its high permeability, the drug can still permeate into the brain in high concentrations that almost equal those of plasma. Interestingly, another study that measured brain concentrations of phenytoin in specific brain regions, showed a 46% increase of phenytoin concentration in hippocampus of Mdr1a/1b knockout (–/–, –/–) mice, compared with concentrations in wildtype mice; while Mdr1 over-expression resulting from kainate-induced seizures in wildtype mice led to a 30% reduction of phenytoin brain/plasma ratio (Rizzi et al., 2002). This indicates that the panorama possibly changes when Pgp is over-expressed in the brain target tissue. In another *in vivo* study in epileptic rats, the Pgp inhibitor tariquidar not only increased phenytoin concentrations in brain, but significantly improved the seizure control in epileptic rats (van Vliet et al., 2006). An up-regulation of Pgp in epileptic brains contributed to a 20% reduction of phenytoin brain levels (van Vliet et al., 2006). The possible explanation for this paradox is that phenytoin is a substrate of Pgp, but its high permeability allows it reaching enough brain concentrations in normal conditions. The localized over-expression of the transporter in the brain target tissue accounts for an additive efflux of the drug where it has to be active, so that the concentration in the site of action is diminished. The impact of Pgp expressed at the BBB on the pharmacological effect of highly permeable substrates that are CNS-active drugs depends on the target receptor affinity of the drug. Some of them that are highly potent at still quite low concentrations are probably less impacted that those which require higher concentrations, e.g. the antipsychotic agent risperidone (Doran et al., 2005). That means, for drugs that need a relative high concentration in brain tissue to exert a pharmacological effect, the over-expression of Pgp, and the consequent over-extrusion of the drug does impair relevantly the drug activity, and this is what probably occurs in the case of phenytoin resistant epileptic rats.

In relation to human Pgp, most data available up to now come from *in vitro* experiments. We have described the *in vitro* transport of phenytoin by human Pgp in CETA with LLC-PK1 cells transfected with MDR1 gene. This model allows predicting whether a drug is transported or not, including highly permeable compounds. On the other hand, in a dynamic, humanized BBB model that replies several characteristics of the human BBB, and thus, may allow for a more translational significance of drug transport, the permeability of phenytoin into the “brain side” of the system was 10 fold lower for endothelial cells derived from pharmacoresistant epileptic patients (with high

expression of Pgp), as compared with endothelial cells from normal individuals (Cucullo et al., 2007). The incubation with the Pgp inhibitor tariquidar increased the permeability of phenytoin by a 3.5 fold factor, but did not change permeability of phenytoin in endothelial cells from normal individuals (Cucullo et al., 2007), meaning that expression of Pgp contributed substantially to the lower phenytoin uptake into the “brain side” of the *in vitro* model, and that a similar situation is plausible to occur in pharmacoresistant epileptic patients. But regarding the *in vivo* situation in human patients, the question still remains open and new techniques such as positron emission tomography using ^{11}C-labeled AEDs will allow for studying the impact of Pgp expression and over-expression on AEDs pharmacokinetics in human patients (Löscher & Potschka, 2005b).

7.3. Multidrug resistance-associated proteins interactions with antiepileptic drugs

In the present study no substrate affinity of human MRP1, MRP2 or MRP5 was observed for the AEDs carbamazepine, lamotrigine, levetiracetam, phenobarbital, phenytoin or topiramate. However, further investigations should be performed before discarding these drugs as MRP substrates. Several characteristics of this model do not allow excluding false negative results. First, these cells express several endogenous transporters that may interfere with the transport mediated by (exogenous) human MRP, and the expression of endogenous transporters may vary among parental cells and transfectants (Goh et al., 2002; Flanagan et al., 2002). Second, while some MRP transporters such as MRP1, MRP3 and MRP5 are expressed on the basolateral membrane of MDCK II transfectants, MRP2 and MRP4, but also Pgp are localized at the apical membrane (Schinkel & Jonker, 2003). If endogenous transporters are differentially expressed in case of overexpression of a particular transporter (Dallas et al., 2006), it will be virtually impossible to dissect the participation of individual MRPs by pharmacological inhibition, since MK571 can inhibit most of them at the concentration we used in this work (i.e., 50 µM). Third, this model has relatively high paracellular permeability in comparison to the BBB *in vivo* (Liu et al., 2008); however, this problem is expected to be ameliorated by means of the CETA method, as it occurs in the LLC-PK1 cells. Fourth, it was demonstrated recently that MDCK II cells have a more fluidic plasmalemma than the endothelial cells of the BBB, thus the transcellular permeability through the former monolayers are higher than in the latter (Di et al., 2009).

The only AED that seems to be effluxed by MRPs is valproic acid, yet this is an observation derived from pharmacological inhibition in LLC-PK1 monolayers. However,

no transport of this compound by human MRP1, MRP2 or MRP5 could be confirmed in the CETA method using MDCK II transfectants.

Several methodologies have been employed in the investigation of substrate specificity of MRPs, including uptake in cells, uptake in vesicles, bidirectional transport assay, and transport in knockout mice; among others (Schinkel & Jonker, 2003; Borst et al., 2007). Here we describe the use of CETA method as additional tool of investigation. Transport of known substrates is easily assessed with this method, as shown for calcein, CMFDA and vinblastine sulphate. Furthermore, it allows for measurement of polarized transport, what implies an advantage over the uptake assay. These observations support the idea that the MDCK II model is more reliable to avoid false positives, but it does not allow discarding false negative results. A similar conclusion was derived from MDCK-MDR1 transfectants, when comparing it with results obtained from other methods (Feng et al., 2008).

7.4. Conclusions

1) The data presented in this work and the data available in the literature regarding Pgp function supports the multidrug transporter hypothesis of pharmacoresistance in epilepsy. Here we demonstrate that the AEDs topiramate, phenytoin, lamotrigine, phenobarbital and levetiracetam are substrates for Pgp *in vitro.* In *in vivo* conditions, these molecules are able to reach enough concentrations in brain target tissue probably because of their high permeability and the normal expression levels of Pgp. On the other hand, the over-expression of the transporter in case of pharmacoresistant epilepsy may have a clinically relevant impact on brain distribution of AEDs in the target tissue.

2) Contrarily, our results do not support the theory that Pgp acts in conjunction with MRP1, MRP2 or MRP5 to extrude AEDs from brain tissue, since none of the AEDs investigated was shown to be substrate for these MRP transporters.

3) While efflux of valproate in LLC-PK1 cells indicates participation of MRPs, no transport of this drug was observed in MDCK II cells expressing MRP1, MRP2 or MRP5. This interesting finding deserves further investigation in order to know whether valproate is a substrate for some MRP transporter whose expression is up-regulated in brain pathologies such as intractable epilepsy. Furthermore, pharmacological inhibition with MK571 in vincristine-stimulated LLC-MDR1 cells indicates that phenobarbital and levetiracetam may also be transported by endogenous MRPs, but as in the case of

valproate, our preliminary results in MDCK II transfectants do exclude a possible role of MRP1, MRP2 and MRP5 in the transport of both AEDs.

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Summary

Carlos Luna Tortós

Evaluation of transport of antiepileptic drugs by efflux transporters (multidrug transporters) of the blood-brain barrier

Epilepsy is a major central nervous system disease affecting about 50 million people worldwide. While the pharmacological treatment is the main therapeutic option for the management of epileptic patients, about one third of them are considered to be refractory to the currently available antiepileptic drugs (AEDs). One of the mostly investigated hypotheses for explaining this phenomenon is the multidrug transporter hypothesis. Over-expression of several members of the ATP-binding cassette (ABC) transporters, such as Pgp and several multidrug resistance-associated proteins (MRPs) has been identified in epileptogenic focus of both drug-refractory human patients and (non responder) animal models of temporal lobe epilepsy. Although the significance of this phenomenon is not completely understood, it has been shown that in animal models of epilepsy efflux transporters reduce the concentration of major AEDs in the epileptogenic brain areas, and the inhibition of these transporters can reverse the nonresponder condition, implying that they play a major role in drug efflux from brain to blood, and reduce AED concentration in the site of action. However, the significance of ABC transporters over-expression in human beings is not known as yet. Hence, it was our aim to investigate whether several human ABC transporters, namely Pgp and MRP1, MRP2 and MRP5 transport AEDs *in vitro.*

First, we were able to validate a modified version of the widely used Transwell assay, demonstrating its functionality for drug-transport experiments. With this model we could demonstrate that the AEDs topiramate, phenytoin, phenobarbital, lamotrigine and levetiracetam are substrates for human Pgp, but not for MRP1, MRP2 or MRP5. This results support the hypothesis that the overexpression of Pgp at the lumen of the blood-brain barrier endothelium may account for increased drug efflux in brain of human patients. Contrarily, carbamazepine and valproic acid do not seem to be substrates for human Pgp.

On the other hand, given the high paracellular and transcellular permeability of the MDCK II cell model (which was used for assessment of MRP transport), further analysis with these transporters should be performed before discarding the former AEDs as substrates for MRPs. Experiments in LLC-PK1 cells also indicated that valproic acid is a substrate for endogenous MRPs, but probably not for human MRP1, MRP2 or MRP5.

Finally, experiments performed with brain capillary endothelial cells show that they can be transfected with human MDR1 and express functional protein. Uptake experiments in GPNT and RBE4 cells confirmed transport of phenytoin by Pgp. However, further standardization of these models need to be performed, in order to employ them for the evaluation of other AEDs.

Zusammenfassung

Carlos Luna Tortós

Untersuchung des Transports von Antiepileptika durch Efflux-Transporter (Multidrug-Transporter) der Bluthirn-Schranke

Epilepsie ist eine der bedeutendsten Krankheiten des Zentralnervensystems, die ca. 50 Millionen Menschen weltweit beeinträchtigt. Die pharmakologische Behandlung gilt als die erste Wahl zur Therapie epileptischer Patienten. Allerdings erweisen sich ca. ein Drittel der Patienten als resistent gegenüber den momentan verfügbaren Antiepileptika. Eine Hypothese, die schon intensiv untersucht wurde, ist die so genannte Multidrug-Transporter-Hypothese. So konnte eine Überexpression von Vertretern der ATP-binding-Cassette (ABC) Transporterproteine, wie zum Beispiel von P-Glycoprotein und von Mitgliedern der Multidrug-resistance-associated Proteine (MRPs), im epileptogenen Fokus von pharmakoresistenten humanen Patienten und in Tiermodellen der Temporallappenepilepsie, sowie bei Tieren, die nicht auf medikamentöse Behandlung ansprachen, nachgewiesen werden. Obgleich die Bedeutung dieses Phänomens nicht vollständig geklärt ist, wurde in Tiermodellen für Epilepsie an der Ratte gezeigt, dass diese Transporterproteine die Konzentration von Antiepileptika in den epileptogenen Hirnregionen verringerten. Die Hemmung der Transporter resultierte in einer wiederhergestellten Ansprechbarkeit der zuvor pharmakoresistenten Ratten gegenüber den Substanzen. Dies deutet darauf hin, dass Transporter eine wichtige Rolle für den Auswärtsstrom von Substanzen aus dem Hirngewebe ins Blut spielen und die Konzentration von Antiepileptika am Ort ihrer Wirkung vermindern. Allerdings ist die Bedeutung der ABC-Transporter-Überexpression bei epileptischen Patienten noch nicht geklärt. Daher war es unser Ziel, *in vitro* zu untersuchen, ob einige humane ABC-Transporter, wie P-Glycoprotein, MRP1, MRP2 und MRP5, Antiepileptika transportieren.

Zunächst etablierten wir eine modifizierte Version des weit verbreiteten Transwell-Assays und zeigten deren Anwendbarkeit für Substanz-Transport-Experimente. Mit Hilfe dieses Models konnten wir nachweisen, dass die Antiepileptika Topiramat, Phenytoin, Phenobarbital, Lamotrigin und Levetiracetam Substrate von humanem P-Glycoprotein, aber nicht von MRP1, MRP2 oder MRP5 sind. Diese Ergebnisse unterstützen die Hypothese, dass die Überexpression von Pgp an der lumenwärts gerichteten Endothelienmembran der Blut-Hirn-Schranke für den erhöhten Substanzefflux im Gehirn epileptischer Patienten verantwortlich sein kann. In Gegensatz dazu scheinen Carbamazepin und Valproinsäure nicht zu den Substraten von P-Glycoprotein zu zählen.

Auf der anderen Seite sollten weitere Untersuchungen zu den MRP-Transportern durchgeführt werden, bevor die genannten Antiepileptika als MRP-Substrate verworfen werden, denn die Versuche zur Bestimmung des Transports durch MRPs wurden mit MDCK II Zellen durchgeführt, die eine hohe parazelluläre und transzelluläre Permeabilität aufweisen. Experimente mit Transporter-Hemmstoffen in LLC-PK1 Zellen zeigten, dass Valproinsäure ein Substrat für endogene MRPs ist, aber möglicherweise nicht für humane MRP1, MRP2 oder MRP5.

Ausserdem konnten Endothelzellen der Blut-Hirn-Schranke mit humanem MDR1 erfolgreich transfiziert werden, so dass sie funktionelles Pgp exprimierten. Uptake-Experimente mit GPNT und RBE4 Zellen bestätigten den Transport von Phenytoin durch P-Glycoprotein. Allerdings sollte eine weitergehende Standardisierung dieser Modelle durchgeführt werden, um sie zur Untersuchung anderer Antiepileptika anzuwenden.

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