Methods of investigating drug-induced increase in the expression of the drug efflux transporter P-glycoprotein in different cell types

Thesis

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Abbreviations

ABC transporters	ATP-binding cassette transporters
AEDs	antiepileptic drugs
ATP	adenosine-5'-triphosphate
BBB	blood-brain barrier
BCRP	breast cancer resistance protein
CAR	constitutive active/androstane receptor
CBZ	carbamazepine
CCRP	cytoplasmic CAR retention protein
cm^2	square centimeter
CNS	central nervous system
CYP	cytochrome P450
CYP2B6	a member of the cytochrome P450
CYP3A	a member of the cytochrome P450
DEX	dexamethasone
DOX	doxorubicin
FDA	Food and Drug Administration
g	gravity
GABA	gamma aminobutyric acid
GABA(A)	GABA(A) receptor
GPNT	rat brain endothelial cell line
h	hour
kDa	kilodalton
LBD	ligand-binding domain
LLC	pig kidney epithelial cells
MDCK	Madin-Darby canine kidney
MDR	multidrug resistance
min	minute
MK571	an inhibitor of multidrug resistance proteins (MRPs)
ml	millilitre
MRPs	multidrug resistance proteins
NBD	nucleotide binding domain

NKCC1	Na-K-Cl cotransporter
OATs	organic anion transporters
OATPs	organic anion-transporting polypeptides
PB	phenobarbital
PCR	polymerase chain reaction
Pgp	P-glycoprotein
PHT	phenytoin
PNGase F	N-Glycosidase F
Pur	puromycin
rpm	revolutions per minute
PXR	pregnane X receptor
qPCR	quantitative real-time RT-PCR
RBE	rat brain endothelial cells
RIF	rifampicin (also called rifampin)
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	single-nucleotide polymorphism
TLE	temporal lobe epilepsy
TMD1/2	transmembrane domain1 or transmembrane domain 2
TQ	tariquidar
WT	wild type
XREM	xenobiotic-responsive enhancer module
µl	microlitre
µM	micromolar

1 Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent unprovoked seizures and can be caused by many factors like for instance: trauma, brain infection, brain tumor, and febrile illness. It leads to multiple pathological conditions like loss of consciousness and disturbances of movement, sensation (vision, hearing and taste), and mood or loss in mental function (Garcia-Morales, et al., 2008). Epilepsy disease affects humans and animals (cats, dogs) (Dewey, 2006).

People suspected of having epilepsy undergo electroencephalogram (EEG) investigation, and after medical check-up pharmacological treatment or surgery are performed. However, the main way of epilepsy treatment is application of antiepileptic drugs (AEDs). The problem is that 30-40% of patients are resistant to pharmacological treatment (Kwan and Brodie, 2000). The mechanism of such pharmacoresistance is poorly understood and is a limiting factor in the treatment of many neurological diseases. There is increasing evidence that overexpression of so-called multidrug transporters is involved in drug resistance in epilepsy and other diseases such as: brain tumours, depression and schizophrenia (Löscher and Potschka, 2005a). The association between enhanced expression of multidrug transporters and drug resistance in epilepsy was first shown by Tishler (Tishler et al., 1995) in patients with drug-resistant epilepsy. One of the most examined multidrug transporters engaged in pharmacoresistance in epilepsy is the efflux transporter P-glycoprotein (Pgp), which limits penetration of drugs into many cell types.

Pgp is a phosphorylated glycoprotein that belongs to the ABC transporter family. Pgp has a molecular weight of 160 kDa and is a product of *ABCB1* gene. It is expressed in brain capillary endothelial cells that form the blood-brain barrier (BBB), but also in peripheral organs such as kidney, liver, and intestine (Schinkel and Jonker, 2003). The action of Pgp in the organism needs to be considered in two ways: positive when preventing the body from intoxication by harmful substances and negative by limiting access of therapeutic drugs that cannot reach efficient concentrations at their target sites.

The research aim was to answer the question whether antiepileptic drugs (AEDs) induce Pgp overexpression, which would facilitate pharmacoresistance in epilepsy. For this aim, we have established methods to analyse drug-induced changes in Pgp expression. We have investigated the differences in Pgp expression after exposure to AEDs (phenobarbital, carbamazepine and phenytoin) and known Pgp inducers (dexamethasone, doxorubicin) for different time periods in various cell types. For our purpose we were using: Madin-Darby Canine Kidney cells (MDCK), pig kidney epithelial cells (LLC) either wild type or transfected with human Pgp (MDCK-MDR1, LLC-MDR1), and rat

brain endothelial cells (GPNT, RBE4). The drug-induced Pgp expression level was analyzed both on protein expression by Western blotting and on functionality level by uptake assay.

2 Overview

3 Epilepsy

3.1 Definition and meaning

Epilepsy is a common neurological disorders characterized by spontaneous recurrent seizures. Epilepsy can be caused by many factors including trauma, brain infection, brain tumor, febrile illness (Shinnar and Glauser, 2002; Theodore et al., 2008; Varoglu et al., 2009). Epilepsy and seizures affect over 3 million Americans of all ages, approximately 200,000 new cases of seizures and epilepsy occur each year (The Epilepsy Foundation of America® website).

The patients suffering from epilepsy have diverse mental and physiological problems (Hermann, et al., 2008). The symptoms depend on the location and extent of brain tissue that is affected (Kandel et al., 2000).

In general, seizures can be classified into two categories: partial (focal) and generalized (The Commission on Classification and Terminology of the International League Against Epilepsy, 1981). Partial seizures are seizures, which affect only a small region of the brain. Simple partial seizures are often precursors to larger seizures, where the abnormal electrical activity spreads to a larger area of the brain. An example of a partial seizure is localized jerking beginning in the right hand and progressing to clonic movements (jerks) of the entire right arm. Symptoms preceding the onset of partial seizure are called auras. Auras include abnormal sensation such as sense of fear, rising feeling in the abdomen. The aura is due to electrical activity originating from the seizure focus and represents the earliest manifestation of a partial seizure (Kandel et al., 2000).

Generalized seizures begin without prior aura and involve both hemispheres. They can be divided into convulsive and nonconvulsive types, depending on whether the seizure is associated with tonic or clonic movement (Kandel et al., 2000).

To investigate the activation of the brain the electroencephalogram (EEG) is performed which allows recording the neurons activity in the special brain regions. Seizures are the results of

excitability changes in the neurons. During the seizure, neurons have characteristic activity that can be recorded and analyzed by EEG. The EEG mainly records the input from excitatory neurons (glutamatergic) or from inhibitory (GABAergic) neurons.

GABA (gamma-aminobutyric acid) and glutamate are the main neurotransmitters in the mammalian central nervous system (CNS). The opposite effects (GABA is inhibitory, glutamate is excitatory neurotransmitter) keep a balance between them and affirms proper action of CNS. Any changes in this neurotransmitter homeostasis can lead to seizures (Löscher and Potschka, 2005b; Bankstahl et al., 2008).

The most common type of epilepsy is temporal lobe epilepsy (TLE) that took the name from the part of the brain involved in disease (Patrikelis et al., 2009). The main feature of TLE is recurrent epileptic seizures arising from one or both temporal lobes of the brain. The major way to treat epilepsy is pharmacotherapy. Unfortunately, despite the introduction of several new-generation AEDs, around 30-40% patents with epilepsy are still refractory to AEDs used in therapy (Kwan and Brodie, 2000). This refractoriness is called pharmacoresistance and its mechanisms are not completely known so far. However, there are several mechanisms that may explain the pharmacoresistance in epilepsy (Remy and Beck, 2006; Lazarowski et al., 2007).

4 Pharmacoresistance

4.1 Definition

Pharmacoresistance to drug treatment is defined as the persistence of significant disease symptoms despite at least two treatment trials with different drugs at the maximum tolerated doses for an adequate time period (Löscher and Potschka, 2005a). Many existing theories propose mechanisms of pharmacoresistance in epilepsy (Remy and Beck, 2006), but it is not clear what can cause such refractoriness.

4.2 Mechanism of pharmacoresistance

There are two hypotheses by which pharmacoresistance in epilepsy treatment can be explained. Drug transporter-related mechanisms (4.2.1) involve specific multidrug transporters that eliminate drugs from the brain to the blood and drug target-related mechanisms are explained by alteration in drug targets (receptors) (4.2.2).

4.2.1 Drug transporter-related hypothesis

Drug transporter-related mechanisms explain pharmacoresistance as result of so called multidrug transporter (MDR) overexpression in the brain. Those proteins are responsible for elimination of harmful substances from the brain to the blood and prevent the accumulation and penetration of drugs to the parenchymal tissues.

All drugs to be effective must reach their target at concentration allowing the successful effect. This optimal concentration level of AEDs provides the adequate properties of the drugs and leads to expected outcome. However, multidrug transporters expressed in BBB decrease the concentration of the chemical compounds in the brain by removing them back to the blood stream. The structure of the BBB is shown by Fig. 3B (BBB is described in detail in chapter 5). A functional BBB restricts the uptake of pharmacological substances into the brain and can contribute to pharmacoresistance.
This active transport of drugs by efflux transporters plays, of course, a protective role against toxic substances, but on the other hand it is a barrier for therapeutic substances. The association between drug resistance and enhanced expression of multidrug transporters in the brain of epileptic patients was shown for the first time by Tishler et al. (Tishler et al., 1995). Multidrug transporters such as Pgp, MRP1 and MRP2 are overexpressed in capillary endothelial cells and astrocytes in epileptogenic brain tissue from patients with refractory epilepsy (Lazarowski et al., 2007). Expression of multidrug transporters in astroglial end-feet covering the blood vessels is found in epileptogenic brain and might function as a “second barrier” (Abbott et al., 2002).

In order to pass BBB, commonly used AEDs have lipophilic nature but they are also substrates for Pgp and MRPs in the BBB (Schinkel et al., 1996; Luna-Tortós et al., 2008). In pathological condition, e.g. epilepsy, overexpressed Pgp and MRPs lead to reduction of drug in the epileptic neurons and contribute to multidrug resistance in epilepsy. Because of many different reports, it is still a

controversial issue which AEDs are substrates for multidrug transporters and differing results from publications can be due to species diversity (Baltes et al., 2007a, 2007b).

Pharmacoresistance might also have genetic background. One of the explanations for resistance in epilepsy treatment is the genome variability, for example gene polymorphism leading to alteration in drug metabolism, drug targets or drug transporters (Löscher and Potschka, 2005a).

The well-known "silent" C3435T polymorphism in MDR1 gene was reported by Kimchi-Sarfaty (Kimchi-Sarfaty et al., 2007) as one reason for changes in Pgp substrate specificity. Synonymous single-nucleotide polymorphisms (SNP) do not alter coding sequence and are usually not expected to change the function of the protein to which they correlate. More than 50 SNPs are known and have been suggested to alter the function of Pgp (Hoffmeyer et al., 2000; Drescher et al., 2002; Goto et al., 2002). However, the evidence for correlation between SNPs in MDR1 and changes in function of Pgp was given in 2007 by Kimchi-Sarfaty (Kimchi-Sarfaty et al., 2007). This research group has proved, for the first time, that synonymous SNP in MDR1 gene in exon 26 (C3435T) affects Pgp activity through the changes in timing of cotranslational folding and insertion of Pgp into the membrane. The changes in function of Pgp were investigated in HeLa cell line wild type and expressing SNP plasmids off MDR1. By uptake assay with fluorescent compounds this group has shown that presence of C3435T in combination with one or more other polymorphisms leads to lower accumulation of fluorescent dye in comparison to the control wild type cells.

Pharmacoresistance can be developed also due to the drug tolerance. Tolerance (loss of efficacy) is the reduction in response to a drug after repeated administration and can be another example for pharmacoresistance related to MDRs. Tolerance is an adaptive response of the body to prolonged exposure to the drug and concerns all discovered drugs. It develops to some drug effects much more rapidly than to others. The extent of tolerance depends on the drug and individual (genetic) factors (Löscher and Schmidt, 2006).

Two major types of tolerance are known. Pharmacokinetic (metabolic) tolerance, due to induction of AED-metabolizing enzymes has been shown for most first-generation AEDs and is easy to overcome by increasing dosage. Pharmacodynamic (functional) tolerance is due to "adaptation" of AED targets (e.g., by loss of receptor sensitivity) and has been experimentally shown for all AEDs that loose activity during prolonged treatment. Functional tolerance may lead to complete loss of AED activity and cross-tolerance to other AEDs. Convincing experimental evidence indicates that almost all first-, second-, and third-generation AEDs loose their antiepileptic activity during prolonged treatment, although to a different extent (Löscher and Schmidt, 2006). Thus, loss of efficacy leads to drug

resistance and it is very important to find out what mechanism stands behind this tolerance and how to overcome this effect.

4.2.2 Drug target hypothesis

Modification of drug targets may attenuate effectiveness of applied AEDs and lead to pharmacoresistance. Target hypothesis suggests that the genetic or acquired (disease-related) alterations to the structure and/or functionality of AED targets in epileptic brain regions lead to reduced drug effects (Löscher and Potschka, 2005a; Remy and Beck, 2006). So far, many targets in the brain for AEDs have been identified that are changed during epilepsy. Reduced sensitivity of drug targets to AEDs in experimental epilepsy models has been suggested for the voltage-gated Na^+ channel and the GABA(A) receptor (Beck, 2007).

Voltage-gated Na^+ channels are one of the targets for AEDs. Those are integral membrane proteins ubiquitously expressed in excitable cells like neurons, myocytes and certain types of glia.
During depolarization of the membrane the channel is activated and that leads to fast 'transient' Na^+ inward current (I_{NaT}) which is responsible for action potentials in the cells and in the next step to a slowly inactivating 'persistent' current (I_{NaP}). Both channels are targets for AEDs like carbamazepine and phenytoin (Meldrum and Rogawski, 2007).
Most AEDs block Na^+ channels in their resting state at hyperpolarized membrane potentials, which leads to downregulation of accessory subunits and altered sensitivity of Na^+ channels in epileptic tissue. In this respect, the down regulation of accessory Na^+ channel ß1 and ß2 subunits following status epilepticus (Ellerkmann et al., 2003).
The drug target hypothesis also involves alteration in target sensitivity as a consequence of changes in transcriptional regulation of ion channel subunit gene (Gastaldi et al., 1997) and polymorphism in targets subunits (Kwan et al., 2008).

GABA is an inhibitory neurotransmitter in the adult brain and plays a critical role in the regulation of excitability of the nervous system (Mody and Pearce, 2004). GABA binds to ionotropic GABA(A) receptors. This binding causes the opening of ion channels to allow the flow of negatively charged chloride ions (Cl^-) into the cell and leads to lower amount of intracellular HCO_3^-.

Experimental studies have shown that changes in the structure and function of inhibitory GABA(A) receptors may contribute to drug resistance in epilepsy (Bethmann et al., 2008).

In adult pyramidal neurons, GABA is an inhibitory neurotransmitter. It motivates the inward of chloride anions to the cells. In maintenance of ion homeostases another transporter, cotransporter KCC2, is involved. It transports the chloride and potassium ions out of the cells in ratio $1K^+/1Cl^-$. Thus, lower concentration of Cl^- in adult neurons results in expression of an inhibitory post-synaptic current that hyperpolarizes the post-synaptic neuronal membrane. Unlike in adult neurons, in neonatal pyramidal neurons another cotransporter called NKCC1 is activated. NKCC1 is responsible for symport of chloride ions, potassium and sodium inside the cells in ratio $2Cl^-/K^+/Na^+$. Apart from this, glutamate receptors are present, which bind the glutamate and increase cellular level of Na^+. Both rising intracellular concentrations of Cl^- and Na^+ lead to excitatory function of GABA. Similar situations occur in neurons during seizures. Enhanced level of sodium and chloride ions results in excitatory post-synaptic current. Thus, there is a shift to neonatal (excitatory) GABA(A) receptors in epileptic hippocampus of adult patients (Fig. 1), which tends to hyperexcitability of pyramidal neurons (Cohen et al., 2002; Köhling, 2002).

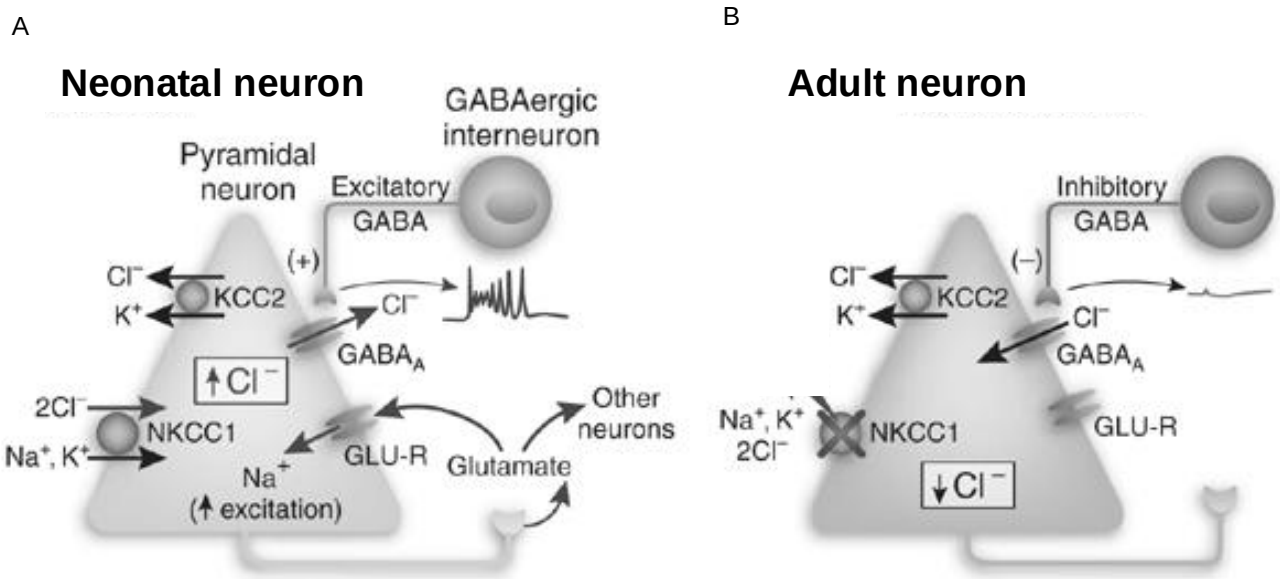


Fig. 1 Comparison of neonatal (excitatory) and adult (inhibitory) GABA(A) receptors. Neonatal neuron and excitatory role of GABA (gamma-aminobutyric acid) are shown in Fig. A. In Fig. B inhibitory GABA is shown. GABA together with glutamate are the main neurotransmitters in the central nervous system. The proper brain activity depends on the homeostasis between those neurotransmitters. In situation, when the balance is disturbed epileptiform discharges occur, which lead to seizures (modified from Fukuda, 2005).

Specific biochemical interaction between a drug and target (enzyme, receptor), which produces its pharmacological effect, gives impact on pharmacoresistance as well.

Ineffective mechanism of drug action can be due to the properties of the drug itself. In order to cross BBB, drugs must be lipophilic with molecular weight less than 500 kDa and not to be a substrate for BBB active efflux transporters. The effectiveness of the drug depends not only on the drug itself but also on the changes in adequate target and substances that can affect this target and AED efficacy (Remy and Beck, 2006). Excitatory role of GABA(A) in epilepsy, for example, is due to altered homeostasis of chloride ions, which results in a changed chloride gradient across the neuronal membrane.

5 Blood-Brain Barrier (BBB)

5.1 Structure and function

BBB is a dynamic interface between brain and blood. The main roles of BBB are elimination of (toxic) substances from the endothelial compartment and provide endogenous nutrition for the brain.
It can be considered as an organ protecting the brain and regulating its homeostasis. Specific transport system expressed in BBB (Fig. 3) plays important role in homeostasis and preserves the proper quality of the BBB (Persidsky et al., 2006).

BBB is composed by endothelial brain capillary cells which differ in structure from endothelial cells in peripheral organs (Löscher and Potschka, 2002). Capillary endothelial cells are surrounded in 90% by astrocytic end-feet that support the function of BBB (Fig. 2). Endothelial cells in BBB have more organized and compact structure because of their protective role in CNS.

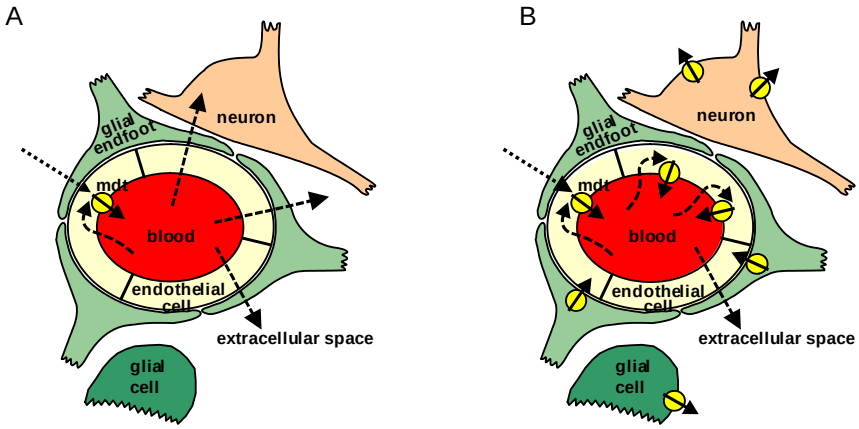


Fig. 2 Structure comparison of capillary endothelial cells showing differences in multidrug transporter (mdt) expression. Structure of endothelial cells in healthy brain (A) and in epileptic brain (B) are shown (Löscher and Potschka, 2002).

Brain endothelial cells are connected between each other by tight junctions (TJ, or zonula occludens), structures that enhance the tightness of the barrier. TJs are connections that join together the cells and preserve a barrier for fluids (Weiss et al., 2009). This compact structure of BBB controls the exchange of compounds between blood and brain and blocks diffusion between cells. TJs prevent migration of integral proteins and provide apical and basolateral orientation of the proteins in the cells (van Meer and Simons, 1986). Tight junctions appear as a belt-like band of anastomosing sealing strands that encircle each cell in the sheet (Alberts et al., 1994). Many transmembrane proteins are involved in formation of TJs, such as occludins and claudins and junctional adhesion molecule-1 (JAM-1) (Förster, 2008).

Occludins were first identified as integral proteins localized in TJs in chicken (Furuse et al., 1993). The occludin transmembrane domain spans the membrane four times with a short cytoplasmic N-terminus and a long corboxyterminal cytoplasmic domain (Förster, 2008). Claudins are also integral proteins involved in TJ formation. Claudins (22 kDa) are a cell specific backbone of TJs and are composed of 4 transmembrane domains. There is no sequence similarity to occludin. JAM-1 molecules are involved in immune cells transmigration and cell adhesion.

As described above the BBB is composed of brain capillary endothelial cells connected by TJs and astroglial end-feet. Expression of multidrug transporters in capillaries helps to maintain proper function of BBB (Abbott, 2002). Capillary endothelial cells do not contain fenestrations and show low rates of pinocytosis (Löscher and Potschka, 2005a). This makes the BBB very leakproof except for those molecules that can cross cell membranes by their lipid solubility (such as oxygen, carbon dioxide, ethanol, and steroid hormones) and those that are allowed to enter by specific transport systems (exogenous nutrition such as sugars and some amino acids).

In the apical (luminal) membrane of capillary endothelial cells, efflux transporters are expressed such as Pgp and MRPs (MRP1, MRP2, MRP5). Apart from them, other transporters are expressed at the BBB including the breast cancer resistance proteins (BCRP, known as ABCG2), the members of the organic anion transporting polypeptide family (OATP family) and the organic anion transporters family (OAT family). Some of these proteins are also expressed in the abluminal membrane of capillary endothelial cells (MRP4, OATP2, OAT3) (Pardridge, 2007).

Above named multidrug transporters have capability to work together and increase the efflux of the drugs from the brain parenchyma.

6 ATP-binding cassette transporters (ABC-transporters)

The ATP (adenosine-5'-triphosphate)-binding cassette (ABC) transporters are a large family of membrane proteins able to transport various compounds through the cell membrane against the concentration gradient. The characteristic feature of this family is highly conserved ATP-binding cassette. In order to transport compounds out of the cell, ABC transporters use energy from hydrolysis of ATP. There were 48 ABC transporters genes found in humans (Borst and Elferink, 2002; Dean and Annilo, 2005). ABC transporters are involved in many protective functions and their expression is associated with many diseases like cancer, epilepsy, brain disorders in humans and pets. ABC transporters have an influence on drug accumulation in brain, fetus, intestine and are involved in disposition and elimination of the drugs (Pérez-Tomás, 2006; Takano et al., 2006).

Within ABC transporters, various members might be distinguished: Pgp, BCRP, and family MRPs (Table 1), which work as efflux proteins and remove drugs outside of the cell. Those proteins can be located both on luminal and abluminal sides of brain capillary endothelial cells (Fig. 3). There are two types of nomenclatures used to name all transporters expressed in the blood-brain barrier (Table 1).

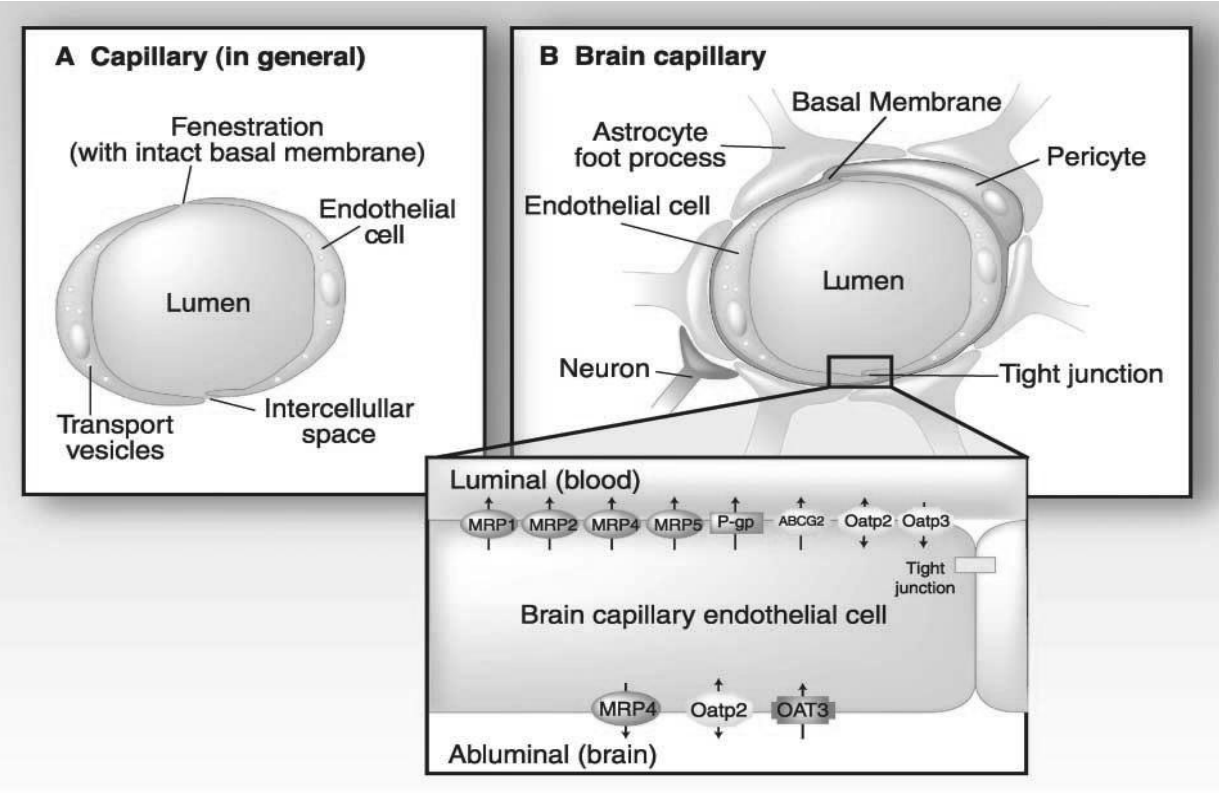


Fig. 3 Comparison between brain capillary endothelial cells (B) that build blood-brain barrier and capillaries from peripheral organs (A) (Deeken and Löscher, 2007).

6.1 P-Glycoprotein (Pgp)

6.1.1 Pgp structure

Pgp belongs to ABC transporters, energy driven efflux pumps. Pgp is phosprorylated glycoprotein with an apparent molecular weight 160 kDa and consists of 1280 amino acids. It is a product of *ABCB1* (known as *MRD1*) gene in humans located on chromosome 7 and has 28 exons. Pgp has two segments each of which contains 6 α-helix transmembrane domains in N-terminal site (transmembrane domain 1, TMD1) followed by a large ATP-binding site (NBD1, nucleotide-binding domain 1) and then next 6 α-helix transmembrane domain (transmembrane domain 2, TMD2) in C-terminal site containing also place for ATP (NBD2, nucleotide-binding domain 2).

Inactivation of one of the two nucleotide-binding domains of Pgp by amino acids substitution blocks drug transport and even ATP hydrolysis by the unaffected NBD. It shows that two NBDs interact strongly and cannot hydrolyze Mg-ATP independently.

Both domains share 65% amino acids similarity. In the outer part in the TMD1, Pgp protein has three glycosylated residues (Asn, asparagine 91, 94, and 99), which play a role in functionality of the protein and protection of Pgp structure against environmental conditions (Gribar et al., 2000). Treatment of Pgp with peptide-N-glycosidase F reduces the apparent molecular weight from 160 kDa to the predicted core weight 140 kDa (Greer and Ivey, 2007).

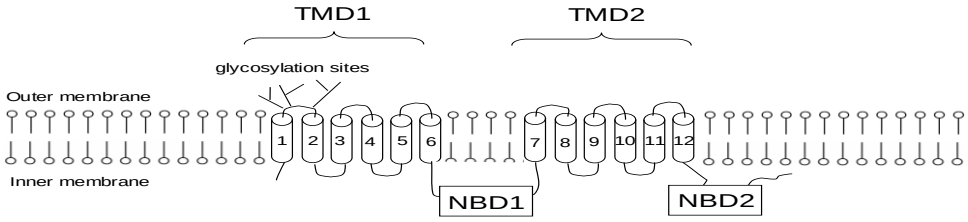


Fig. 4 Schematic structure of P-glycoprotein. Two transmembrane domains are shown (TMD1 transmembrane domain 1, TMD2 transmembrane domain 2) each of them contains 6 transmembrane α-helix. On the outer membrane three glycosylation sites are present which responsible for maturation of Pgp. In the inner part of the membrane nucleotide binding domains are present (NBD1 nucleotide binding domain 1, NBD2 nucleotide binding domain 2) that interact with ATP in order to produce energy needed for the transport of compounds against concentration gradient (modified from Loo et al., 2004).

6.1.2 Pgp function

Pgp uses energy from ATP hydrolysis to transport different substances through the cellular membranes. Pgp was discovered for the first time in 1970s as a transporter involved in multidrug resistance in cancer cells (Juliano and Ling, 1976) and a first multidrug transporter discovered in BBB in endothelial cells (Thiebaut et al., 1989). It is localized in apical (luminal) membrane of brain capillary endothelial cells. Pgp is responsible for protection of the brain against the toxic compounds (e.g. drugs) that could be harmful for the brain. Pgp removes toxic compounds from the brain parenchyma back to the blood stream by active transport with ATP hydrolysis process. It has been shown in Pgp-knockout mice that lack of MDR1 gene leads to enhanced neurotoxicity of different

drugs which is evidence that Pgp expression plays important role in protection of the brain against harmful compounds (Schinkel et al., 1994; Schinkel, 1999).

Pgp is expressed not only in brain capillary endothelial cells but also in other organs like liver (Fig. 5), kidney, intestine, and everywhere else where physiological barrier exist. It protects the body against harmful effect of substances (Schinkel and Jonker, 2003; Marzolini et al., 2004).

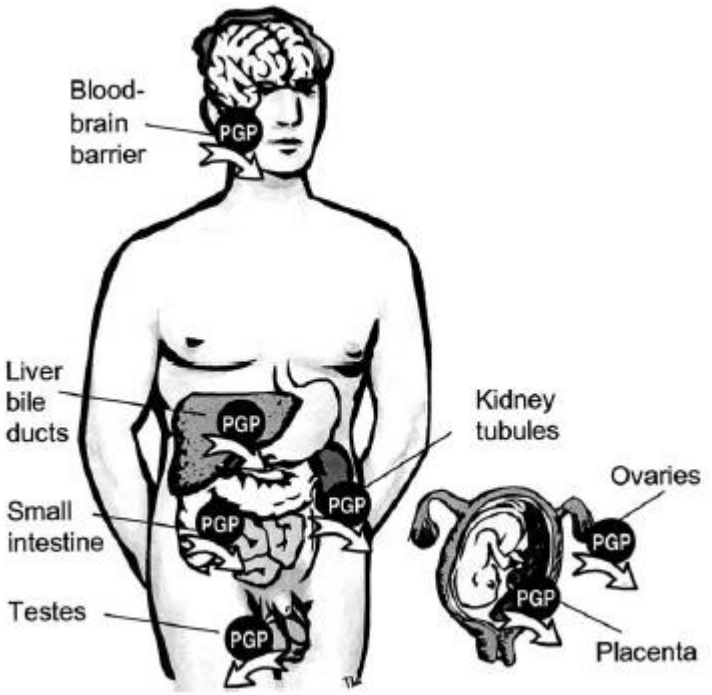


Fig. 5 Scheme showing P-glycoprotein (Pgp) expression in different organs. Pgp expression is not restricted to blood-brain barrier, but also occurs in many organs, where it limits the accumulation of toxic compounds (Marzolini et al., 2004).

6.1.3 The potential way in which Pgp can work

The observation that Pgp actively transports a variety of hydrophobic drugs is still under investigation. The ability to bind various drugs by one protein was examined by many researchers. Analysis of bacterial transcription regulator BmrR, a soluble protein that can tightly bind very different drugs can serve as an example to understand this phenomenon. The key element in this protein is a central flexible cavity that contains negatively charged residues in a hydrophobic

environment. Drugs can bind to this flexible cavity by Van der Waals interactions and do not require precise position. This model is not directly applicable to Pgp protein but can help to understand the possibility of binding the miscellaneous drugs by one protein (Vazquez-Laslop et al., 2000).

Another concept about transport of drugs by Pgp is called 'vacuum cleaner' according to which drugs are targeted from the aqueous medium to the membrane and then are transported out of the cells.

Pgp can also function as a flippase, moving hydrophobic molecules from the inner to the outer leaflet of the plasma membrane (Higgins and Gottesman, 1992). This idea was supported by the discovery that MDR3 isoform (that shares more than 75% similarity with MDR1) is a phosphatydylcholine (PC) flippase in liver canalicular cells (Ruetz and Gros, 1994). This theory was confirmed by the experiments with Pgp reconstituted into proteoliposomes (Romsicki and Sharom, 2001). Affinity of Pgp to binding several substances depends on drugs' lipid-water partition coefficient (Romsicki and Sharom, 1999).

6.1.4 How to overcome the pharmacoresistance?

Upregulation of ABC transporters which occurs in many diseases and leads to pharmacoresistance is treated pharmacologically with specific Pgp transporter inhibitors like tariquidar, cyclosporin A but those substances are used only in experimental trials (Robey et al., 2008; Hughes, 2008). Up till now there is no transporter inhibitor which is used regularly in epilepsy treatment.

Because Pgp, one of the multidrug transporters, is expressed in many organs, treatment with inhibitors can affect the expression and action of transporters in healthy tissue leading to undesirable effects.

Interrupting Pgp function by using inhibitors involves very often toxic side effects. Therefore, it is very important to invent a method that could apply the inhibitors directly to the destination or to find inhibitors that could work without such toxic side effect.

Cyclosporin A, for example, has been shown to be a potent inhibitor of Pgp both in cell lines (Nobili et al., 2006) and in animal models (Slater et al., 1986; Sikic et al., 1997; Eyal et al., 2009) but because of its immunosupressive effect it is rather used during organ transplantation than in epilepsy treatment (Liu et al., 2007).

Previously, a new method of inhibitor application has been described and is still investigated. To limit the undesirable effect inhibitors are enclosed in nanoparticles (Fisher and Ho, 2002; Fricker and Miller, 2004) called liposomes. Those small (<500 nm) vesicles are composed by different lipids. Because inhibitors or specific drugs are enclosed inside the vesicles, drugs are released very slowly and the time of acting is prolonged. Such nanoparticles can be also marked with specific antibodies against proteins present in target tissue. This modification allows them to migrate directly to the target organs, without toxic effect on other tissues (Huwyler et al., 1996; Huwyler et al., 2002).

6.2 Multidrug resistance associated proteins (MRPs)

Harmful compounds which enter the body are modified by oxidation (phase I metabolism), and to make them more water soluble by conjugation to gluthatione (GSH), sulfate or glucuronate (phase II metabolism). Those conjugates are very hydrophilic and need special transporters to efflux them out of the cell. Those transporters are called MRPs and unlike to Pgp (that favorites non-anionic substrates) transport substrate conjugated to glutathione (de Boer et al., 2003).

There are various MRPs that differ in substrate specificity, intracellular localization, structure (Borst and Elferink, 2002). Within ABC transporter family, the most examined are Pgp, MRP1 and MRP2 and BCRP. MRPs with following numbers (from MRP3 to MRP6) were discovered later and there is a need to investigate more their role. Short characterization of MRPs is described below.

6.2.1 Multidrug resistance protein 1 (MRP1)

MRP1 was the first multidrug resistance protein identified by Cole in cells selected for MDR (Cole et al., 1992). Its substrates are drugs conjugated to GSH, sulfate, glucuronate, but also anionic and neutral drugs. MRP1 consists of two NBDs that are not equivalent. An inactivation of NBD1 does not stop the transport completely but blocking the NBD2 leads to complete transport inactivation (Gao et al., 2000). MRP1 is expressed in basolateral membrane of capillary endothelial cells. However, a recent study has shown expression of MRP1 also in apical membrane endothelial cells (Zhang et al., 2004).

6.2.2 Multidrug resistance protein 2 (MRP2)

MRP2 and MRP1 have very similar size and membrane topology. The substrate specifities for MRP2 and MRP1 are overlapping. MRP2 transports a large range of organic anions. The distribution of MRP2 is restricted to the apical membrane of cells, including capillary endothelial cells (Hoffmann et al., 2006). The main function of MRP2 is biliary excretion of endogenous metabolites and it plays a role in reducing the food-derived carcinogens (Dietrich et al., 2001). MRP2 is expressed in brain, liver, kidney and the intestine of rats and humans.

6.2.3 Multidrug resistance protein 3 (MRP3)

MRP3 is an organic anion transporter. MRP3 is located in basolateral membrane of capillary endothelial cells and is expressed in liver, gut, and kidney (Scheffer et al., 2002). Up regulation of MRP3 expression was found in the liver under some cholestatic conditions. That could lead to role of MRP3 in removing the toxins from hepatocytes under the cholestatic conditions (Borst and Elferink, 2002).

6.2.4 Multidrug resistance protein 4 and 5 (MRP4 and MRP5)

Both MRP4 and MRP5 are organic anion pumps able to transport cyclic nucleotides and nucleotides analogs. This characteristic function could be a reason for resistance to clinically used bases, nucleotides and nucleotide analogs. Ability to transport the cyclic nucleotides MRP4 and MRP5 can affect the signal transduction by removing the cGMP (cyclic guanosine monophosphate) from the cells (Borst and Elferink, 2002). MRP4 and MRP5 are widely expressed in many organs, such as kidney heart and prostate (Borst et al., 2007). Recent study by Zhang (Zhang et al., 2004) has shown predominantly apical plasma membrane distribution for MRP5 and an almost equal distribution of MRP4 on the apical and basolateral plasma membrane in primary culture bovine brain microvessel endothelial cells.

6.2.5 Multidrug resistance protein 6 (MRP6)

MRP6 is mainly expressed in liver and kidney. MRP6 is localized in basolateral membranes of polarized cells. Substrate specificity is still under investigation and it is difficult to say which substances are transported by MRP6 (Borst and Elferink, 2002; de Boer et al., 2002).

6.3 Breast cancer resistance protein (BCRP)

Breast cancer resistance protein (BCRP), also called breast cancer related protein, and belongs to ABCG superfamily of ABC transporters. Unlike Pgp and MRP1 it has only one nucleotide binding domain followed by membrane spanning domain. Probably BCRP functions as a homodimer (Borst and Elferink, 2002).

Overexpression of BCRP in cancer is associated with high pharmacoresistance to various anticancer agents (anthracyclines, mitoxantrone). High expression of BCRP was detected in many different tumors where it implicates resistance and limits the efficacy of used chemotherapy (Doyle and Ross, 2003). For the first time BCRP was identified as overproduced protein in MCF7 breast cancer cells by Allikmets et al. (Allikmets et al., 1998).

The number of drugs to which BCRP confers pharmacoresistance is much lower than that for Pgp. BCRP is expressed in many organs such as: small intestine, liver and in luminal membrane of capillary endothelial cells in the brain (Borst and Elferink, 2002).

Table 1 ABC transporter nomenclature and localization		
name according to the old nomenclature	name according to the new nomenclature	localization
Pgp (MDR1)	ABCB1	apical membrane of brain capillary endothelial cells, kidney, intestine, liver
MRP1	ABCC1	basolateral membrane of brain capillary endothelial cells, also in apical membrane endothelial cells
MRP2	ABCC2	apical membrane of brain capillary endothelial cells, also expressed in liver and kidney and the intestine of rats and humans.
MRP3	ABCC3	basolateral membrane of brain capillary endothelial cells and is expressed in liver, gut, and kidney
MRP4	ABCC4	apical and basolateral membrane of brain capillary endothelial cells, also widely expressed in many organs, such as kidney heart and prostate
MRP5	ABCC5	apical membrane of brain capillary endothelial cells, also widely expressed in many organs, such as kidney heart and prostate
MRP6	ABCC6	expressed in liver and kidney, is localized in basolateral membranes of polarized cells
BCRP	ABCG2	apical membrane of brain capillary endothelial cells, also expressed in placenta, in small intestine, liver

Table 1 Table showing the ABC transporter names in both used nomenclatures and their main localization.

7 Other transporters: members of the organic anion transporting polypeptide family (OATP family) and the organic anion transporter family (OAT family)

Those two families of proteins are expressed in the brain in the capillary endothelial cells. They play a role in drug efflux at the BBB and blood-CSF barrier. The characteristic feature of those transporters is that they do not hydrolyze ATP and cannot transport drugs against the concentration gradient. Thus, they function as exchangers: exchange the drug for another molecule or ion by drug gradient without energy consuming. Another characteristic is that those transporters are reversible and can transport the drugs into and out of the brain, which depends on the existing drug gradient. Thus, they work bidirectional (Löscher and Potschka, 2005b). They can be localized both apically (Oatp3) and basolaterally (OAT3) and can be also expressed in both membranes (Oatp2) (Löscher and Potschka, 2005a).

All ABC transporters work together and protect the body against toxic endogenous substances and affect drug's accumulation in brain, fetus, testis, etc. It can happen that one transporter can overtake the role of another in excretion of toxins. To investigate a role of certain transporter, specific inhibitors must be available. Application of the inhibitors in studies on multidrug resistance allows checking, which transporter is functionally active. In case of Pgp studies, Pgp inhibitor tariquidar is often used.

It has been done lot of research on Pgp and BCRP, but there is still a need for investigation of MRPs functions. Unfortunately, the study on certain MRP members is limited because of lack of specific inhibitors.

8 Regulation of drug efflux transporters

8.1 Orphan nuclear receptors, the pregnane X receptor (PXR) and the constitutive active/androstane receptor (CAR)

All xenobiotics that get to the body must be processed and extruded as metabolized substances. Members of the NR1I (Orphan Nuclear Receptor) family, the nuclear pregnane X receptor (PXR) and the constitutive active/androstane receptor (CAR) are involved in regulation of toxic substances and

protective mechanisms against chemical insult. Both PXR and CAR are activated by diverse lipophilic chemicals both exogenous xenobiotics and endogenous substances (like bile acid). PXR and CAR regulate the expression of cytochromes P450 (CYP), a large family of heme-containing monooxygenases, and represent a first line of body's defenses. The most abundant CYP is CYP3A expressed in the liver and intestine (Kliewer and Willson, 2002).

Expression of multidrug transporters like Pgp is under tight transcriptional regulation by nuclear receptors (Synold et al., 2001; Wang and LeCluyse, 2003; Timsit and Negishi, 2007).

PXR was reported in 1998 as a novel murine member of the steroid-retinoid-thyroid hormone receptor family of ligand-activated transcription factors. The name PXR is based on its activation by both natural and synthetic C21 steroids (pregnanes). C21 refers to number of carbon atoms (Kliewer et al., 1998). PXR is highly expressed in liver and intestine of humans, rabbits, rats and mice. CYP3A genes are expressed and induced in response to xenobiotics in the same tissue (Bertilsson et al., 1998; Cheng and Klaassen, 2006). PXR can be activated by both endogenous (corticosteroids) and exogenous (dexamethasone) steroids. However, there are marked differences in PXR activation profiles between species (Zhou, et al., 2009), which are due to differences in ligand binding domain (Fig. 6) (Kliewer and Willson, 2002).

CAR together with PXR are involved in drug metabolism. However, there are also members of nuclear receptor family, NR1I, called estrogen receptor (ER) and glucocorticoid receptor (GR) that are involved in other functions like cholesterol homeostasis (Fig. 7) (Konno, et al., 2008).

8.2 Structure of pregnane X receptor (PXR) and constitutive active/androstane receptor (CAR)

PXR and CAR structures have specific regions for nuclear receptor like: highly conserved DNA binding domain and ligand-binding domain (LBD in C-terminal portion of the protein). There are species-specific differences in the receptor in similarity of amino acids in LBD. The most comparable species to human PXR is rhesus PXR (95% of amino acid similarity), then pig PXR (87% of amino acid similarity) and dog PXR (83% of amino acid similarity).

First, a cDNA encoding mouse PXR was cloned (Kliewer et al., 1998), then human, monkey, dog, rabbit, and rat PXR have been cloned as well (Bertilsson et al., 1998; Zhang et al., 1999; Jones et al., 2000).

Nuclear receptor most related to PXR is the constitutive active/androstane receptor (CAR). These two receptors share approximately 70% and 50% amino acid identity in their DBDs and LBDs, respectively (Fig. 6) (Blumberg et al., 1998).

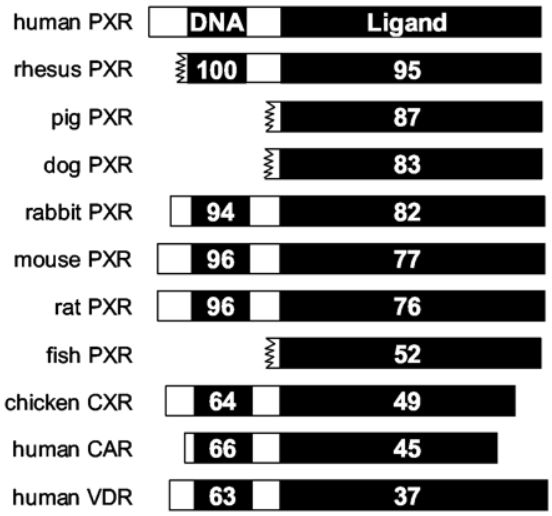


Fig. 6 Sequence comparison of PXR across species. The similarity is expressed as percent of identity of amino acids in the DNA-binding (DNA) and ligand-binding (Ligand) domains (Kliewer et al., 2002).

8.3 Mechanism of activation of pregnane X receptor and constitutive active/androstane receptor (CAR)

Both PXR and CAR are activated by many substances. The mechanisms of activation of PXR and CAR are shortly described below.

After the exposure to the substrates (e.g. rifampicin, dexamethasone) PXR is released from the co-chaperone protein, called Hsp90 (heat shock protein 90) and cytoplasmic CAR retention protein, CCRP). Then PXR is translocated to the nucleus where it binds to the retinoid X receptor (RXR). The whole complex binds to the specific promoter region (xenobiotic-responsive enhancer module, XREM) which activates the genes responsible for the elimination of the toxins (Fig. 7) (Timsit and Negishi, 2007).

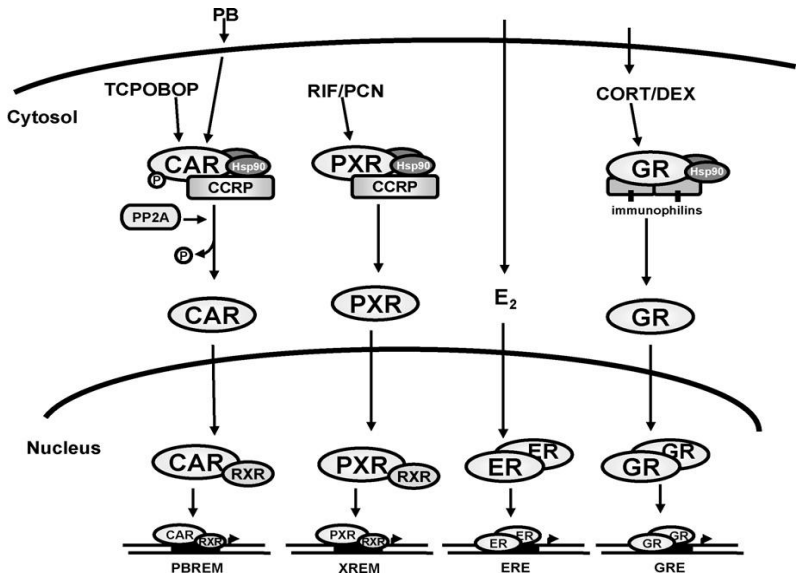


Fig. 7 Summary of signaling pathways for CAR, PXR and the nuclear steroid receptors ER, GR (Timsit and Negishi, 2007).

For instance, CAR is regulated by phenobarbital. The activation of CAR upon phenobarbital exposure, results in its dissociation from co-chaperon proteins HSP90 and CCRP. Released CAR is translocated to the nucleus. This translocation depends on activity of protein phosphatase PP2A and it is followed by binding to the retinoid X receptor (RXR) and to PBREM, which starts transcriptional activation of genes involved in phase I, phase II elimination pathways. CAR is fundamental different from PXR, exactly, with a regard to its cellular regulation. Thus, regulation of CAR nuclear translocation depends on phosphorylation and dephosphorylation of receptor (Moore et al., 2002).

Among the genes that are regulated by PXR in the liver and/or intestine are genes encoding phase I monooxygenases (CYP3A, CYP2B6, etc) and genes encoding phase II enzymes involved in conjugation of xenobiotics (members of the glutathione-S-transferase, sulfotransferase and UDP-glucuronosyltransferase) or involved in transport of xenobiotics (MRP1, MRP2, OATP2) (Gerbal-Chaloin et al., 2001; Falkner et al., 2001; Synold et al., 2001; Nagaoka et al., 2006; Martin et al., 2008).

9 Introduction to methods used in this thesis

9.1 Western blotting (modified from Gallagher, 2006)

9.1.1 Principle of the technique

Western blotting (immunoblotting) method allows analyzing protein expression in tissue and cells. Immunoblotting method was discovered by E. Southern. Because of the analogy between name and geographical directions method for protein separation was called Western blotting.

Western blotting is a transfer of protein molecules separated first in polyacrylamide gel into the surface of an immobilizing membrane (nitrocellulose or PVDF membranes). Electrophoretic separation of proteins is performed with detergent sodium dodecyl sulfate (SDS), which denatures protein structures and gives negative charge to proteins, which are then separated by their molecular weight. Proteins with lower molecular weight move faster in the gel than proteins with higher molecular weight.

There are two types of gels used to separate proteins: a stacking (focusing) gel and resolving (separating) gel. The role of stacking gel is concentrating the samples that they can run from the same place of the gel. Resolving gel is more concentrated polyacrylamide gel that allows the samples to be separated according to the size.

Immunodetection of separated proteins is made by specific antibodies and adequate methods are used to identify the antigen-antibody complex. After electrophoresis proteins are transferred from the gel to the nitrocellulose membrane or PVDF (polyvinylidene fluoride membrane). PVDF membrane is a high sensitive, hydrophobic membrane with high capacity to bind proteins and it needs to be first washed in methanol for re-hydratation.

In order to check protein transfer blot can be stained in reversible protein Ponceau dye and the gel can be incubated with Coomassie solution. To block unspecific bindings a membrane is blocked with skim milk. After that membrane is incubated with specific primary antibody, followed by incubation with secondary antibody. Secondary antibody can be conjugated to the horseradish peroxidase (HRP) enzyme to determine the presence of molecular target.

Identification of the antigen-antibody complex is done by using ECL (enhanced luminol-based chemiluminescent substrate) working solution. This high sensitive solution is for use with HRP-conjugated secondary antibodies and gives fluorescent signal that can be detected on X-ray film.

Protein concentrations are normalized on loading controls (tubulin, actin). The expression of such internal markers should be stable and should not be affected by drugs used in the treatment of

investigated cells or tissues. Examined proteins are recognized by expected molecular weight shown by marker ladder (standard weights are expressed in kDa).
Blots are analyzed by densitometry using special software. The representative protein bands are obtained on the same Western blot on one X-ray film.

9.2 Quantitative real-time RT-PCR (qPCR)

qRT-PCR is the most sensitive technique for mRNA detection and quantification currently available. In this method, mRNA must be first isolated from cells or tissues. Isolated mRNA is then copied to cDNA (complementary DNA) by reverse transcriptase using an oligo dT primer.

Samples are incubated with PCR mix containing a heat-stable polymerase (such as Taq polymerase), specific primers for the gene of interest, deoxynucleotides and a suitable buffer to form double DNA. Next, cDNA is denatured at 94^{o}C so that the two strands are separated. The whole mixture is cooled to 50-60^{o}C and specific primers are annealed that are complementary to a site on each strand. The primers may be up to 600 bases but are often about 100 bases. Primer design must be adequate, otherwise it might influence on specific amplification.

Next step is to increase the temperature up to 72^{o}C so that heat-stable Taq DNA polymerase is able to extend the DNA from the primers. Now four cDNA strands (from the original two) exist. These are denatured again at approximately 94^{o}C. Again, the primers are annealed at a suitable temperature; Taq DNA polymerase binds and extends from the primer to the end of the cDNA strand so there are now eight cDNA strands. Again, the strands are denatured by raising the temperature to 94^{o}C and then the primers are annealed at 60^{o}C. Amplified products are detected and measured by PCR cycler (Alberts et al., 1994; Protocol Online, httP//www.protocol-online.org/).

9.3 Uptake assay

Functionality of Pgp can be investigated by accumulation test (also called uptake assay). Uptake assay uses the ability of Pgp to actively efflux the substrates out of the cells. The more active Pgp is, the fewer amounts of substrates are accumulated within the cells (Pgp removes the substrates from the cells).

In such tests, cells are incubated with a Pgp substrate (for example digoxin, vinblastine, rhodamine) for certain time. For a control of assay, specific Pgp inhibitor (tariquidar) is used, which blocks Pgp function and results in higher intracellular accumulation of Pgp substrates.

Samples representing cell lysates are analyzed by β-counter when substrates are radioactive (for example ^{3}H-digoxin) or by spectrofluorometer in case of fluorescent substrates (for example rhodamine 123). Measurement of protein concentration is perfomed and data are expressed in DPM/mg protein (Luna-Tortós et al., 2008).

10 Summary and Purpose

Epilepsy which is characterized by recurrent spontaneous seizures is a very common neurological disorder that affects people and animals (Dewey, 2006). Despite various advanced AEDs about one third of the patients develop pharmacoresistance (Löscher and Potschka, 2002). The consequences of uncontrolled epilepsy are very serious, and it is very important to find out what are the mechanisms behind the resistance in the epilepsy treatment. Because of very distinct effects in epileptic patients, like loss of consciousness and disturbances of movement, sensation or mood or mental function, epilepsy is termed as multiple or complex disease (Garcia-Morales et al., 2008). The phenomenon of resistance is intriguing because of the fact that patients with refractory epilepsy do not respond to all applied AEDs in spite of the different mechanism of these drugs (Lazarowski et al., 2007).

Up to now, the mechanisms of pharmacoresistance are poorly understood and are a limiting factor in the treatment of many diseases. There is increasing evidence that overexpression of multidrug transporters is involved in drug resistance in epilepsy. One of the best examined multidrug transporters engaged in pharmacoresistance in epilepsy is the efflux transporter Pgp that limits penetration of drugs into many cell types and tissues (Schinkel, 1999; Takano et al., 2006).

Pgp is a phosphorylated glycoprotein with molecular weight of 160 kDa and is a product of *ABCB1* gene. Pgp is expressed in many tissues, including the intestine, liver, kidney and brain, and is involved in drug absorption, distribution and excretion (Schinkel and Jonker, 2003). In CNS, Pgp contributes to the BBB function and is localized in the luminal (apical) membrane of brain capillary endothelial cells. The role of Pgp is protecting the brain from potentially harmful xenobiotics by effluxing them from the brain, so that the concentration of drugs in brain may become insufficient for

therapeutic activity. If such overexpression of Pgp enhances the efflux of AEDs from the brain, they cannot reach level needed for effective work. It has been shown that in epileptic patients with refractory epilepsy Pgp is overexpressed in brain endothelial capillary cells (Tishler et al., 1995). However the aetiology of such overexpression and pharmacoresistance is unkown. Pgp overexpression might be caused by disease itself or might be a result of treatment with antiepileptic drugs.

This work was meant to answer the question whether AEDs induce Pgp overexpression, which would facilitate pharmacoresistance in epilepsy. For that reason investigations of Pgp expression after exposure to AEDs (phenobarbital, carbamazepine, phenytoin, topiramate) and known Pgp inducers (dexamethasone, doxorubicin) for different time periods in various cell types were done.

All cell lines, which were used in following study, are considered as suitable models of the BBB. Additionally, immortalized rat brain endothelial cells were used to verify whether they constitute as a good complacement for research in rats. For our purpose various cell lines were used like: epithelial cells Madin-Darby Canine Kidney (MDCKII) or porcine epithelial cells (LLC), both wild type (MDCK-WT, LLC-WT) and transfected with human Pgp (MDCK-MDR1, LLC-MDR1) and immortalized rat brain endothelial cells: GPNT and RBE 4 cells.

11 Materials and Methods

11.1 Cell culture

For the experiments, all cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2/95% atmosphere.

11.1.1 Epithelial cell lines

Epithelial cell lines (MDCKII, LLC) used in experiments were renal epithelial cell lines derived from canine and porcine kidney. Those cell lines were chosen because of the presence of tight junctions (TJs), which are structures that block the movement of integral proteins and provide functionaly polarized membranes: apical and basolateral. Because of the presence of TJs the transcellular transport is preserved. MDCK and LLC cell lines are also accepted as a model of the

BBB. Furthermore, MDCK and LLC lines express Pgp (canine, porcine) and also can be easily transfected with Pgp from another species (human MDR1, mouse mdr1a).

11.1.1.1 MDCKII

MDCK cells transfected with human MDR1 (MDCK–MDR1) and respective wildtype (WT) MDCK cells were kindly provided by Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands). MDCK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco™/Invitrogen Corporation, Eggenstein, Germany) supplemented with 10% fetal calf serum. The cells were grown on 78 cm^2 plates and the passaging was performed twice a week with subconfluent monolayers using Trypsin/EDTA (Gibco™/Invitrogen). Since it is known (Polli et al., 2001) that MDCK cells express lower Pgp level during first three passages, MDCK cells were passaged at least three times before using in experiments. Experiments were performed with cells within 10 passages.

11.1.1.2 LLC-PK1

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). LLC cells were cultured in Medium 199 (Gibco™/Invitrogen Corporation, Eggenstein, Germany) supplemented with 10% fetal calf serum (Gibco™/Invitrogen). The cells were grown on 78 cm^2 plates and the passaging was performed twice a week with subconfluent monolayers using Trypsin/EDTA (Gibco™/Invitrogen). Cells were passaged at least three times before using in experiments.

11.1.2 The immortalized rat brain endothelial (RBE) cell lines

Other cell lines used in experiments were immortalized rat brain endothelial cells: GPNT and RBE4. Those cell lines express whole set of BBB markers and are used in studies of the BBB permeability and transport functions. Additionally, GPNT express high level of mdr1a RNA. Pgp expression in those immortalized cell lines can be enhanced by routine treatment with puromycin (Demeuse et al., 2004).

11.1.2.1 GP8/GPNT

GPNT cells were kindly provided by Prof. F. Roux (INSERM U26, Hôpital Fernand Widal, Paris, France). GPNT were obtained from a previously characterized rat brain endothelial cell line, GP8 (Greenwood et al., 1996) that were transfected by lipofection with pcDNA3-RSV plasmid containing the puromycin resistance gene. The specific clone named GPNT (for GP8 and the company NeuroTech S.A.) was selected using morphological criteria of BBB characteristics.

GPNT cells were grown on collagen type I-coated plates (Collagen Type I from rat tail, Roche Molecular Biochemicals) and were maintained in α-Minimal Essential Medium/Ham's F10 (1/1 vol./vol.) (Invitrogen Corporation, Eggenstein, Germany**)** supplemented with Glutamax-I, 1 ng/ml basic fibroblast growth factor and 10% heat-inactivated fetal calf serum. For some experiments, GPNT medium was supplemented additionally with 5 µg/ml transferrin, 5 µg/ml insulin, 5 µg/ml selenium and 5 µg/ml puromycin. The cells were grown on 78 cm^2 plates and the passaging was performed twice a week with subconfluent monolayers using Trypsin/EDTA (Gibco™/Invitrogen). Cells between passages 30-40 were used in experiments.

11.1.2.2 RBE4

RBE4 cells were kindly provided by Prof. F. Roux (Hôpital Fernand Widal, Paris, France).

RBE cells were transfected by the calcium phosphate coprecipitation procedure with the plasmid pE1A/neo containing the E1A region of Adenovirus 2 and the neomycin-resistance gene for selection in resistance to G418.

RBE4 cells were grown on collagen type I-coated plates (Collagen Type I from rat tail, Roche Molecular Biochemicals) and were maintained in α-Minimal Essential Medium/Ham's F10(1/1 vol./vol.) (Invitrogen Corporation, Eggenstein, Germany**)** supplemented with Glutamax-I, 1 ng/ml basic fibroblast growth factor, 10% heat-inactivated fetal calf serum, and additionally with 300 µg/ml geneticin (G418). The cells were grown on 78 cm^2 plates and the passaging was performed twice a week with subconfluent monolayers using Trypsin/EDTA (Gibco™/Invitrogen).

11.2 Western blotting

Cells monolayer were first washed with ice-cold PBS, collected with cell scraper and centrifuged at 800-1000 g at +4°C for 5 minutes to pellet the cells. In some experiments, aliquots for RNA isolation were taken and freezed as pellets without PBS at -20°C. After that, cells were suspended in ice-cold lysis buffer (25 mM Tris, 50 mM NaCl, 0.5% Na-deoxycholate, 0.5% Triton X-100, 1x Roche Protease Inhibitor Cocktail Complete®, pH=8.0) and incubated on ice for 30 min After lysation, cells were centrifuged at 13000g at +4°C for 15 minutes. Supernatants (protein lysates) were collected to new tubes and were stored at -20°C until examination. Protein concentration was measured using BCA (bicinchoninic acid) assay. This method combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) using a unique reagent containing bicinchoninic acid (Smith et al., 1985; www.thermo.com/pierce).

25-100 µg total cell proteins (depending on the cell line) were taken for loading on the gel. Proteins were transferred either to nitrocellulose membrane or to PVDF-membrane for 2 h. Next, blots were incubated with primary Pgp antibody C219 (from different brands: Calbiochem, Alexis, Signet) for overnight and with primary for actin or tubulin (Sigma). Next, the incubation with secondary antibody either rabbit-anti mouse-HRP (Dako) for Pgp or goat-anti-rabbit-HRP (Dako) for tubulin and actin was done. Blots were then incubated with luminal kit (Pierce) according to the commercial instruction. Blots were exposed to X-ray film and analyzed by Scion Image (Scion Corporation, Maryland, USA). For the details in Western blotting protocol see Appendix.

Pgp expression was quantified and normalized on α-tubulin or β-actin.

11.2.1 Comparison of different protocols used for Western blotting method

modification	protocol no. 1	protocol no. 2
preparation of cell lysis	scrubbed cells were sonificated 3x15 seconds, then centrifugated at 1000 g for 10 min; liquid part contains protein fraction	scrubbed cells were centrifuged at 1000 g for 10 min; cell pellet was incubated with lysis buffer on ice and then centrifugated again at 13000-15000 g for 15 min; protein fraction was in supernatant
lysis buffer	20 mM Tris, 10% SDS, protease inhibitor, water	25 mM Tris, 50 mM NaCl, 0.5% Na-deoxycholate, 0.5% Triton X-100, protease inhibitor
Primary Pgp antibody	mouse C219, Alexis	mouse C219, Signet,
	mouse C219, Calbiochem	
primary antibody for loading controls	rabbit tubulin, Sigma	rabbit actin, Sigma
secondary antibody	secondary rabbit-anti mouse-HRP, Dako	secondary rabbit-anti mouse-HRP, Dako
	secondary goat-anti-rabbit-HRP, Dako	secondary goat-anti-rabbit-HRP, Dako
solution for dissolving the antibody	5% milk in PBS	2% milk in PBS-T*
type of membrane	0.2 μm nitrocellulose membranes	PVDF-membrane
transfer buffer	3 buffers system	one buffer system (25 mM Tris, 192 mM glycine, 10% methanol)
% of separation gel	7.5%	7%
Ponceau S staining	no	yes

*PBS-T PBS with 0.05 %Tween

Table 2 Table represents changes in Western blotting protocol. Protocol no. 1 contained settings used at initial work, new protocol was modified and was used as optimaized protocol for experiments.

11.3 Uptake assay

Functionality of P-glycoprotein was monitored by accumulation (uptake) test using selected radioactive Pgp substrates. On the day of the assay cells were washed with PBS buffer and preincubated in serum-free Optimem® medium for 1 hour with or without Pgp inhibitor tariquidar (0.5 µM). ^{3}H-Digoxin, a specific Pgp substrate, was then added for 2 hours. The plates were shaking during both preincubation and incubation periods to preserve equal concentration of drugs on the plates. The uptake was stopped by rapidly removing the medium and single washing of the cell monolayers with ice-cold PBS. Cells were then scrubbed and lysed with lysis buffer (25 mM Tris, 50 mM NaCl, 0.5% Na-deoxycholate, 0.5% Triton X-100). Protein concentration was measured as well (for protocol see Appendix). The amount of tritiated drug retained in the cells was measured by ß-scintillation counter. The aliquots of cell lysates were analyzed in parallel by Western blotting. For the details in uptake assay protocol see Appendix.

The intracellular amount of digoxin was expressed in DPM/mg protein.

11.4 Quantitative real time RT-PCR (qPCR)

Total RNA was isolated from cells using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The concentration and purity of RNA were estimated spectrophotometrically at 260 nm and 280 nm, and the integrity of the RNA was checked by electrophoresis on 1% agarose gel. RNA was reverse transcribed with oligo(dT) and random primers using SuperScript III First-Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Amplification was detected by SYBR® Green fluorescence MX3005P instrument (Stratagene, La Jolla, CA, USA). cDNA of each sample was diluted 1:24 in RNase free water. Thermocycling was carried out in a final volume of 20 µl containing 10 µl Precision™-MX-SY MasterMix 2x (PrimerDesign Ltd, Southampton, UK), 1 µl of each forward and reverse primer (final concentration 0.5 µM), and 8 µl diluted cDNA. The thermal cycling conditions were 10 min at 95^{o}C to activate DNA polymerase, followed by 45 amplification cycles at 94^{o}C for 10 s, 58^{o}C for 10 s, and 72^{o}C for 30 s, with the last cycle finished by 3 min elongation at 72^{o}C and dissociation curve analysis to ensure the specificity of the PCR product. For each primer pair, we performed no template control (NTC) and no-reverse-transcriptase control assays, which produced negligible signals (usually >40 in Ct value), suggesting that primer–dimmer formation and genomic DNA contamination effects were negligible.

Dissociation curve analysis revealed that no side products have been formed. The GAPDH gene was used as an endogenous reference for normalizing target gene mRNAs. All primers were designed using PRIMER3 software (web-based version httP//fokker.wi.mit.edu, Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and checked for specificity using GenBank databases by BLAST tool (httP//blast.ncbi.nlm.nih.gov, National Center for Biotechnology Information, Bethesda, MD, USA). To ensure that all qPCR products are generated from cDNA but not from genomic DNA, all primers were analyzed by UCSC IN-SILICO PCR software (Webbased version httP//genome.ucsc.edu/cgi-bin/hgPcr, University of California, Santa Cruz, CA, USA), and products of PCR reaction were checked on 2% agarose gel (Kuteykin-Teplyakov et al., 2009).

Table 3 Sequences of primers used for qPCR

Protein name	Target gene name	GenBank accession number[a]	Amplicon length, *bp*	Forward primer, 5'→3'	Reverse primer, 5'→3'
Pgp (MDR1), human	*ABCB1*	NM_000927.3	217	CCGAACACATTGGAAGGAA	CTTTGCCATCAAGCAGCAC
Mrp1, dog	*Abcc1*	NM_001002971.1	165	GGCTCTGCTTCCCCTTCTAC	GGATTTTGCCCCAACTTCTT
Mrp2, dog	*Abcc2*	NM_001003081.1	187	TTGGCTTACTCCTGCCTGTT	CCAGTGTCAGAGGTTGCTTG
Mrp5, dog	*Abcc5*	DQ985741.1	204	CCTACAACAAAGGGCAGGAA	AGATGGCAAGACCCGAATAG
Mdr1a, rat	*Abcb1a*	AF257746	152	TACATCTTGGCGGACCTTAC	CGCTGGTTTCTTTTCTTTCTTC
Mdr1b, rat	*Abcb1b*	NM_012623.2	211	TTTCTGCTGTTGTCTTTGGTG	GGTCGGGTGGGATAGTTGA
GAPDH, dog	*Gapdh*	AB038240.1	117	ATTCCACGGCACAGTCAAG	TACTCAGCACCAGCATCACC
GAPDH, rat	*Gapdh*	NM_017008.3	136	ATGACTCTACCCACGGCAAG	TACTCAGCACCAGCATCACC

[a]Gene accession number at www.ncbi.nlm.nih.gov/Genbank/ (table based on data from Dr. K. Kuteykin-Teplyakov)

11.5 Confocal microscopy

Confocal microcopy investigation was done in GPNT cells in order to define a proper time for the treatment with AEDs. Cells were cultured in medium composition mentioned above. Cells on the

day of the confluence, or 3 and 6 days after confluence were chosen for the microscopy examination of Pgp expression level. First, cells were seeded on sterile cover slips in 24 well plates. On the proper day of confluence cells were washed twice with PBS and fixed first with 3 ml/well paraformaldehyde solution for 15–20 min at room temperature and for 5 min with 50 mM NH_4Cl. In order to destabilize the biological membrane and enhance penetration of macromolecules, cells were incubated with detergents: 5 min with saponin/PBS and for 5 min with 1% Triton-X100 in PBS. After that, cover slips with the cells were incubated in blocking solution for at least 30 min with BSA/saponin/PBS. Next step was incubation with primary antibody. Anti-Pgp in dilution 1:20 (10 µl/cover slip) and anti-α-tubulin in dilution 1:1000 (10 µl/cover slip) were applied for 2 h at room temperature. After incubation with primary antibody, cover slips were washed in blocking solution twice for 5 min and secondary antibody dissolved in blocking solution was used. This incubation was done in darkness for 1 h at room temperature. In this experiment secondary antibody goat anti-mouse Cy2 1:50 (10 µl/ cover slip) was used. To remove unbounded antibodies cells were washed twice with saponine/PBS and twice with PBS. After that, cover slips were mounted to the glass slide with Mowiol, let it dry and analyzed with confocal microscopy. All washing steps and applying solutions were done very slowly to do not disturb cell monolayer (modified from Volk et al., 2005).

11.6 Influence of different medium compositions on Pgp expression in rat brain endothelial cell lines

In order to check whether there is an influence of medium composition on Pgp expression two protocols were tested in GPNT and RBE4 cell lines.

Table 4 Medium compositions for GPNT cell line		
medium component	**medium type**	
	medium no. 1	medium no. 2
α-Minimal Essential Medium/Ham's F-10 (1:1, vol/vol)	**+**	**+**
10% heat-inactivated FCS	**+**	**+**
2 mM glutamine	**+**	**media already supplemented with Glutamax-I**
bFGF	**2 ng/ml**	**1 ng/ml**
5 µg/ml transferrin	**+**	**-**
5 µg/ml insulin	**+**	**-**
5 ng/ml elenium	**+**	**-**
1% Pen/Strep	**-**	**+**
5 µg/ml puromycin	**+**	**+**
collagen type I-coated plates	**+**	**+**

Table 5 Medium compositions for RBE4 cell line		
medium component	**medium type**	
	medium no. 1	medium no. 2
α-Minimal Essential Medium/Ham's F-10 (1:1, vol/vol)	**+**	**+**
10% heat-inactivated FCS	**+**	**+**
2 mM glutamine	**+**	**+**
300 µg/ml geneticin	**+**	**-**
bFGF	**1 ng/ml**	**1 ng/ml**
1% Pen/Strep	**-**	**+**
collagen type I-coated plates	**+**	**+**

Table 4 and 5 Comparison of two medium compositions for GPNT and RBE4 cells culturing. Prescription for medium 1 was taken from the literature (Régina et al., 1999), protocol for medium 2 preparation was received from F. Roux (as a protocol for cell culturing GPNT and RBE4 cells).

11.7 Deglycosylation of Pgp (investigation of changes in Pgp glycosylation after using PNGase F by Western blotting)

This work was meant to check whether upper band (app. 160 kDa) and lower band (app. 140 kDa), both recognized by Pgp antibody in MDCK cells, belonged to the same protein.

Distinction between core-glycosylated and glycosylated form of Pgp in MDCK wild type was possible by using PNGase F enzyme (kindly provided by Prof. W. Baumgärtner) that removes carbohydrates from proteins. N-Glycosidase F, also known as PNGase F, is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (Fig. 8) (Maley et al., 1989). Extracted proteins from MDCK wild type cells were first denaturated and then incubated with 1 µl of PNGase F enzyme for 1 h in 37°C. After the incubation, samples were loaded into the gel and electrophoresis was performed.

Incubation with PNGase F enzyme should result in only one band recognized by C219 antibody, which corresponds to core-glycosylated protein (Loo and Clarke, 1999b).

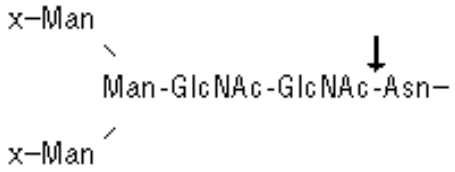


Fig. 8 Schematic representation of place cleaved by PNGase F (cleaved place is marked with an arrow). Enzyme PNGase F cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (modified from New England BioLabs®Inc. website).

12 Statistics

Data were statistically analyzed by one-way analysis of variance (ANOVA), followed by the Bonferroni Multiple Comparison test or unpaired t-test, using PRISM 5 software (GraphPad Inc, La Jolla, CA, USA). Value $p<0.05$ was considered statistically significant. For several preliminary experiments statistical analysis was not performed because of too low sample size (see legends below the graphs).

13 Results

13.1 Western blotting results

First Western blotting experiments in MDCKII cells were performed with two different protocols in order to establish a reliable Western blotting method for Pgp expression analysis. For the details of those two protocols see Table 2.

The changes in the protocols were a result of improvement the Western blotting methods and were done to elaborate optimized conditions for Pgp protein analysis in our laboratory. Enhancement in the protocol was needed in order to solve problems with unstable loading control, position of default Pgp bands and to adapt method to our conditions.

All experiments in GPNT and MDCK cells are presented in Tables 6, 7 & 8 (see Appendix), which contain data from Western blotting experiments according to protocols no. 1 and 2. Data from Western blotting done in parallel to uptake assays are also shown in those tables.

13.2 Part I. Experiments in MDCKII cells according to the protocol no. 1

Study of Pgp expression after AEDs treatments was began by Prof. H. Potschka (currently at Ludwig-Maximilians-University, Munich) and students L. Lombardo and S. Jahn, who found out possible Pgp upregulation after AEDs. After that, study on Pgp expression was continued.

Pgp antibodies from Alexis and Calbiochem were used as described in Table 2. The purpose of this trial was to check in a first screening, whether AEDs can induce Pgp expression in MDCKII wild type cells. Protein expression was analyzed and the results were shown as percent of DMSO control (Fig. 9A) and as an immunoblot (Fig. 9B). Data shown in Fig. 9 did not indicate that used AEDs might upregulate Pgp expression. However, statistical analysis could not be performed because of too low sample size.

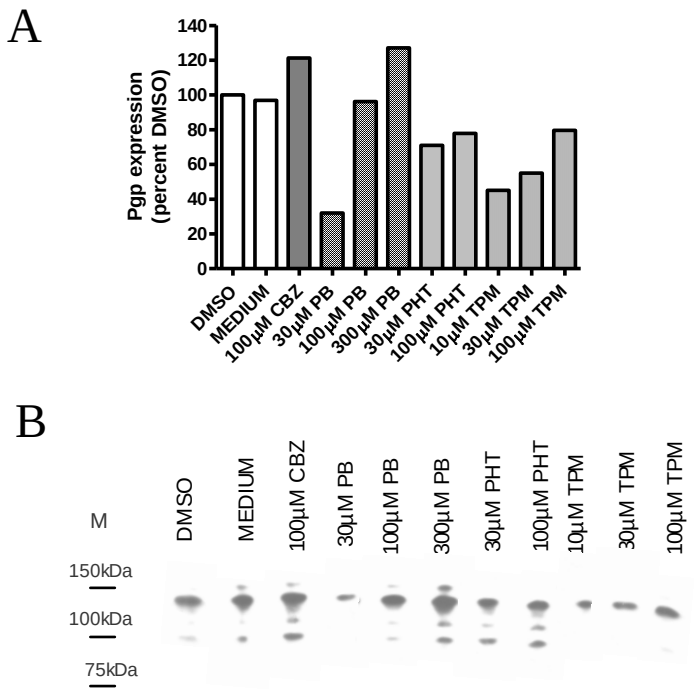


Fig. 9 Pgp expression in MDCKII wild type cells P13 after the treatment with different antiepileptic drugs (drug concentrations indicated below the graph). Representative immunoblot (B) and analysis of Western blots (A) of Pgp protein levels in MDCKII are shown. Cells were treated for 5 days starting on the day of confluence. All drugs were dissolved in DMSO up to final concentration of 0.1%. Only the Pgp signal around 140 kDa was used for quantification. Pgp expression was normalized on percent DMSO control. There was no trend in data suggesting enhanced Pgp expression after AEDs treatment. Statistical analysis was not performed because of too low sample size.

The purpose of the experiment shown in Fig. 9 was to get a first impression about possible induction of Pgp expression after the treatment with AEDs. The data could give hint for enhanced Pgp expression after after carbamazepine (100 µM) and phenobarbital (300µM) treatment. However, statistical analysis could not be performed, because of limited sample size.

Thus, for next trial MDCKII wild type were treated with AEDs and known Pgp inducer rifampicin (25µM) (Haslam et al., 2008). Rifampicin was used as a positive control. Graph and representative immunblot are shown in Fig. 10A and 10B.

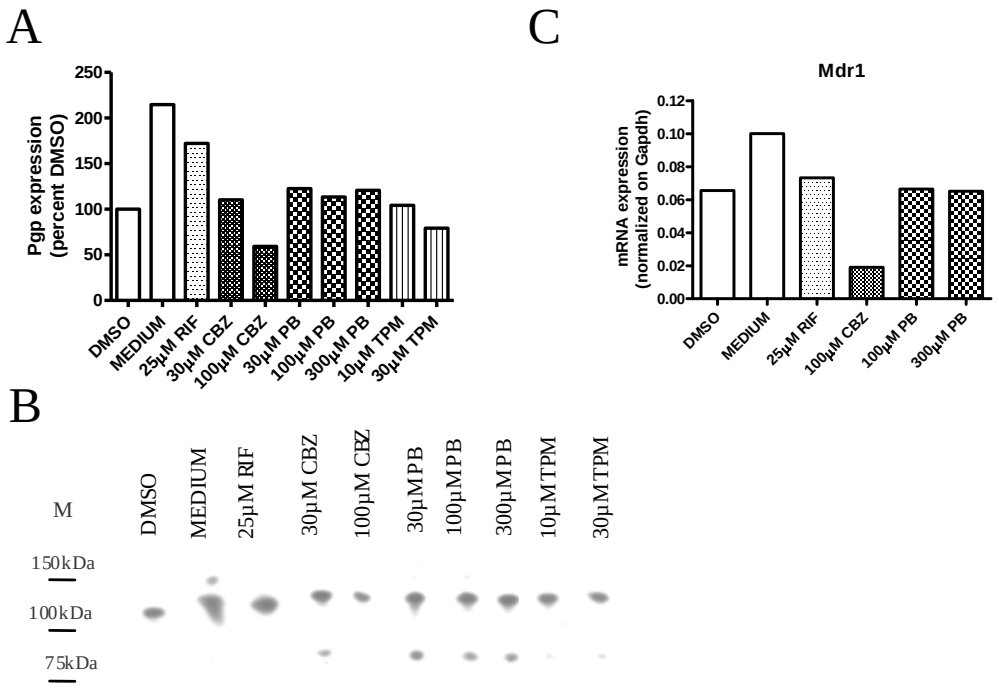


Fig. 10 Pgp expression in MDCKII wild type cells P17 after the treatment with different antiepileptic drugs (drug concentrations indicated below the graph). Representative immunoblot (B) and analysis of Western blots (A) of Pgp protein levels in MDCKII are shown. Additionally, mRNA level was investigated (C). Cells were treated for 5 days starting on the day of confluence. Rifampicin (RIF, 25 µM) was used as a positive control. All drugs were dissolved in DMSO up to final concentration of 0.1%. Only the signal around 120 kDa was used for quantification. Pgp expression was normalized on percent DMSO control. There was no trend in data suggesting enhanced Pgp expression after AEDs treatment. Statistical analysis was not performed because of too low sample size.

To complete the data and see whether used AEDs can affect multidrug transporter genes expression, PCR was performed. mRNA level of Mdr1 gene (Fig. 10C) and other transporter genes like, MRP1, MRP2 and MRP5 (Fig. 11A, B & C) were analyzed.

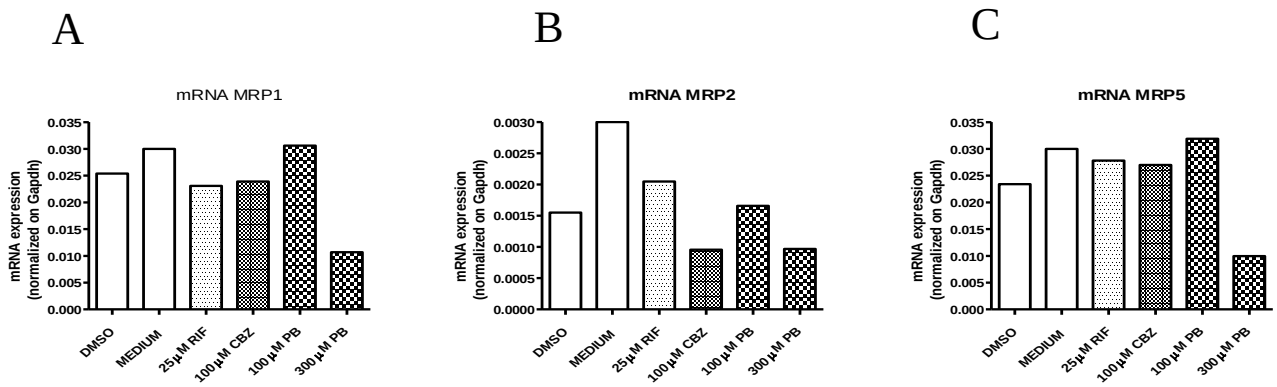


Fig. 11 mRNA expression level of MRPs in MDCKII wild type cells P17. Cells were treated for 5 days starting on the day of confluence. Rifampicin (RIF, 25 μM) was used as a positive control. All drugs were dissolved in DMSO up to final concentration of 0.1%. MRP1 (A), MRP2 (B), MRP5 (C) were analyzed. Data were normalized on Gapdh. There was no trend in data suggesting enhanced Pgp expression after AEDs treatment. Statistical analysis was not performed because of only one sample repetition. mRNA analysis was performed by Konstantin Kuteykin-Teplyakov.

The data could not be statistically analyzed because of only one sample per drug; however there was no indication for Pgp enhanced expression after treatment with AEDs. Rifampicin treatment showed tendency in enhanced Pgp expression (Fig. 10A) and slightly on mRNA level (Fig. 10C).

Because of unstable results from expreriments presented above, in next trial two sets of the same samples were done in order to check variability of method.

MDCKII wild type cells were treated with rifampicin (RIF) at concentration 25 μM and with phenobarbital at concentration 300 μM. The experiment was planned in two sets (repetition of separately prepared samples). Pgp antibody has recognized as more than one band but only the strongest around 120 kDa was used for quantification of the blots (Fig. 12A & B for Set I and Fig. 12C & D for Set II).

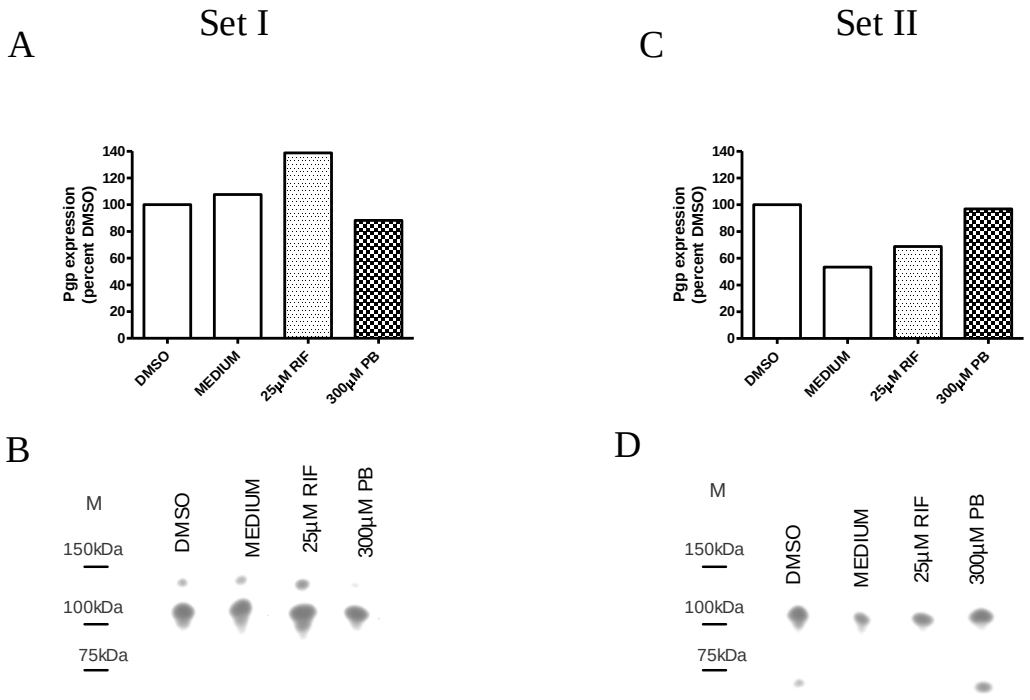


Fig. 12 Pgp expression in MDCKII wild type cells P9. This is the same experiment made in two sets. Representative immunoblots (B & D) and analysis of Western blots (A & C) of Pgp protein levels in MDCKII are shown. Cells were treated for 5 days starting on the day of confluence with phenobarbital (PB, 300 µM) and rifampicin (RIF, 25 µM). RIF was used as a positive control. Drugs were dissolved in DMSO up to final concentration of 0.1%. Only the signal around 120 kDa was used for quantification. Pgp expression was normalized on percent DMSO control. Data represent reverse trend in Pgp expression after RIF. Statistical analysis was not performed because of small sample size.

In order to check the repeatability of Western blotting method two set of samples were analysed (Fig. 12). Samples were loaded on the same gel twice (Set I & Set II). However, data presented aboved showed contradictory results after the treatment with rifampicin (Fig. 12A *vs* Fig. 12C).

All presented graphs and immunoblots lacked of loading protein. This fact is due to the ongoing problems with tubulin used as a protein for blots normalization. In presented experiments, tubulin antibody did not always provide a strong and stable signal.

That is why instead of tubulin, villin was used as a loading control (Robine et al., 1985) in next experiment (Fig. 13).

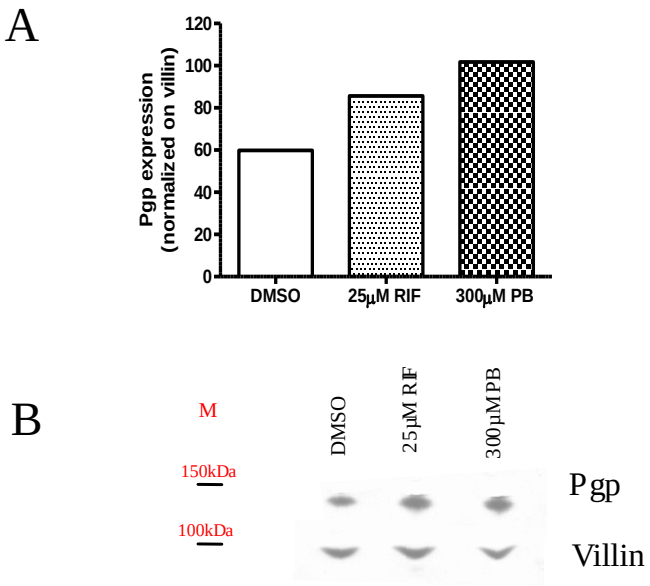


Fig. 13 Pgp expression in MDCKII wild type cells P7. Representative immunoblot (B) and analysis of Western blots (A) of Pgp protein levels in MDCKII wild type (WT) are shown. Cells were treated with phenobarbital (PB, 300 μM) and rifampicin (RIF, 25 μM) for 2 days starting before cells have reached confluence. RIF was used as a positive control). Drugs were dissolved in DMSO up to final concentration of 0.1%. Signal around 140 kDa was used for quantification. Pgp expression was normalized on villin. There was a trend in data suggesting enhanced Pgp expression after rifampicin and phenobarbital treatment. Statistical analysis was not performed because of too low sample size.

MDCKII wild type cells were again treated with known Pgp inducer rifampicin (RIF, 25 μM) and phenobarbital (PB, 300 μM). Loading control gave strong signal and the data could be normalized on villin. Pgp expression was increased after PB and RIF, but because of only one sample per drug data could not be evaluated statistically.

All experiments, shown in part I in Results chapter, were done under suboptimal condition according to the protocol no.1. The aim of following work was to check different conditions (Pgp antibody, loading controls etc.) and to improve the protocol. In order to optimalize Western blotting technique many changes were brought in the protocol (see Table 2).

The main idea behind presented experiments was to establish the optimized condition for Western blotting method in MDCK cell line. During those experiments a lot of technical problems have occurred. Pgp signals had positions different to what was expected. Tubulin provided very weak or variable signals (data not shown) Because of such unstable signals from loading control, tubulin was replaced by villin (Fig. 13) or data in selected experiments (Fig. 9, 10 & 12) had to be normalized on total protein content (expressed as percent DMSO control). In order to test the reliability of method two sets of samples were used (Fig. 12), but they provided the reverse effect of used rifampicin. Such variations between the data could be the result of suboptimal condition for Western blotting method and were a reason to improve the protocol.

Another important purpose was to check whether AEDs can induce Pgp expression under those conditions. Many different drugs like phenobarbital, carbamazepine, phenytoin and topiramate were tested in various concentrations; additionally known Pgp inducer rifampicin was used. All used drugs were dissolved in DMSO, which could also affect Pgp expression and have an impact on results.

In order to check the possibility that gene expression could be affected by drugs independently of protein induction, mRNA levels for different multidrug transporters were analyzed (Fig. 10C & 12).

However, taking into account all the data, there was no clear trend in Pgp induction after AEDs and rifampicin, both on mRNA and protein level.

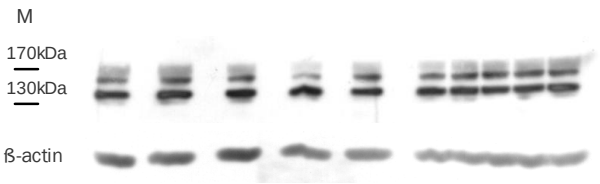
13.3 Summary of protocol changes in Western blotting method for Pgp detection

A short comparison between used protocols is presented in Fig. 14.

Western blotting in MDCK cells according to the protocol no. 1

- Pgp antibody recognized more that one band and none of them had expected molecular weight of Pgp (app.160 kDa)
- Tubulin signals were very unstable and weak
- Signals after incubation with Pgp and tubulin antibodies resembled more dots than bands

Western blotting in MDCK cells according to the protocol no. 2



- Pgp antibody recognized bands with approximately molecular weight of 140 kDa and 160 kDa
- Anti-actin antibody was used instead of anti-tubulin which provided strong, equal signals
- All proteins were well separated and had position with expected molecular weight

Fig. 14 Comparison of representative data obtained in Western blotting according to the two protocols. Figure presents the progress in Western blotting method. The main remarks are described on the right panel of the figures.

After many changes in the protocols, testing various antibodies for Pgp and loading controls, evaluation of buffers, solution compositions, and the protocol for Pgp detection by Western blotting that works in our laboratory was finally established.

Pgp antibody recognized still more than one band (bands with approximately molecular weights 140 kDa & 160 kDa) but it has been proved later that both bands corresponded to Pgp protein that differed in glycosylation. Core-glycosylated form of Pgp protein occured as a band around 140 kDa and mature

glycosylated Pgp had molecular weight of app. 160 kDa. This result was proved by the experiment with PNGase F, an enzyme able to remove the glycosylated residues from the proteins.

In addition, expected Pgp position on immunoblot was confirmed by experiments with various wild type cell lines (LLC, GPNT) and cells transfected with human Pgp, in which Pgp signals had molecular weight below 160 kDa.

Finally, the protocol for Pgp detection in various cell lines by Western blotting was established.

13.4 Part II. Experiments in MDCKII cells under optimized conditions according to modified protocol

In Part II of Western blotting results all data obtained by modified protocol are shown. The changes in protocol are presented in Table 2. Modifications were done together with Dr. Konstantin Kuteykin-Teplyakov and were the results of many trials with different concentrations and sources of antibodies and modifications of recipes for used solutions like lysis buffer, transfer buffer, etc.

In the next experiments, treatments with 300 µM phenobarbital or 70 µM carbamazepine were performed with different starting points and periods of treatments, which are explained in Fig. 15.

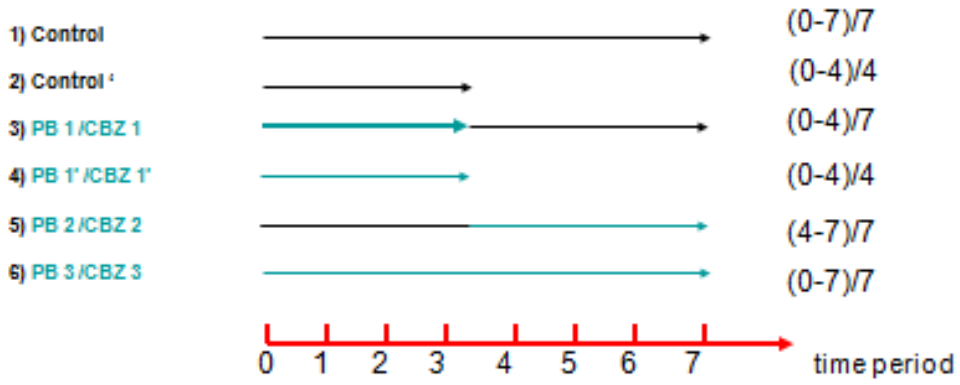


Fig. 15 Scheme representing the way of the treatment MDCKII wild type cells with phenobarbital (PB, 300 µM) and carbamazepine (CBZ, 70 µM). Scheme presents the differences in beginning and period of the cell treatment. Numbers in brackets represent the beginning of the treatment and its duration and last numbers indicate the whole period of experiment. For instance: (0-7)/7, means that cells were treated from the day of confluence (point 0) for six days and protein determination was done on seventh day in culture.

In next experiments, which were performed, DMSO was avoided. If drugs were not dissolved in medium or water, other solvent like methanol or ethanol was used.

The graphs presenting the results after treatment MDCKII wild type cells with 300 µM phenobarbital are shown in Fig. 16.

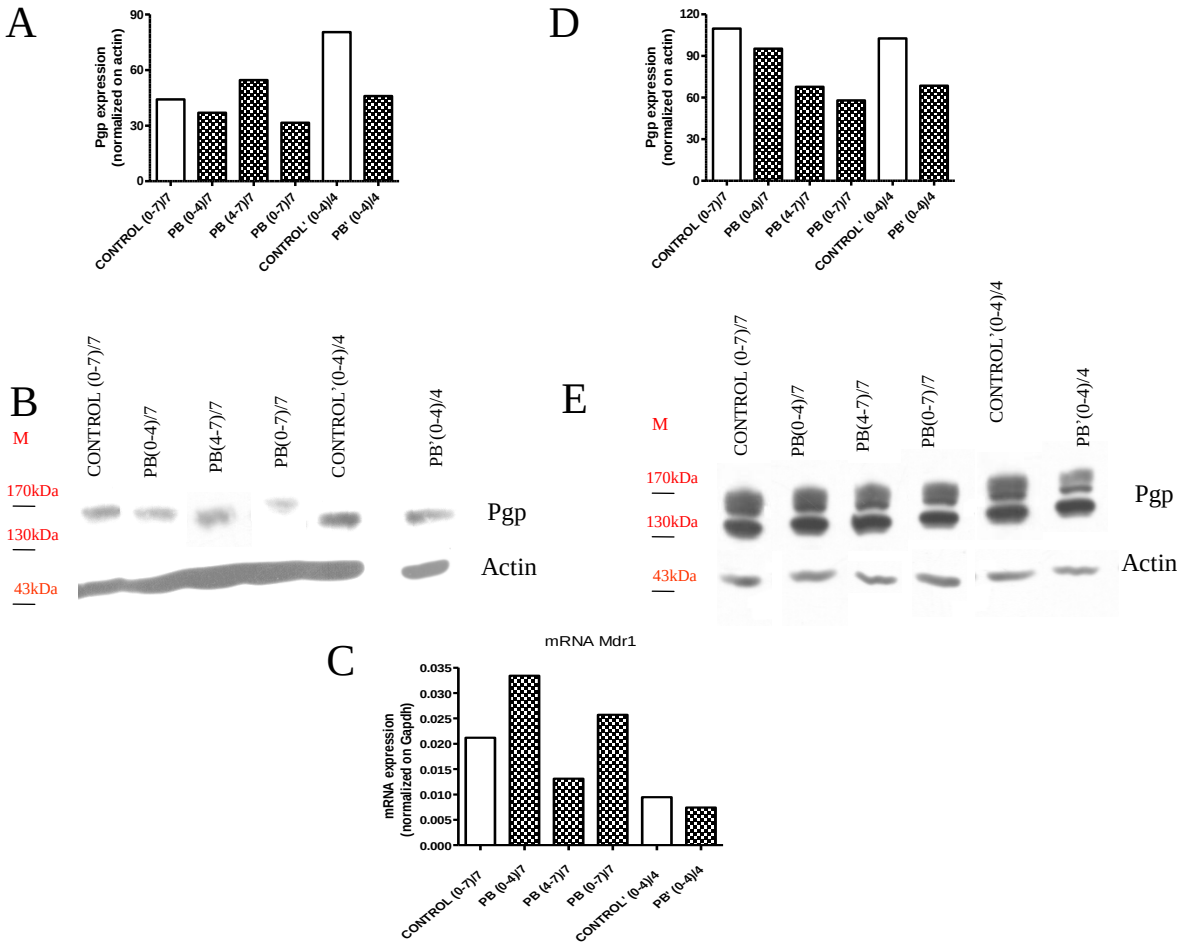


Fig. 16 Pgp expression in MDCKII wild type cells P12 and P16 from two independent experiments after treatment with 300 µM phenobarbital. Representative immunoblots (B & E) and analysis of Western blots (A & D) of Pgp protein levels in MDCKII wild type (WT) are shown. Additionally, mRNA level was investigated (C). Cells were treated at different time points and for various periods (Fig. 15). Cells were treated with 300 µM phenobarbital dissolved in medium. Signal around 160 kDa was used for quantification. Pgp expression was normalized on actin. mRNA data were normalized on Gapdh. There was no trend in data suggesting enhanced Pgp expression after phenobarbital treatment. Statistical analysis was not performed because of only two samples per drug. mRNA analysis was performed by Konstantin Kuteykin-Teplyakov.

Experiment was done in two separated trials. For the first experiment, in addition to protein (Fig. 16A & B), mRNA for Mdr1 gene and other gene transporters (Fig. 17A, B, & C) were quantified.

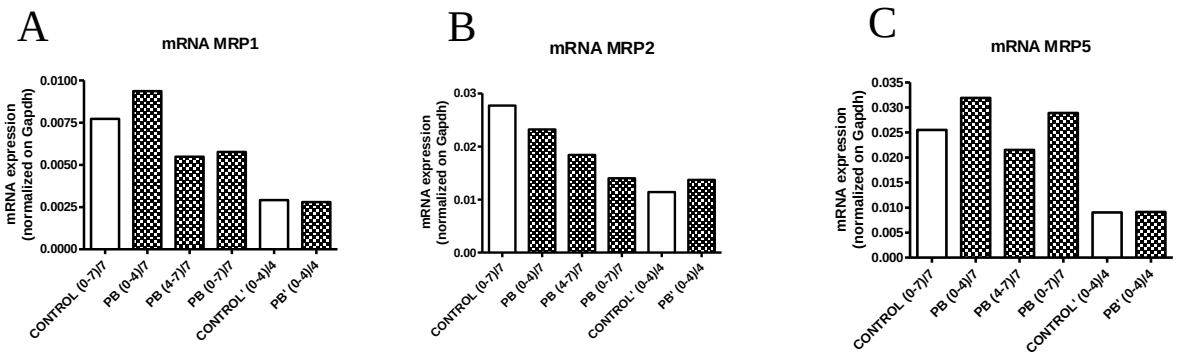


Fig. 17 mRNA expression level of MRPs in MDCKII wild type cells P16. Cells were treated with 300 µM phenobarbital dissolved in medium. Scheme of the treatment is shown in Fig. 15. MRP1 (A), MRP2 (B), MRP5 (C) were analyzed. Data were normalized on Gapdh. There was no trend in data suggesting enhanced Pgp expression after phenobarbital treatment. Statistical analysis was not performed because of only one sample repetition (n=1). mRNA analysis was performed by Konstantin Kuteykin-Teplyakov.

Pgp antibody in first experiment recognized bands with approximately molecular weight below 160 kDa (Fig. 16B) and in second trial both bands below 160 kDa and 140 kDa (Fig. 16E). Both trials were performed under the same conditions. However, actin bands were strong and actin could be used as a loading control without any problem with its stability. Thus, Pgp expression was normalized on actin.

There was no indication for Pgp overexpression on protein and mRNA level after the treatment with 300 µM phenobarbital and statistical analysis could not be performed because of only two samples per drug.

In next trial, similar experiment but with 70 µM carbamazepine as a treatment in MDCK cells was performed and is shown in Fig. 18.

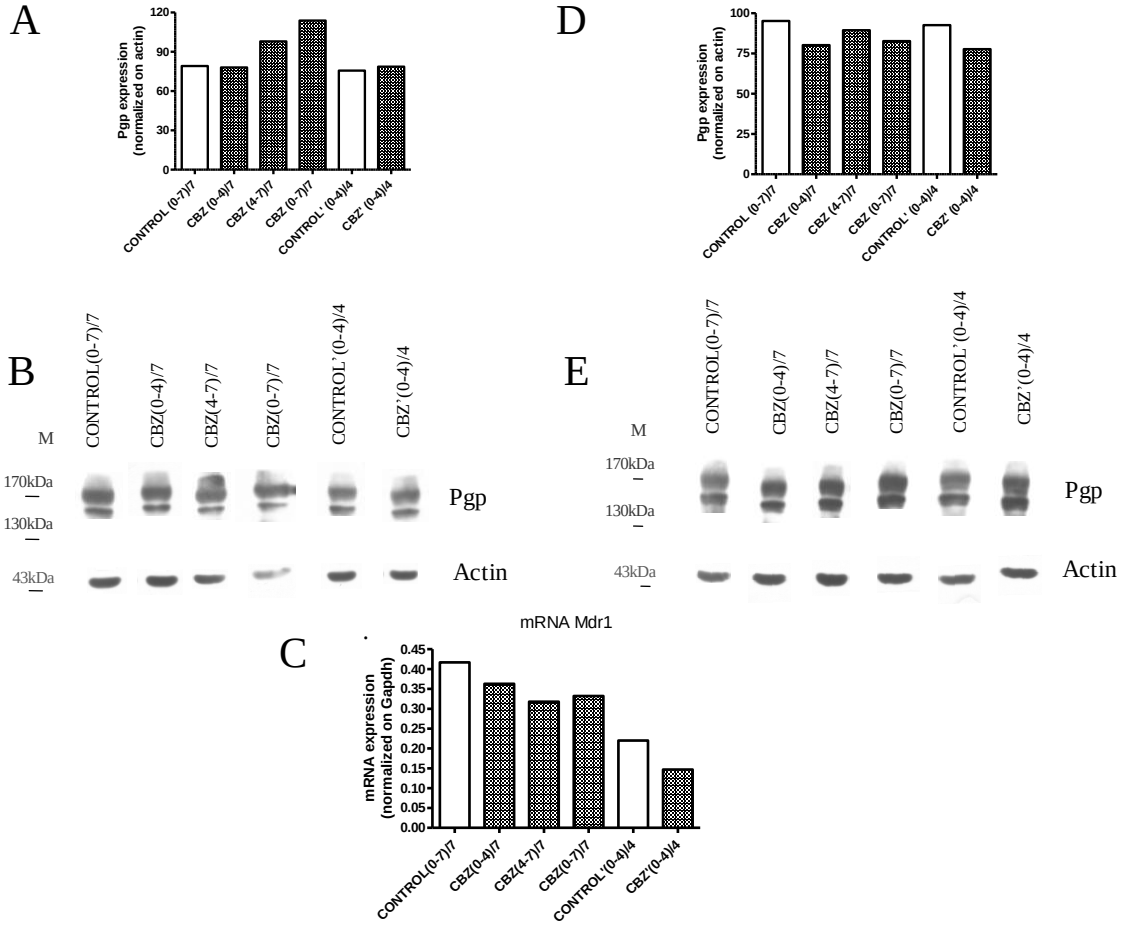


Fig. 18 Pgp expression in MDCKII wild type cells P12 and P11 from two independent experiments after the treatment with 70 µM carbamazepine. Representative immunoblots (B & E) and analysis of Western blots (A & D) of Pgp protein levels in MDCKII are shown. Additionally, mRNA level was investigated (C). Cells were treated at different time points and for various periods (Fig. 15). Cells were treated with 70 µM carbamazepine dissolved in medium. Signal around 160 kDa was used for quantification. Pgp expression was normalized on actin. mRNA data were normalized on Gapdh. There was no trend in data suggesting enhanced Pgp expression after carbamazepine treatment. Statistical analysis was not performed because of only two samples per drug. mRNA analysis was performed by Konstantin Kuteykin-Teplyakov.

For the first trial, Pgp expression (Fig. 18A & B) and mRNA level of Mdr1 (Fig. 19C) and other transporter genes (Fig. 18A, B, C) were analyzed. For second trial only protein level was investigated (Fig. 18D & E). Because of two samples per drug statistical analysis was not performed; however there was no clear trend indicating Pgp overexpression after the treatment with carbamazepine at concentration 70 μM.

Pgp antibody has recognized bands with approximately molecular weights of 140 kDa and below 160 kDa. This pattern of Pgp signals probably represented different glycosylated forms of Pgp proteins. Experiment showing the differences in glycosylation is presented in Fig. 23.

Multiple signals recognized by Pgp antibody was a problem in context of quantification the immunoblots. Which band should be used in quantification of the blots, around 140 kDa or 160 kDa, was under the question. To answer this question and to analyze the proper band, affected by the treatment with AEDs, the following experiment was performed. MDCKII wild type cells were treated with AEDs (100 μM phenobarbital, 30 μM phenytoin and 100 μM phenytoin) for 3 days. The Pgp expression was then quantified separately for both bands recognized by Pgp antibody (Fig. 19A & B). Representative immunoblot is shown in Fig. 19C.

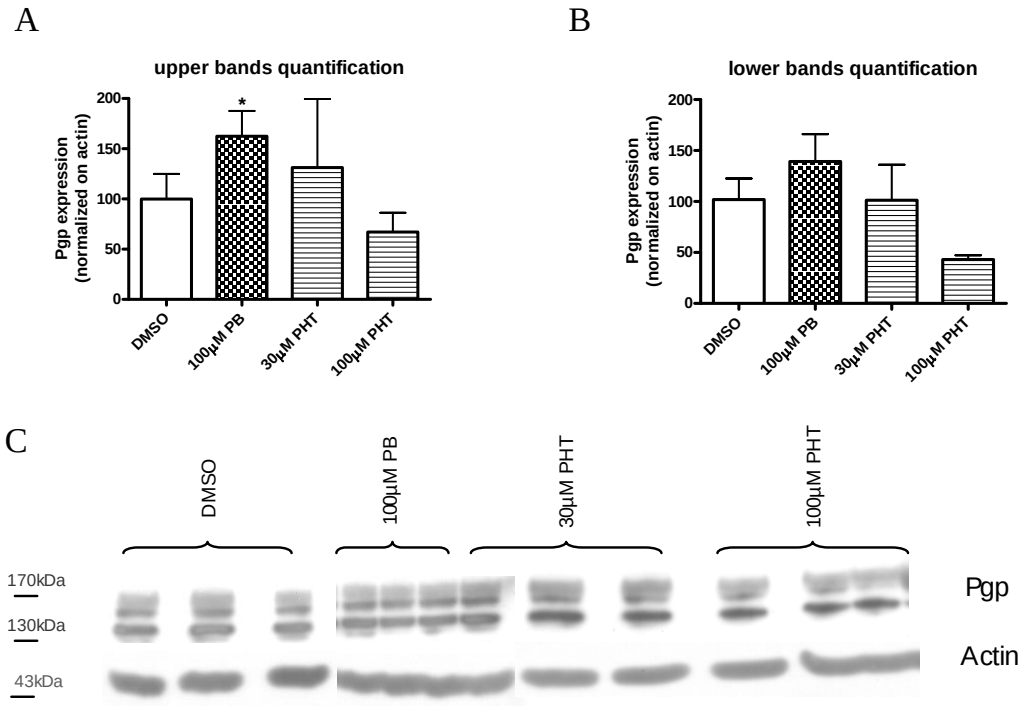


Fig. 19 Pgp expression level in MDCKII wild type cells P10. Cells were treated with antiepileptic drugs phenobarbital and phenytoin (drug concentrations indicated below the graph) for 3 days starting 3 days after the cells have reached confluence. Analysis of Western blots for upper band recognized by Pgp antibody (A) and lower band (B) are shown. Representative immunoblot (C) is shown below. Both bands recognized by anti-Pgp antibody (C219, Signet) were quantified separately. Drugs were dissolved in DMSO up to final concentration of 0.1%. Pgp expression was normalized on actin. Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA) followed by post-hoc t-test. Significant differences between individual groups of data are indicated by one asterisk (P<0.05).

As it is shown in Fig. 19A, there was a significant Pgp induction after phenobarbital, but no induction after phenytoin treatment in data presenting the upper bands quantification. In Fig. 19B, there was no Pgp induction after phenobarbital and phenytoin treatment in data showing the lower bands quantification.

Quantification of upper band and lower band gave the same trend but Pgp expression was significantly increased when upper band was calculated. These results suggested that both bands recognized by Pgp antibody correlated with each other but for our purpose analysis of the upper bands should be performed.

Another argument to affirm the soundness of quantification the upper bands was coming from the next experiment with various cell lines transfected with human Pgp that served as positive controls (Fig. 20, 21, 22).

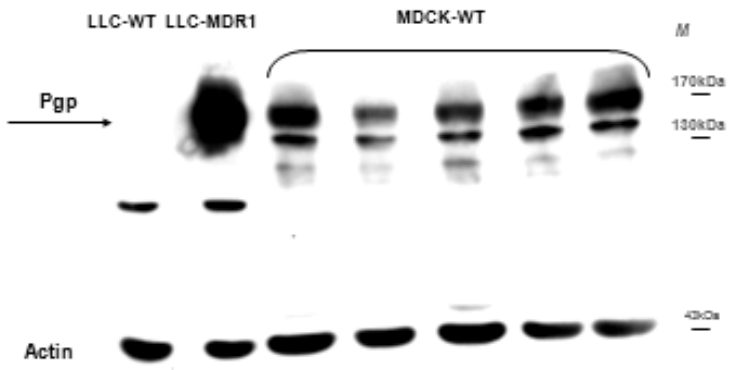


Fig. 20 Comparison of Pgp expression in MDCKII wild type (MDCK-WT) and LLC wild type (LLC-WT) or transfected LLC (LLC-MDR1) cell lines. In MDCKII cells, Pgp antibody (C219, Signet) has recognized two bands around 140 kDa and 160 kDa. In LLC-MDR1 cells, Pgp antibody recognized only one band with app. molecular weight 160 kDa. LLC-MDR1 cells were used as a control in order to define Pgp band position in MDCK cells.

Western blotting was performed with MDCKII wild type (MDCK-WT) and LLC wild type (LLC-WT) and transfected with human Pgp (LLC-MDR1). Pgp antibody has recognized bands with molecular weight around 140 kDa and 160 kDa. As positive controls LLC cells were used. Pgp antibody in LLC-MDR1 cells recognized one band with approximately molecular weight 160 kDa. This position corresponded to the upper band obtained from MDCK-WT analysis. The same position of the Pgp bands, both in MDCK-WT and LLC-MDR1, was another indication that the upper band should be quantified as it was done in our experiments. This immunoblot supported also the fact that MDCK-WT cells expressed more Pgp in comparison to LLC-WT cells, where Pgp expression was so low that it could not be detected by Western blotting.

In Fig. 21, comparison of different cells lines is presented in context of Pgp expression. Again, in order to define right position of Pgp protein different cells lines were used both wild type and transfected with Mdr1 gene. Additionally, MDCK treated with doxorubicin were used as another

positive control. MDCK-WT (1) and MDCK-WT (2) were derived from two sources. The first one was the gift from Prof. Borst and the second were cells from ATCC The Global Biosource Center™ (kindly provided by Dr. M. Al-Falah). The reason for using distinct sources of MDCK-WT cells was to compare the Pgp expression pattern. As it is shown in Fig. 21 Pgp signals occured with approximately molecular weight 160 kDa in all cell lines and constitute as another cause to quantify upper bands in MDCK cells.

As it is shown, cells transfected with Mdr1 gene gave stronger signal from Pgp protein; thereby they expressed higher level of protein. MDCK-MDR1 cells treated with doxorubicin (MDCK-MDR1+DOX) expressed even more Pgp in comparison to unstimulated MDCK-MDR1 cells. This enhanced expression of Pgp was due to the treatment with doxorubicin, which is known as Pgp inducer.

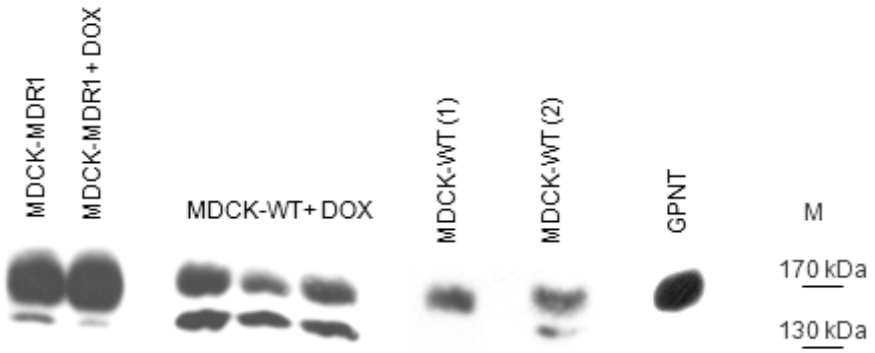


Fig. 21 Comparison of Pgp expression in MDCKII wild type cells (MDCK-WT), transfected MDCKII (MDCK-MDR1) or MDCKII treated with Pgp inducer doxorubicin (MDCK-WT+DOX, MDCK-MDR1+DOX) with GPNT cells. MDCK-WT cells were obtained from two sources: from Prof. Borst (1) and from ATCC (2). In MDCKII cells Pgp antibody (C219, Signet) has recognized two bands around 140 kDa and 160 kDa. In GPNT cells, only one band was recognized by Pgp antibody (C219, Sgnet) with app. molecular weight 160 kDa.

The position of Pgp band was confirmed also by next experiment with several different cell lines (Fig. 22). MDCK and LLC cells were used both wild type (MDCK-WT, LLC-WT), transfected with human Pgp (MDCK-MDR1, LLC-MDR1) and transfected with other transporters like MDCK-MRP1, MDCK-MRP2 and MDCK-MRP5. In addition, rat brain endothelial cell line GPNT was used. In all cell lines Pgp confirmed its position around 160 kDa. MDCK wild type and MDCK transfected with human Pgp gene were treated with doxorubicin (MDCK-WT+DOX and MDCK-MDR1+DOX)

for 3 days. MDCK transfected cells and cells treated with DOX expressed more Pgp proteins compared to untreated MDCK cells (enhanced signal with app. molecular wight of 160 kDa) (Fig. 21).

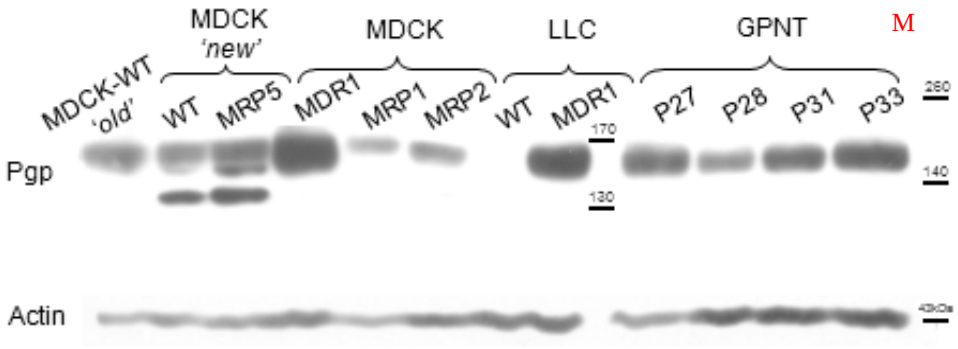


Fig. 22 Comparison of Pgp expressions in different cell lines. The correct Pgp position was confirmed by using transfected cell lines MDCK-MDR1 and LLC-MDR1. MDCK cells maned 'old' were kindly provided by Prof. P. Borst in 2002, the 'new' MDCK were received in 2008. Both cell charges were used in order to compare Pgp expression pattern.

Pgp expression in LLC-WT was so low that detection with C219 antibody was not possible.

13.5 Deglycosylation of Pgp (investigation of changes in Pgp glycosylation after using PNGase F by Western blotting)

In order to prove that both bands (140 kDa and 160 kDa) in MDCK cells belonged to the same protein, but just one is core-glycosylated and another glycosylated form, MDCK-WT cells were incubated with PNGase F enzyme that removes all types of N-linked (Asn linked) glycosylation (Greer and Ivey**,** 2007) (Fig. 23). MDCK-WT with various passage numbers was incubated with the enzyme as described in Matherials and Methods.

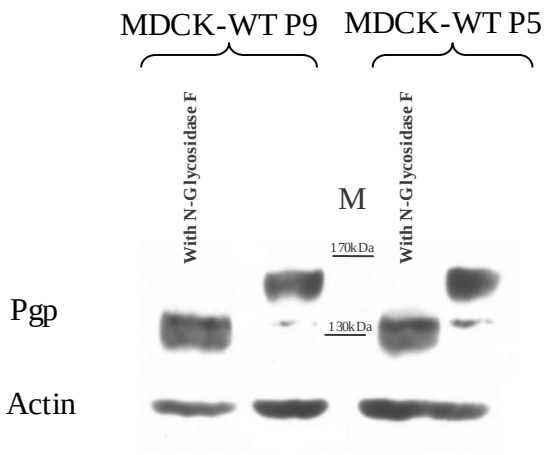


Fig. 23 Investigation of changes in Pgp glycosylation after using PNGase F by Western blotting in MDCK wild type. MDCKII protein lysates were treated with PNGasaF as described in Materials and Methods. Cells incubated with the enzyme gave only one band (app. 140 kDa) on the blots after incubation with Pgp antibody. Cells without the enzyme had usual Pgp band patterns and Pgp antibody recognized two bands (app. 140 kDa & 160 kDa). In order to check Pgp expression, experiment was done with two different batches of MDCK cells. MDCK-WT P9 were cells received from Prof. Borst in old batch, MDCK-WT P5 cells were received from Prof. Borst in 2008. In both old and new batches of MDCK cells Pgp antibody recognized the same pattern of bands.

In cells incubated with the enzyme, Pgp antibody has recognized only one band with approximately molecular weight 140 kDa. In cells that have not been incubated with PNGase F, Pgp antibody has recognized two bands (around 140 kDa and 160 kDa). Those results indicate that both bands belonged to Pgp protein. Band around 140 kDa might be core-glycosylated form of P-glycoprotein and band around 160 kDa could correspond to a completely glycosylated form of mature protein.

Those findings affirmed the soundness why only the upper band was used for quantification the immunoblots.

13.6 Part III. Experiments in GPNT cells under optimized conditions according to modified protocol

13.6.1 Influence of different medium compositions on Pgp expression in GPNT cells

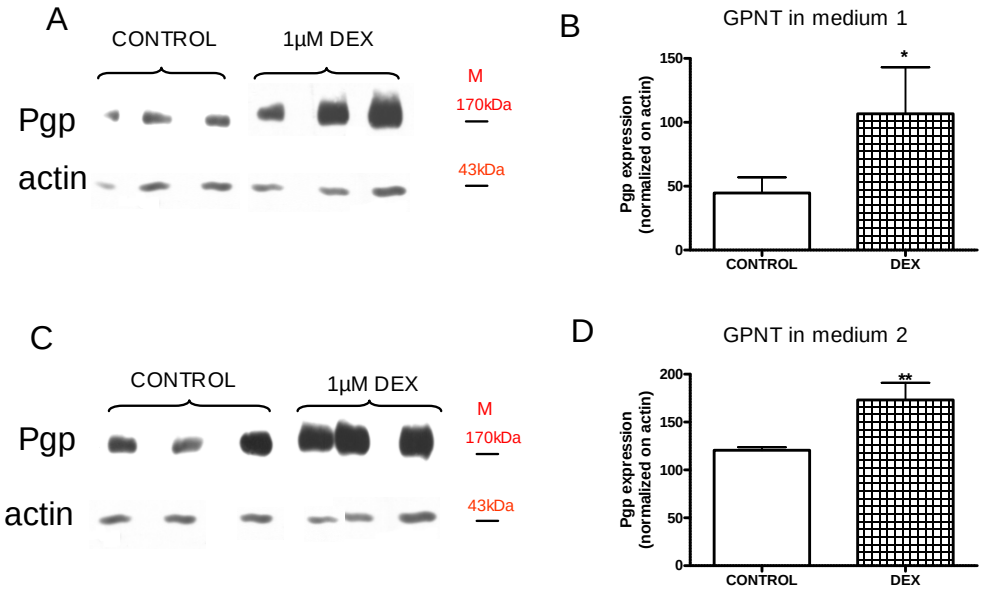


Fig. 24 Changes in Pgp expression in GPNT cells after 3 days of treatment with dexamethasone (DEX, 1 µM) in different medium compositions. Representative immunoblots (A & C), analysis of Western blots (B & D) of Pgp protein levels in GPNT are shown. Fig. A & B show Pgp expression in cells cultured in in medium 1. Fig. C & D show Pgp expression in cells cultured in medium 2. For medium compositions see Table 3. Data are shown as means ± SD of three samples. Data were statistically compared by unpaired t-test. Significant differences between compared data are indicated by one asterik (P<0.05) or by two asterisk (P<0.01).

GPNT cells were cultured in two medium compositions. One medium composition (medium 1) was taken from the literature (Régina et al., 1999), another medium prescription (medium 2) was received from F. Roux with the GPNT cells. Those two media differed between each other (Table 4). GPNT were cultured in two different medium compositions and Pgp protein level was analyzed by Western blotting. The idea of this experiment was to check whether differences in medium protocol can influence Pgp expression level in presence of Pgp inducer dexamethasone. As it is shown in Fig.

24, Pgp expression was significantly enhanced after dexamethasone treatment in cells cultured in both medium compositions. Therefore, those two medium compositions gave the same effect on Pgp expression and both could be used to culture GPNT cells. In presented work medium 2 was chosen as a culture medium for GPNT cells.

13.7 Confocal microscopy

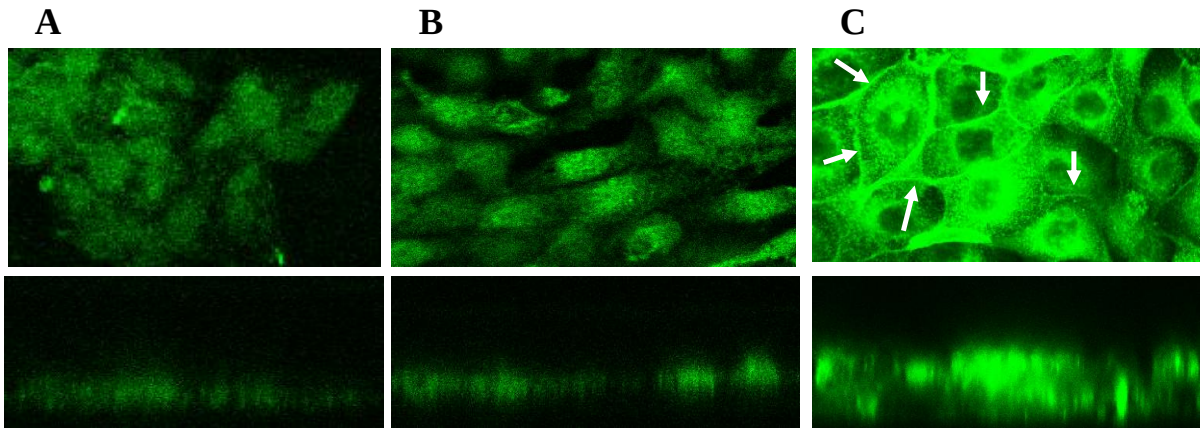


Fig. 25 Examination of Pgp expression in GPNT cell line by confocal microscopy. Representative pictures are shown in GPNT on the day of confluence (A), 3 days after confluence (B) and 6 days after confluence. Pictures in Fig. A & B were done with different magnification than picture in Fig. C. In contrast to Fig. A & B, Pgp in Fig. C was localized in cell membrane. Upper panel of pictures represents xy-scans showing cell structure, lower panel represents z-scans showing apical membrane expression of Pgp in GPNT cells.

Preliminary confocal studies on Pgp expression were performed in GPNT cells.

As it is shown in Fig. 25, Pgp expression enhanced during culturing the cells. Moreover, during the extension of culturing, Pgp occured in membrane surface (Fig. 25C). As it was shown (Loo and Clarke, 1999a) functionally active Pgp is localized in membrane surface as fully glycosylated, mature protein. Pictures presented in Fig. 25 were done in different magnifications. In Fig. A and B magnification was distinct from Fig. C. In Fig. A & B, Pgp was localized within the cells, but after 6 days of confluence (Fig. C) Pgp was targeted to cell membrane.

The findings that Pgp expression was changing during culturing of GPNT cells served as a clue for futher study on Pgp induction, because it allowed to decide when GPNT treatment should begin.

All Western blotting experiments in GPNT cells were done according to protocol no. 2 (see Table 2).

Rat brain endothelial cell line, GPNT, had an advantage to investigate changes in Pgp expression, because in comparison to MDCK cells, they provided only one and sharp band recognized by Pgp antibody. Pgp band, in GPNT, appeared with molecular weight around 160 kDa. The figure below (Fig. 26) shows representative immunoblots (Fig. 26B & E) from the two independent experiments, in which GPNT cells were treated with puromycin, a known Pgp inducer (Demeuse et al., 2004), alone or in combination with phenobarbital (Fig. 26A & B). Because of lack of puromycin effect on Pgp expression, in next experiments GPNT were additionally treated with another inducer, doxorubicin (Fig. 26D & E).

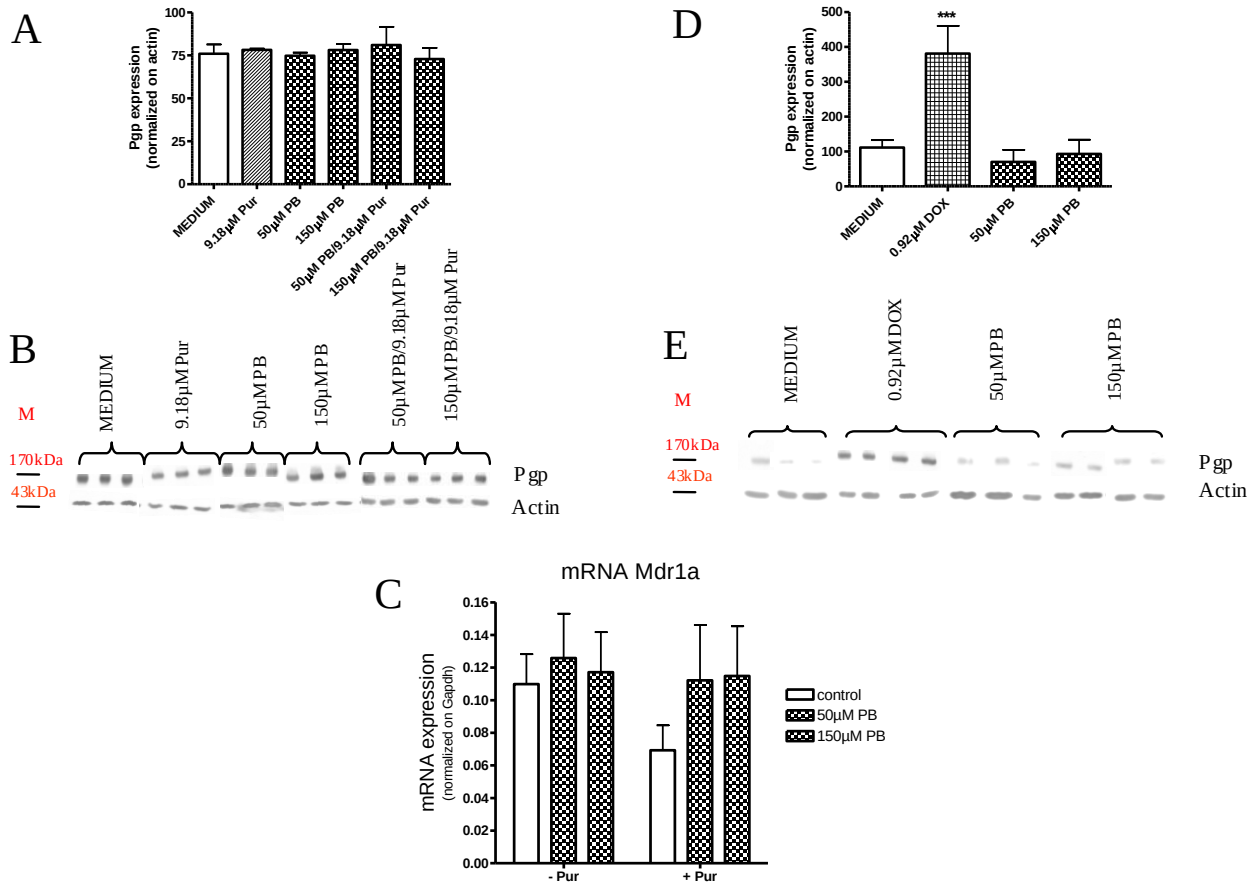


Fig. 26 Pgp expression level in GPNT cells P30 and P36 in two independent experiments. Representative immunoblots (B & E), analysis of Western blots (A & D) of Pgp protein levels in GPNT are shown. Additionally, mRNA analysis was investigated (C). Cells were treated with phenobarbital (PB), puromycin (Pur) and doxorubicin (DOX) (drug concentrations are indicated below the graph) for 3 days starting on the day of the confluence. Pur and DOX were used as positive controls. Pgp expression was normalized on actin. mRNA data were normalized on Gapdh. Data are shown as means ± SD of three to four samples. Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by three asterisks (P < 0.001). mRNA analysis was performed by Konstantin Kuteykin-Teplyakov.

For the experiment shown in Fig. 26A & B, mRNA levels of Mdr1a (Fig. 26C), Mdr1b and MRP1 (Fig. 27A & B) were investigated.

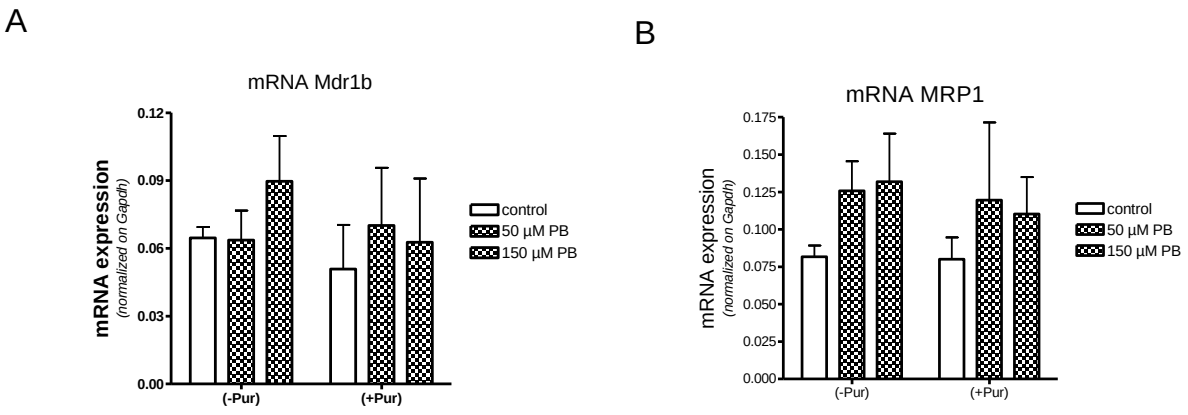


Fig. 27 mRNA expression level of Mdr1b and MRP1 in GPNT cells P30. Cells were treated with phenobarbital (PB) alone or in combination with puromycin (Pur). Mdr1b (A), MRP1 (B) were analyzed. Data were normalized on Gapdh. Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA) and were not significant. mRNA analysis was performed by Konstantin Kuteykin-Teplyakov.

Treatment with puromycin alone or in combination with phenobabital did not affect Pgp expression both on protein and mRNA level, whereas doxorubicin considerably enhanced Pgp expression as it is shown in Fig. 26D & E.

Since some research groups have shown Pgp overexpression after phenobarbital and phenytoin treatment (Lombardo et al., 2008; Wen et al., 2008) the GPNT cells were treated again with phenobarbital (30 µM and 100 µM) and with phenytoin (30 µM, 100 µM) (Fig. 28A & B). In addition, GPNT were treated with puromycin (9.18 µM) and doxorubicin (0.92 µM).

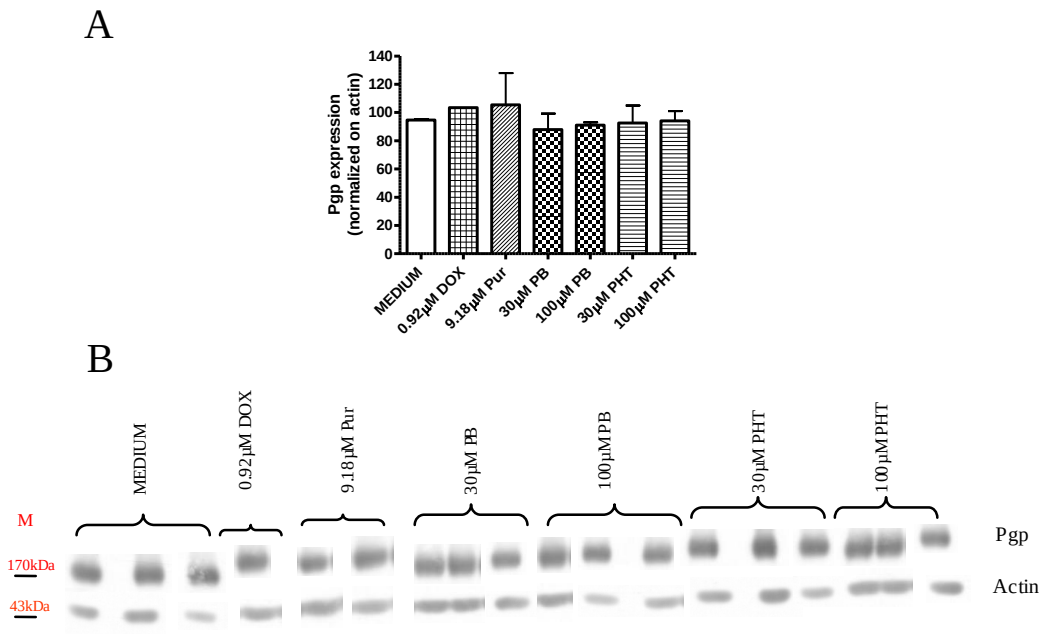


Fig. 28 Pgp expression level in GPNT cells P27. Representative immunoblot (B) and analysis of Western blots (A) of Pgp protein levels in GPNT are shown. Cells were treated with antiepileptic drugs: phenobarbital (PB), phenytoin (PHT) and doxorubicin (DOX) (a positive control) for 3 days starting 3 days after the cells have reached confluence (drug concentrations are indicated under the graphs). Pgp expression was normalized on actin. Data are shown as means ± SD of three samples (except doxorubicin n=1 and puromycin n=2). Data were statistically compared by one-way analysis of variance (ANOVA) and were not significant.

As it is shown in Fig. 28A and B, there were no significant overexpressions after AEDs treatment and Pgp inducer doxorubincin.

Due to lack of reliable Pgp induction in performed experiments, in next trials dexamethasone was used as a known Pgp upregulator (Régina et al., 1999). Additionally, in following experiments Pgp functionality and expression were analyzed. As it is shown in Fig. 29 dexamethasone increased Pgp expression significantly, but phenobarbital and doxorubicine had no effect on Pgp expression (Fig. 29 A & B). Those results corresponded to mRNA analysis of Mdr1a gene, which was significantly upregulated as well. As a complacement, results from uptake assay shown decrease in digoxin uptake after dexamethasone treatment. These findings proved that Pgp functionality and expression were altered by Pgp inducer dexamethasone. Next evidence for Pgp functionality was inhibition of Pgp

activity by inhibitor tariquidar (Fig. 29D). Blocked functionality of Pgp resulted in significant digoxin uptake within the cells.

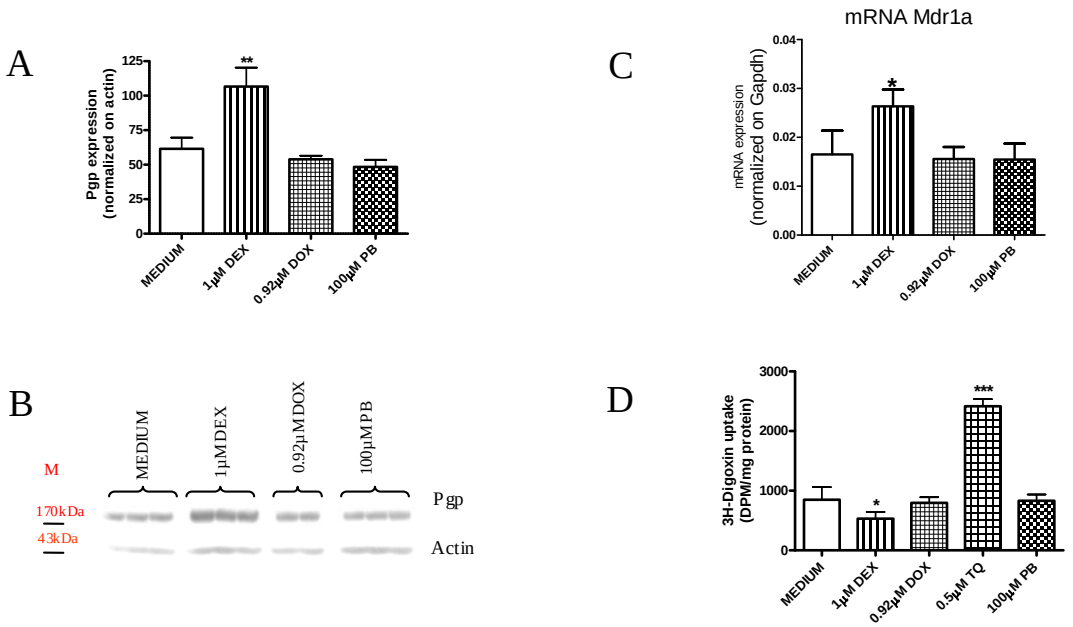


Fig. 29 Pgp expression level in GPNT cells P30. Representative immunoblot (B), analysis of Western blots (A) of Pgp protein levels in GPNT are shown. Additionally, the level of mRNA (C) was investigated and functionality of Pgp in GPNT cells by digoxin uptake assay (D) was done. Cells were treated with phenobarbital (PB) and Pgp inducers: dexamethasone (DEX), doxorubicin (DOX) for 3 days starting 6 to 7 days after confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay (drug concentrations are indicated under the graphs). Pgp expression was normalized on actin; mRNA data were normalized on Gapdh and digoxin uptake on DPM/mg protein. Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by one asterisk (P < 0.05) or two, three asteriks (P<0.01, P<0.001). mRNA analysis was performed by Konstantin Kuteykin-Teplyakov. Uptake assay was performed by Carlos Luna Tórtos.

In addition Pgp function was examined by digoxin uptake assay after treatment with dexamethasone, doxorubicin and phenobarbital at concentrations indicated under the graphs in Fig. 29D. Digoxin, which is specific substrate for Pgp protein, accumulated much less in cells treated with dexamethasone. This result indicated that dexamethasone influenced Pgp function (Fig. 29D) and

amount (Fig. 19A & B). Pgp was more active and removed the substrates out of the cells. In contrast GPNT cells treated with specific Pgp inhibitor tariquidar accumulated more radioactive substrate inside the cells, because Pgp proper function was abolished (Fig. 29D).

Additionally, gene expressions were investigated for Mdr1b, MRP1 and MRP5, resulting in upregulated Mdr1b gene after dexamethasone (Fig. 30A), and no changes in MRP1 and MRP5 gene expression (Fig. 30B & C).

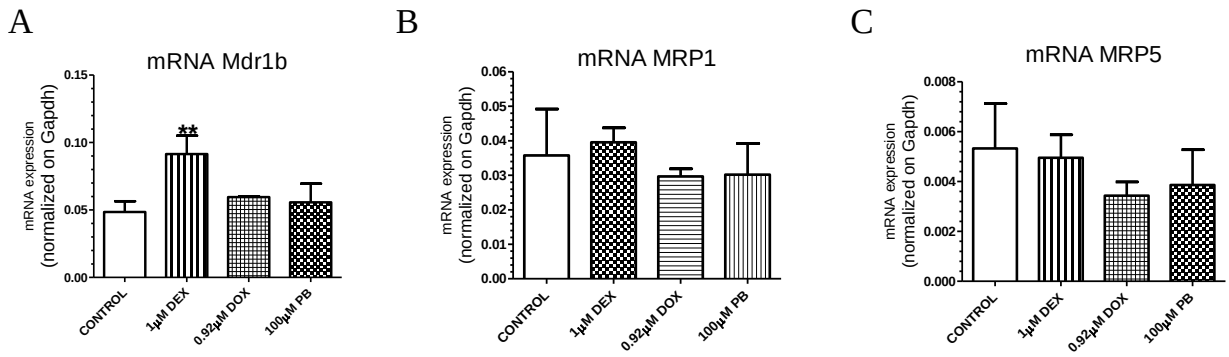


Fig. 30 mRNA expression level of Mdr1b and MRPs in GPNT cells. Cells were treated with phenobarbital (PB) and Pgp inducers: dexamethasone (DEX) and doxorubicin (DOX) for 3 days starting 7 days after confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay (drug concentrations are indicated under the graphs). Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by asteriks (P<0.01). mRNA analysis was performed by Konstantin Kuteykin-Teplyakov.

As it is shown, dexamethasone treatment caused upregulation in Pgp expression (Fig. 29A & B), which was correlated with enhanced Pgp function (Fig. 29D) and with increase in Mdr1a and Mdr1b mRNA level.

In next trial, GPNT cells were treated with dexamethasone, doxorubicin, phenobarbital and tariquidar starting from the day of confluence (Fig. 31). Treatment with tariquidar was performed for 3 days, in contrast to other experiments, in which cells were only stimulated with tariquidar for 2 h before uptake assay was performed.

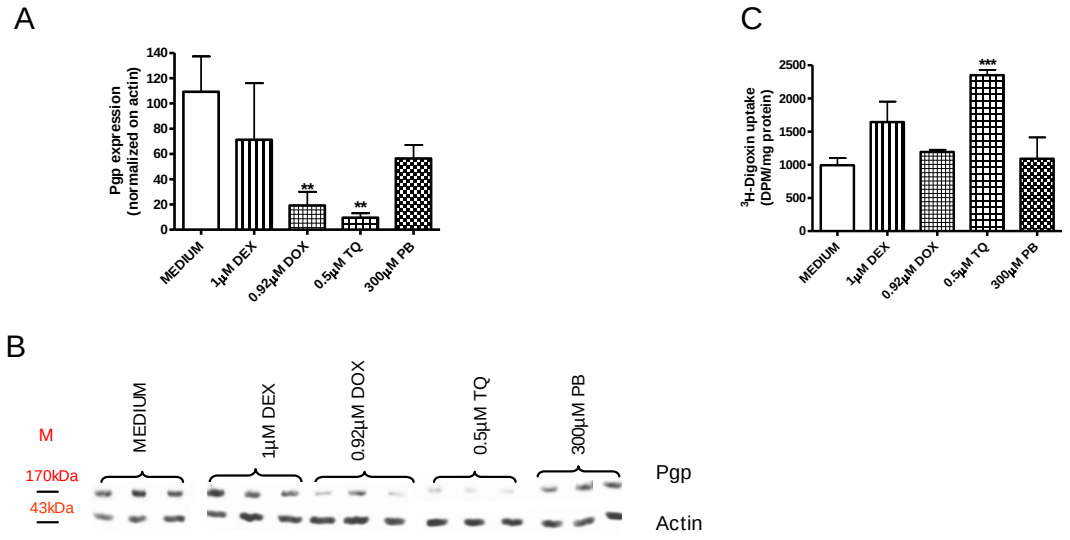


Fig. 31 Pgp expression level in GPNT cells P35. Representative immunoblot (B), analysis of Western blots (A) of Pgp protein levels in GPNT are shown. Additionally the functionality of Pgp in GPNT cells was investigated by digoxin uptake assay (C). Cells were treated with phenobarbital (PB) and Pgp inducers: dexamethasone (DEX), doxorubicin (DOX) and additionally with Pgp inhibitor tariquidar (TQ) for 3 days starting on the day of the confluence (drug concentrations are indicated under the graphs). Pgp expression was normalized on actin. Digoxin uptake was normalized on DPM/mg protein. Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by two or three asteriks (P<0.01, P<0.001).

Results obtained in experiments presented above were unexpected: significant Pgp downregulation (Fig. 31A) after treatment with doxorubicin and tariquidar and no induction after phenobarbital and dexamethasone treatment. The immunoblot is presented in Fig. 31B. Those striking findings will be discussed later.

In Fig. 31C, uptake assay results are presented. There were no changes in Pgp expression (Fig. 31A & B) and functionality (Fig. 31C) after any of used drugs except tariquidar. Cells stimulated with tariquidar accumulated significantly more digoxin compared with cells treated with other drugs, which was an evidence for correctness of the assay.

Next experiment was performed again 6 days after the cells have reached confluence, similar to experiment in Fig. 29 but higher concentration of phenobarbital was applied (300 μM phenobarbital

instead of 100 µM). Pgp expression levels (Fig. 32A) and Pgp functionality (Fig. 32C) were investigated. Exceptionally, cells were also treated with tariquidar for 3 days.

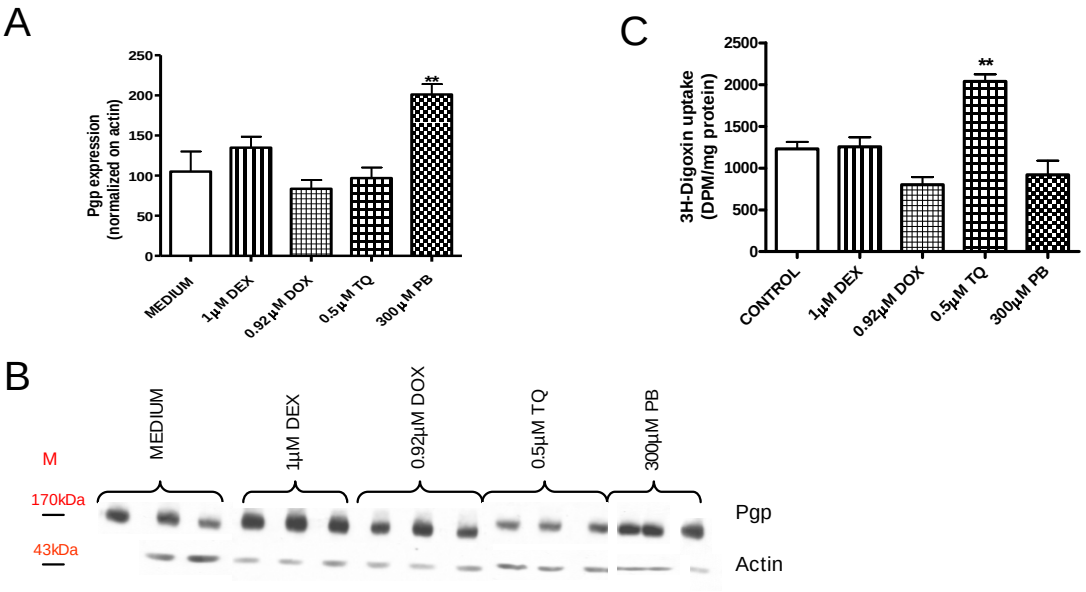


Fig. 32 Pgp expression level and functionality in GPNT cells P35. Representative immunoblot (B), analysis of Western blots (A) of Pgp protein levels in GPNT are shown. Additionally, the functionality of Pgp in GPNT cells was investigated by digoxin uptake assay (C). Cells were treated with phenobarbital (PB) and Pgp inducers: dexamethasone (DEX), doxorubicin (DOX) and additionally with Pgp inhibitor tariquidar (TQ) for 3 days starting 6 days after the cells have reached confluence (drug concentrations are indicated under the graphs). Pgp expression was normalized on actin. Digoxin uptake was normalized on DPM/mg protein. Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by asteriks (P<0.01).

Enhanced concentration of phenobarbital led to Pgp upregulation (Fig. 32A & B), which was not correlated with functionality assay (Fig. 32C).

Pgp inducers dexamethasone and doxorubicin did not affect Pgp expression (Fig. 32A & B). Tariquidar treatment had no effect on Pgp protein level as well, but it influenced on Pgp activity by significantly increased digoxin uptake (Fig. 32C).

Various AEDs were used in next experiment presented in Fig. 33.

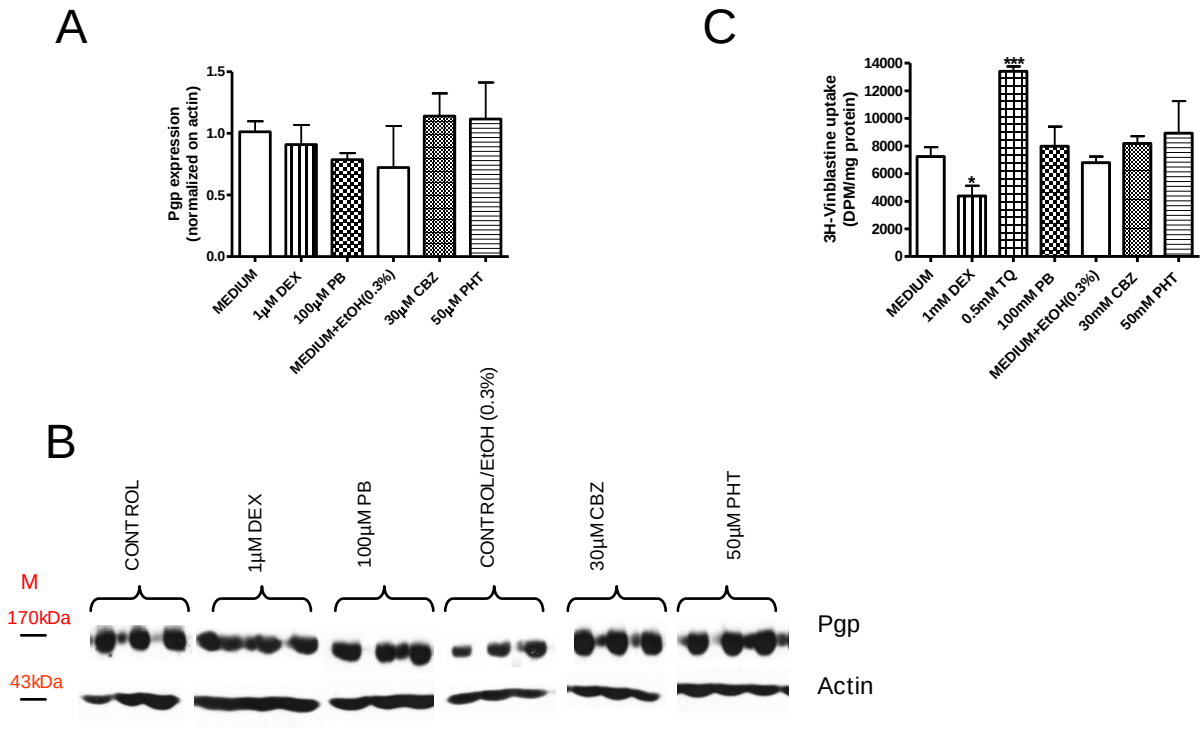


Fig. 33 Pgp expression level in GPNT cells P36. Representative immunoblot (B), analysis of Western blots (A) of Pgp protein levels in GPNT are shown. Additionally, the functionality of Pgp in GPNT cells was investigated by digoxin uptake assay (C). Cells were treated with antiepileptic drugs: phenobarbital (PB), phenytoin (PHT) and carbamazepine (CBZ) and Pgp inducer dexamethasone (DEX) for 3 days starting on the day of confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay (drug concentrations are indicated under the graphs). Data are shown as means ± SD of three samples. Pgp expression was normalized on actin. Vinblastine uptake was normalized on DPM/mg protein. Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by one asterik (P<0.05) or asteriks (P<0.01). Western blotting was performed by Konstantin Kuteykin-Teplyakov. Uptake assay was performed by Carlos Luna Tórtos.

GPNT cells were treated with dexamethasone and different AEDs: phenobarbital (100 µM), carbamazepine (30 µM) and phenytoin (50 µM) for 3 days starting from the day of confluence. No effects of used AEDs and dexamethasone on Pgp expression were observed (Fig. 33A & B). In addition uptake assay was performed, but as a Pgp substrate vinblastine was used. Again no effect after AEDs treatment on functionality of Pgp was observed. Significant vinblastine accumulation was

observed after dexamethasone treatment. Tariquidar stimulation affected the functionality of Pgp by increasing vinblastine uptake.

All experiments shown above led to conclusion that AEDs did not affect Pgp expression and functionality in GPNT cells.

13.8 Uptake assay results

All experiments in GPNT and MDCK cells are collected in Table 9 and 10 (see Appendix), which contain data from uptake assay experiments.

13.8.1 Establishment of uptake assay in different cell lines

As described in Materials and Methods, uptake assays allow investigating functionality of proteins. This chapter was meant to specify the uptake assay conditions. Different cell lines were involved to optimize the protocol from uptake assay and various experimental concentrations of Pgp substrates were tried, before the protocol was fully established. The cells were not treated with any of AEDs. Cells were treated with radioactive substrate ^{3}H-digoxin and specific Pgp inhibitor tariquidar. In cells expressing functional Pgp, tariquidar should inhibit Pgp and more radioactive substrate must be accumulated by the cells compared to control group not stimulated with inhibitor.

In order to formulate proper conditions of uptake assay experiments, two Pgp substrates were used: radioactive digoxin and fluorescent rhodamine. Those substrates were tested in different cell lines like GPNT, RBE4 and MDCK in various differentiation states of cells (Fig. 34).

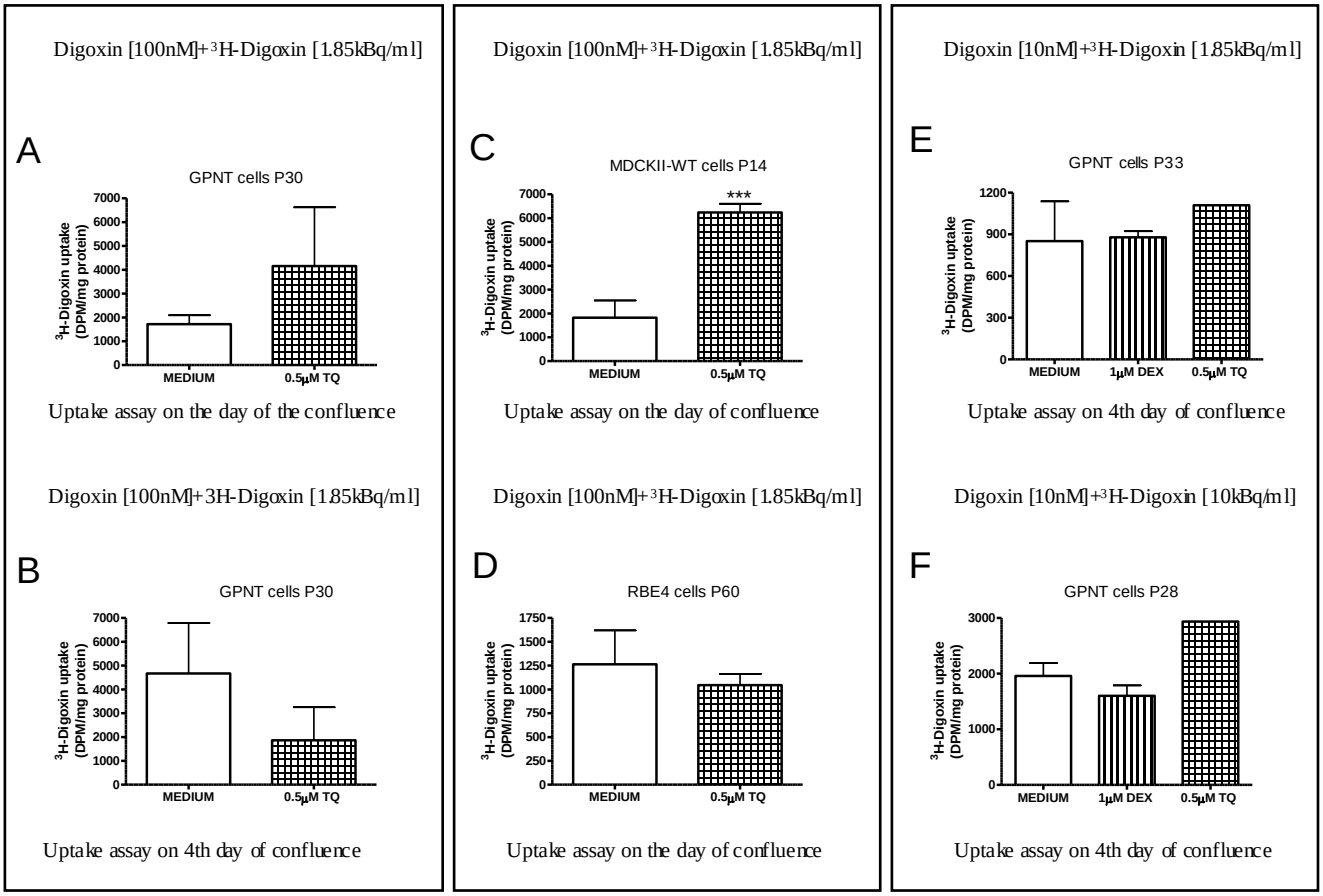


Fig. 34 Graph representing digoxin uptake assays in different cell lines. Cells were treated with Pgp inhibitor tariquidar (TQ) at concentration 0.5 μM and additionally with Pgp inducer dexamethasone (DEX) at concentration 1 μM. GPNT cells (Fig. 34A & B) were treated with digoxin [100 nM]+^{3}H-digoxin [1.85 kBq/ml] in distinct times of differentiation (Fig. 34A digoxin uptake assay on the day of confluence; Fig. 34B digoxin uptake assay on 4th day of cell confluence). No significant differences between control cells and cells treated with tariquidar were observed. Digoxin uptake assay in MDCK-WT and RBE4 cells is shown in Fig. 34C & D, where digoxin [100 nM]+^{3}H-digoxin [1.85 kBq/ml] was used. Only MDCK-WT cells accumulated significantly more digoxin after stimulation with TQ. GPNT cells treated with DEX and TQ are shown in Fig. 34E & F where digoxin concentration was changed to digoxin [10 nM]+^{3}H-digoxin [1.85 kBq/ml] in Fig 34E and to digoxin [10 nM]+^{3}H-digoxin [10 kBq/ml] in Fig. 34F. Digoxin uptake was normalized on DPM/mg protein. Data are shown as means ± SD of three samples (except TQ in Fig. 34E & F; only one sample). Data were statistically compared by unpaired t-test. Significant differences between compared data are indicated by three asteriks (P<0.001).

For GPNT cells (Fig. 34A, B, E & F) no significant accumulation of digoxin was observed at any time point of treatment. However, there was a trend in enhanced digoxin uptake in Fig. 34A after tariquidar.

In Fig. 34B there was even a decreased uptake of digoxin after tariquidar. In Fig. 34C MDCK wild type cells accumulated significantly more digoxin after tariquidar treatment. Unlike to MDCK, RBE4 cells did not show any changes after tariquidar stimulation. The concentration of 'cold' (non radioactive) digoxin was changed to 10 nM (Fig. 34E) and 'hot' (radioactive) digoxin up to 10 kBq/ml (Fig. 34F) in next experiments. In addition known Pgp inducer dexamethasone was used as a positive control. Under any of those conditions digoxin uptake was not changed neither after tariquidar nor dexamethasone.

Another Pgp substrate that was used to investigate Pgp functionality was fluorescent rhodamine 123 (Fig. 35).

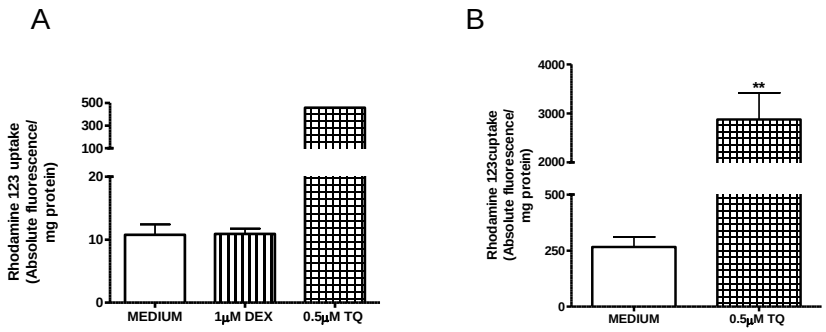


Fig. 35 Rhodamine 123 uptake assays in GPNT cells P33 and P28 in two independent experiments performed on 4th day of confluence. Cells were treated with 3 μM rhodamine 123 (Fig. 35A) or with 10 μM rhodamine 123 (Fig. 35B). Data are shown as means ± SD of three samples (except TQ in Fig. 35A, only one sample). Rhodamine uptake was normalized on DPM/mg protein. Data were statistically compared by unpaired t-test. Significant differences between compared data are indicated by two asterisks (P < 0.01).

Two concentrations of rhodamine 123 were tried: 3 μM (Fig. 35A) and 10 μM (Fig. 35B). Those experiments were done to check whether Pgp in GPNT cell line is functional and whether rhodamine is a better substrate in accumulation test. As it is shown in Fig. 35B tariquidar could inhibit Pgp (cells accumulated significantly more rhodamine 123). There was a trend to increase accumulation of fluorescent dye in cells, but because of only one sample trial with tariquidar this effect could not be analyzed with statistical tests (Fig. 35A). Dexamethasone did not affect digoxin accumulation (Fig. 35A).

In next experiments digoxin was chosen as a more reliable and specific substrate for Pgp. To obtain robust effect of investigated substances, concentration of hot and cold digoxin was increased and digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml] were used.

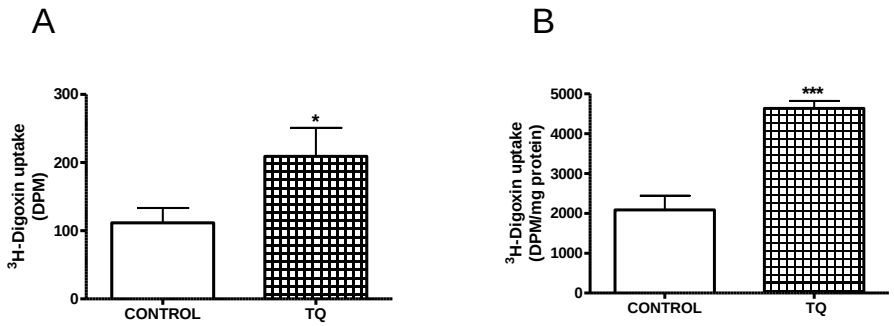


Fig. 36 Digoxin uptake assays in GPNT cells P38 and P32 in two independent experiments performed on 4th day of confluence. Cells were treated with tariquidar (TQ) at concentration 0.5 μM and accumulation of digoxin was investigated under the condition: digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml]. Digoxin uptake was normalized on DPM (Fig. 36A) and DPM/mg protein (Fig. 36B). Data are shown as means ± SD of three samples. Data were statistically compared by unpaired t-test. Significant differences between compared data are indicated by one asterisk (P<0.5) or three asterisks (P<0.001).

For our purpose, concentration of digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml] worked well and allowed to investigate the changes in accumulation of radioactive substance. It showed significantly increased accumulation after treatment with Pgp inhibitor tariquidar in GPNT cells. Because of this, concentrations of 'hot' and 'cold' digoxin were modified in next experiments.

Due to differences in medium composition for uptake assay in literature, next experiments were done in two difference media for accumulation test. The aim was to check whether medium composition can influence on results of uptake assays.

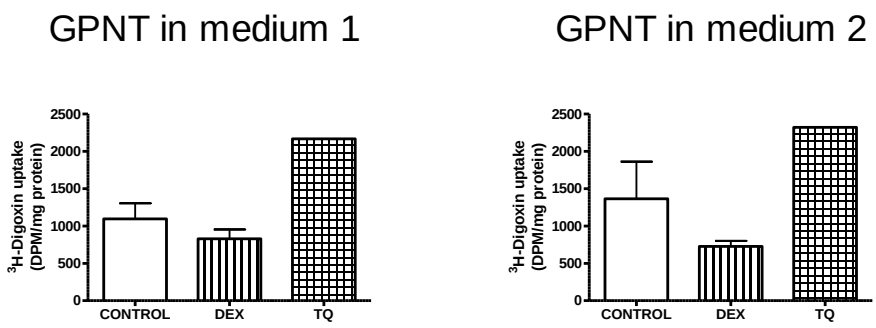


Fig. 37 Comparison of two medium compositions (Table 4) in GPNT cells P30. Cells were treated with dexamethasone (DEX, 1 μM) for 3 days starting on the day of cell confluence and were stimulated with tariquidar (TQ, 0.5 μM) for 2 h on the day of the assay. Uptake assay was performed under the condition digoxin [1 μM]+ ^{3}H-digoxin [10 kBq/ml]. Digoxin uptake assay was normalized on DPM/mg protein. Data are shown as means ± SD of three samples (except TQ, n=1). Data were statistically compared by unpaired t-test. There were no significant differences after DEX treatment although there was a trend showing decreased digoxin uptake after DEX and increase after TQ stimulation.

Data in Fig. 37 indicate that there was rather no influence of medium composition on digoxin uptake and both types of medium can be used to investigate Pgp functionality on GPNT cells. However, slow digoxin uptake in cells treated with tariquidar was a reason for trying another Pgp substrate, vinblastine. Vinblastine is a Pgp substrate but it is less specific than digoxin and was used at concentration vinblastine [1 μM]+^{3}H-vinblastine [10 kBq/ml] (Fig. 38).

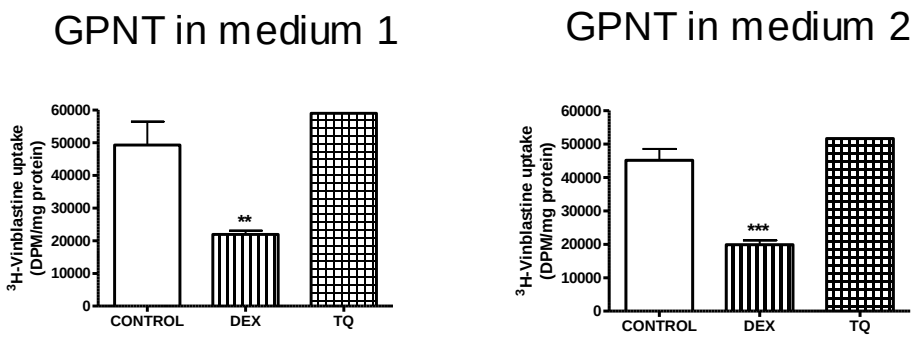


Fig. 38 Comparison of two medium compositions (Table 4) in GPNT cells P32. Cells were treated with dexamethasone (DEX, 1 µM) for 3 days starting on the day of cell confluence and were stimulated with tariquidar (TQ, 0.5 µM) for 2 h on the day of the assay. Uptake assay was performed under the condition vinblastine [1 µM]+^{3}H-vinblastine [10 kBq/ml]. Viblastine uptake assay was normalized on DPM/mg protein. Data are shown as means ± SD of three samples (except TQ, n=1). Data were statistically compared by unpaired t-test. Significant differences between compared data are indicated by two asterisks (P<0.01) or three asterisks (P<0.001).

As it was shown in Fig. 38, vinblastine uptake could be investigated in both medium compositions. Treatment of GPNTcells with dexamethasone resulted in decreased vinblastine accumulation in the cells that was an evidence for increased Pgp function. More vinblastine was removing out of the cells in GPNT treated with Pgp inducer dexamethasone.

The aim of experiments presented above was to find out the proper conditions for uptake assay. Various modifications of protocol (changes in medium composition, concentration of digoxin, time point of the treatment) were tried in cell lines like GPNT, MDCK and RBE4.

Cells were not treated with any of AEDs but just treated with known Pgp inducer dexamethasone and Pgp inhibitor tariquidar. To optimalize condition different Pgp substrates were used (vinblastine and digoxin in various concentrations). Next step was to apply tested experimental conditions in relation to AEDs. For the next experiments, digoxin was chosen as a Pgp substrate, because it is recommended by FDA for use in transport experiments (FDA, 2006) and is a specific substrate for Pgp protein.

13.8.2 Experiments in GPNT cells treated with antiepileptic drugs

For the uptake assays in GPNT cells after the treatment with AEDs specific Pgp substrate digoxin was chosen at concentration digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml].

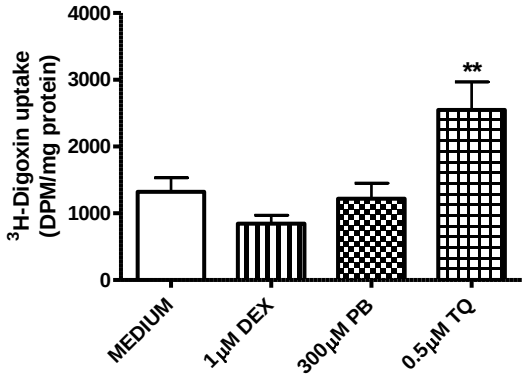


Fig. 39 Digoxin uptake assay in GPNT cells P35. Cells were treated with antiepiletic drug phenobarbital (PB), Pgp inducer dexamethasone (DEX) for 3 days starting on the day of cell confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of digoxin was investigated under the condition: digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by two asterisks (P<0.01).

GPNT cells were treated with AED phenobarbital at concentration 300 μM and with dexamethasone for three days starting on the day of confluence (Fig. 39). Statistical analysis showed significantly increased uptake in cells stimulated with tariquidar.

This experiment was performed when cells were treated with high concentration of phenobarbital (300 μM), in next trial lower concentration (100 μM) was tried which is in range of therapeutic concentrations. Treatment was prolonged up to seven days.

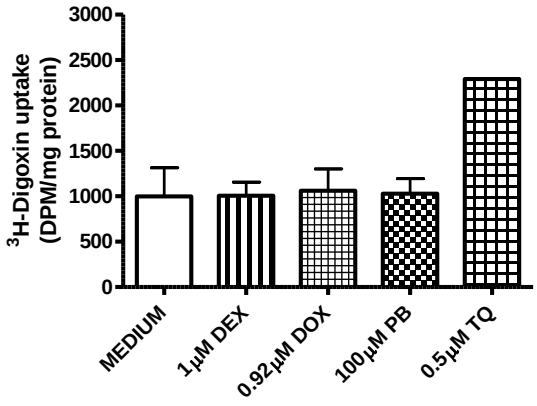


Fig. 40 Digoxin uptake assay in GPNT cells P32. Cells were treated with antiepileptic drug phenobarbital (PB), Pgp inducers dexamethasone (DEX) and doxorubicin (DOX) for 7 days starting on the day of cell confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of digoxin was investigated under the condition: digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples. Data were statistically compared by one-way analysis of variance (ANOVA). There were no significant differences between samples.

As a first AED, phenobarbital was used at concentration 100 μM (Fig. 39) and 300 μM (Fig. 40). As Pgp inducer dexamethasone or doxorubicin were used. No Pgp induction was observed either after dexamethasone, doxorubicin or phenobarbital. Even, when the treatment period was extended (from 3 day up to 7 days, Fig. 39 & 40) no Pgp alteration in Pgp function was observed.However, increased accumulation of digoxin, after treatment with Pgp inhibitor tariquidar, indicates that Pgp was functionally active and inhibited by tariquidar.

Next AED that was tested in uptake assay in GPNT cells was phenytoin at concentration 50 μM (Fig. 41).

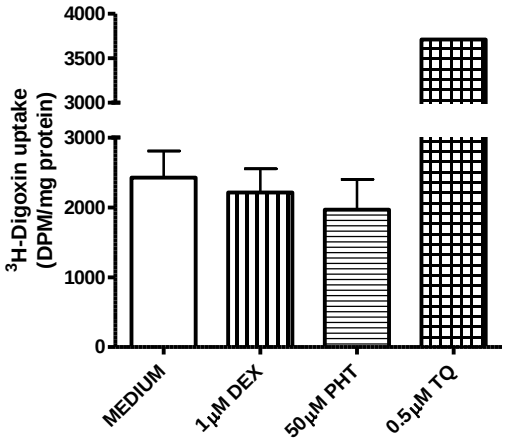


Fig. 41 Digoxin uptake assay in GPNT cells P31. Cells were treated with antiepileptic drug phenytoin (PHT), Pgp inducer dexamethasone (DEX) for 3 days starting on the day of cell confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of digoxin was investigated under the condition: digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples (except TQ, n=1). Data were statistically compared by one-way analysis of variance (ANOVA). There were no significant differences between samples.

In this experiment no significant differences were observed. Anyhow, cells stimulated with tariquidar showed an increase in digoxin uptake assay but because of only one sample per treatment statistical analysis could not be performed. Another used AED was carbamazepine at concentration 30 μM (Fig. 42).

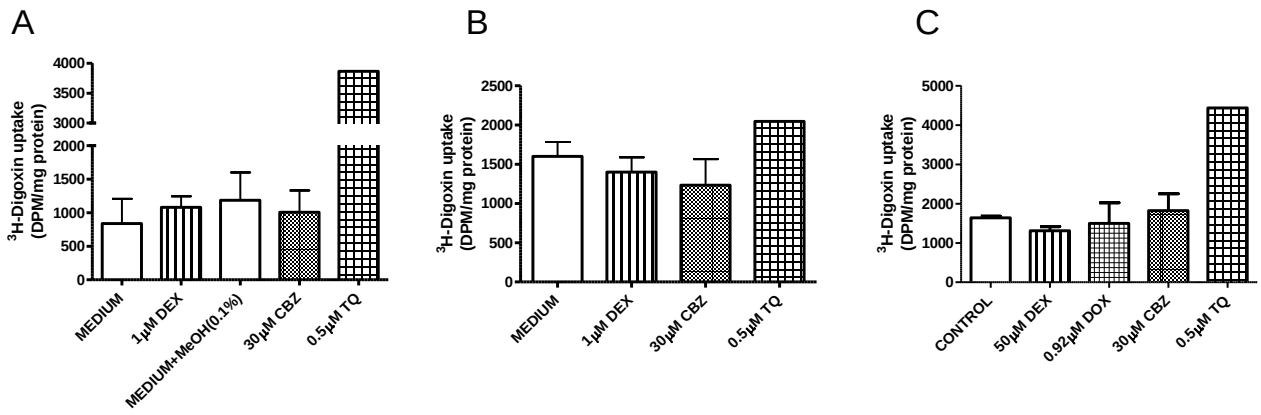


Fig. 42 Digoxin uptake assay in GPNT cells P30, P32, P31 in three independent experiments. Cells were treated with antiepileptic drug carbamazepine (CBZ), Pgp inducer dexamethasone (DEX) for 3 days starting on the day of confluence and were stimulated with tariquidar (TQ, 0.5 µM) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of digoxin was investigated under the condition: digoxin [1µM]+^{3}H-digoxin [10kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples (except TQ, n=1). Data were statistically compared by one-way analysis of variance (ANOVA). There were no significant differences between samples.

In previous experiments dexamethasone at concentration 1 µM did not significantly influence Pgp expression. One of the explanations could be the concentration. For that reason in experiment shown in Fig. 42C, concentration of dexamethasone was increased up to 50 µM. Nonetheless, no significant effect on Pgp functionality was observed.

In the experiments presented above, GPNT cells were treated with various AEDs. Treatment was started on the day of the confluence and lasted for 3 days. As a control Pgp inducers doxorubicin and dexamethasone were used. However, no increase in Pgp expression was found in uptake assays with digoxin. Thus, in next trial digoxin was replaced by vinblastine.

Vinblastine is not so specific like digoxin and could be a better substrate for Pgp in presented experiments. As it is shown in Fig. 39 vinblastine as a substrate for Pgp in uptake assay worked well and provided significant results after the treatment with Pgp inducers. In next trials with vinblastine applied concentration was vinblastine [1 µM]+^{3}H-vinblastine [4.2 kBq/ml].

Next experiments were performed either on the day of confluence (Fig. 43) or 6 days after confluence (Fig. 44). GPNT cells were treated with several AEDs: phenobarbital, carbamazepine and phenytoin. As positive control dexamethasone was used. Functionality of assays was confirmed by tariquidar.

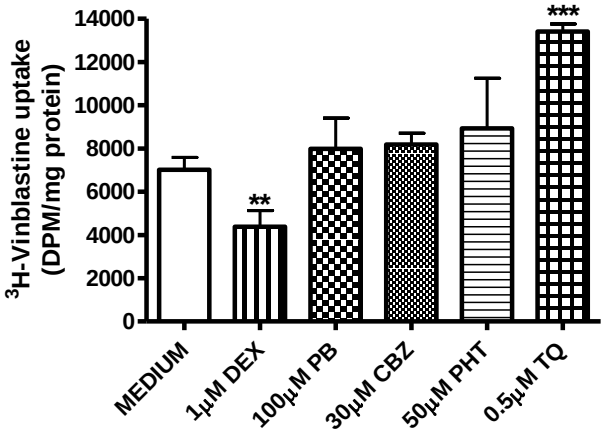


Fig. 43 Vinblastine uptake assay in GPNT cells P40. Cells were treated with antiepileptic drugs phenobarbital (PB), carbamazepine (CBZ), phenytoin (PHT) and Pgp inducer dexamethasone (DEX) for 3 days starting on the day of confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of vinblastine was investigated under the condition: vinblastine [1 µM]+^{3}H-vinblastine [4.2 kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples (except MEDIUM, n=6). Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by two asterisks (P<0.01) and three asterisks (P < 0.001). This uptake assay was performed by Carlos Luna Tórtos.

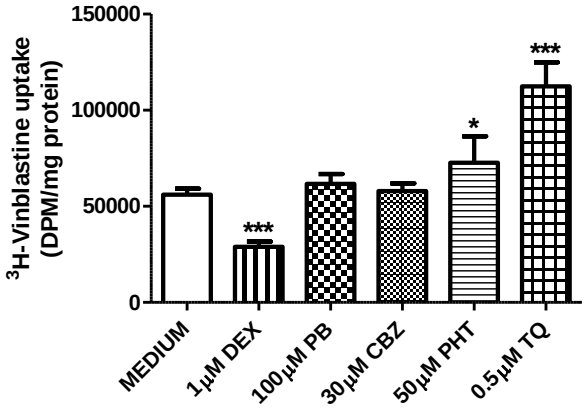


Fig. 44 Vinblastine uptake assay in GPNT cells P40. Cells were treated with antiepileptic drugs phenobarbital (PB), carbamazepine (CBZ), phenytoin (PHT) and Pgp inducer dexamethasone (DEX) for 3 days starting 6 days after confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of vinblastine was investigated under the condition: vinblastine [1 µM]+^{3}H-vinblastine [4.2 kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples (except MEDIUM, n=6). Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by one asterisk (P<0.05), two asterisks (P<0.01) and three asterisks (P < 0.001). This uptake assay was performed by Carlos Luna Tórtos.

In both trials (Fig. 43 & 44) dexamethasone affected Pgp function and resulted in significant decrease in vinblastine uptake. Additionally, treatment with phenytoin also gave significant effect on Pgp functionality (Fig. 34). The correctness of the assays was proved by Pgp inhibitor tariquidar. Stimulation with Pgp inducer dexamethasone caused lower accumulation of radioactive vinblastine compared to controls. In cells, where tariquidar was used, transport of vinblastine was inhibited and resulted in higher accumulation of substrate in the cells.

Apart from GPNT cells, MDCK-WT cells were also used in uptake assay. Uptake assays were performed with both Pgp substrates digoxin (Fig. 46) and vinblastine (Fig. 45). Vinblastine is not as specific as digoxin and can be transported also by multidrug resistance-associated proteins (MRPs). Thus, to confirm that vinblastine uptake was due to Pgp induction, specific inhibitor of MRPs called MK571 was used (Fig. 45).

In both experiments treatment was initated on the day of the cell confluence and was performed for three days. Uptake assays were made on 4th day of confluence.

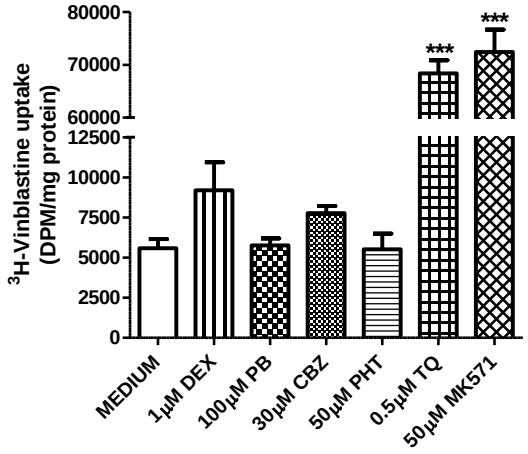


Fig. 45 Vinblastine uptake assay in MDCK-WT cells P9. Cells were treated with antiepileptic drugs phenobarbital (PB), carbamazepine (CBZ), phenytoin (PHT) and Pgp inducer dexamethasone (DEX) for 3 days starting on the day of confluence and were stimulated with Pgp inhibitor tariquidar (TQ) and MRPs inhibitor MK571 for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of vinblastine was investigated under the condition: vinblastine [1 μM]+^{3}H-vinblastine [4.2 kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples (except MEDIUM, n=6). Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by three asterisks (P<0.001). This uptake assay was performed by Carlos Luna Tórtos.

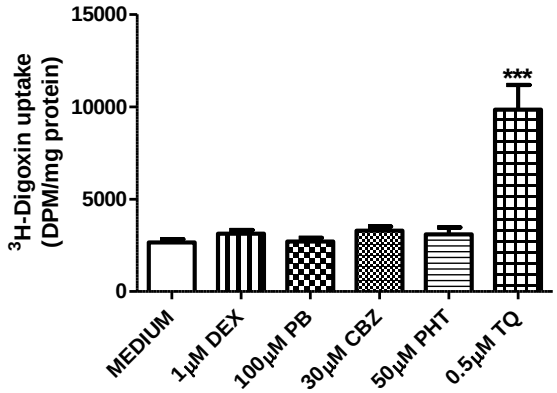


Fig. 46 Digoxin uptake assay in MDCK-WT cells P16. Cells were treated with antiepileptic drugs phenobarbital (PB), carbamazepine (CBZ), phenytoin (PHT) and Pgp inducer dexamethasone (DEX) for 3 days starting on the day of confluence and were stimulated with tariquidar (TQ) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of digoxin was investigated under the condition: digoxin [1 µM]+ ^{3}H-digoxin [10 kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of three samples (except MEDIUM, n=6). Data were statistically compared by one-way analysis of variance (ANOVA) followed by Bonferrroni's Multiple Comparison Test. Significant differences between compared groups of data are indicated by three asterisks (P < 0.001). This uptake assay was performed by Carlos Luna Tórtos.

Uptake assays in MDCK cells have revealed no alternations in Pgp expression after dexamethasone and AEDs treatment. There was significantly increased vinblastine and digoxin uptake after Pgp inhibitor tariquidar and MRP inhibitor MK571.

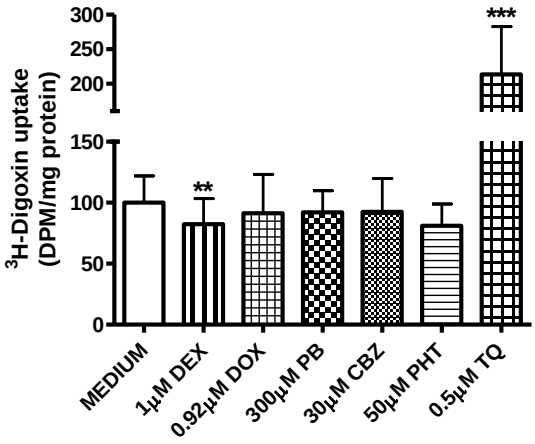


Fig. 47 Digoxin uptake assay in GPNT cells within P30 & P35 in seven indenpendent experiments. Cells were treated with antiepiletic drugs phenobarbital (PHT), carbamazepine (CBZ), phenytoin (PHT) and Pgp inducer dexamethasone (DEX) and doxorubicin (DOX) for 3 days starting on the day of confluence and were stimulated with tariquidar (TQ, 0.5 µM) for 2 h on the day of the assay. Drug concentrations are indicated under the graph. Accumulation of digoxin was investigated under the condition: digoxin [1µM]+^{3}H-digoxin [10kBq/ml] and was normalized on DMP/mg protein. Data are shown as means ± SD of samples (MEDIUM, n=28; DEX, n=19; DOX, n=3; PB, n=3; CBZ, n=11; PHT, n=5; TQ, n=11). Data were statistically compared by one-way analysis of variance (ANOVA) and post test t-test (MEDIUM *vs* DEX & MEDIUM *vs* TQ). Significant differences between compared groups of data are indicated by two asterisks (P<0.01) and three asterisks (P < 0.001).

In Fig. 47, data from 7 independent xperiements in GPNT cells are summarized. Overall, there was significantly decreased uptake with Pgp substrate digoxin after dexamethasone and no changes in Pgp functionality after AED treatments. Functionality of Pgp was proven by using specific Pgp inhibitor tariquidar, which significantly inhibited accumulation of digoxin (Fig. 47).

14 Discussion

The main question addressed in presented thesis dealt with possible Pgp induction after AEDs. First suggestion that AEDs can be substrates for Pgp came from experiments by Tishler (Tishler et al., 1995). It has been reported that several major AEDs (carbamazepine, phenobarbital, phenytoin) are transported either by Pgp or MRPs or both (Löscher and Potschka, 2002). This would explain lower concentration of AEDs in the brain, which would be a result of active transport of drugs by multidrug transporters. However, which AEDs are substrates for Pgp is still very controversial issue because of contradictory data about multidrug transporter substrates (Owen et al., 2001; Crowe and Teoh, 2006; Luna-Tórtos et al., 2008).

The purpose of presented PhD thesis was to check whether AEDs can induce Pgp expression in different cells lines. However, in order to start answering this question, funtional protocols for methods used in thesis had to be established. Western blotting method, used for Pgp expression and uptake assay, applied for Pgp functionality, had to be specified.

14.1 Methodological discussion

14.1.1 Establishment of Western blotting protocol

As a beginning, MDCKII wild type (MDCK-WT) cells were used and various AEDs were tested. Among examined drugs was phenobarbital, because it was reported to be a Pgp substrate (West and Mealey, 2007; Luna-Tórtos et al., 2008) and others like carbamazepine, phenytoin and topiramate, for which the possible induction had to be investigated. Initial experiment was performed in order to get first impression about possible Pgp induction after the treatment with AEDs. Obtained data suggested no induction in Pgp expression after used drugs, whereas a known Pgp inducer rifampicin has shown a tendency to influence Pgp expression (Fig. 10). However, the statistical analysis could not be performed because of only one sample per drug. PCR data showed no indication for Pgp alterations (Fig. 11).

To check correctness of Western blotting conditions, two sets of samples were prepared in parallel and used for Western blotting (Fig. 12). MDCK-WT cells, in both sets, were treated with phenobarbital (PB, 300 μM) and rifampicin (RIF, 25 μM). As it is shown in Fig. 12, data from two

sets differed in response to rifampicin. The explanation of those diverse results might be improper condition of Western blotting, which led to false results. Thus, all experiments presented aboved were performed under suboptimal conditions.

Methodological problems that have occurred during Western blotting were: unstable signals from loading control, signal from Pgp antibody, which resambled dots not bands and unexpected positions of signals recognized by C219 antibody.

First problem was as mentioned above unstable tubulin signal, which could not be recommended as a believable loading control. As a confirmation for this finding the unpublished personal comment from Dr. U. Mönnig (Bayer Schering Pharma AG, Berlin) can serve, that tubulin is not a good but often variable internal control, and it is adviced to use another, like actin, which is more credible.

Tubulin, a commonly used internal control, was applied to control and correct the loading error. However, it did not provide a stable signal and protein normalization on that protein was not possible. The unstable tubulin signal could be due to not optimized Western blotting condition, in which cytoskeleton protein structure would be affected during sample preparation.

To check, whether unstable tubulin signal was the effect of improper Western blotting condition, tubulin was replaced by villin. Villin is cytoskeletal protein with molecular weight of 95 kDa. Villin was used by some groups (Robine et al., 1985) as an internal loading control. As it is shown in Fig. 13, villin was detected on immunolot and provided strong signal. Nonetheless, in case of MDCK cells, in which Pgp antibody recognized multiple bands, villin with its high molecular weight, might interfere with Pgp signals. Thus, in the next experiments as a loading control actin was used. Actin provided strong, equal signals, which allowed normalizing of protein expression on this loading control.

Another intriguing issue was that C219 Pgp antibody has recognized more that one signal, which actually did not resemble a sharp band, but rather a dot or dots (Fig. 9, 10 & 12). Such shapes of signals could be due to improper preparation of electrophoresis or transfer buffers, in which concentration of ions and salts were disturbed. Moreover, the position of Pgp signals did not have expected and reported molecular weights (Loo and Clarke, 1999b). Pgp signals have appeared either with approximately molecular weight of 120 kDa (Fig. 10 & 12) or 140 kDa (Fig. 9 & 13). Signals recognized by Pgp antibody with different than expected molecular weights might represent the fragments of degradated Pgp protein. It could happen, that during the preparation of proteins used sonification (Table 2) warmed up and destroyed the samples.

Several primary anti-Pgp antibodies were tested. Clone C219 of antibody was used. It recognized an internal, highly conserved amino acid sequence: VQEALD and VQAALD, corresponding to the

C-terminal and N-terminal regions, respectively, found in Mdr1 P-glycoprotein. Anti-Pgp antibodies from Alexis, Calbiochem were tried. Finally, after the recommendation from Dr. U. Mönnig, C219 Pgp antibody from Signet was used in all experiments. Anti-Pgp antibody from all tested brands provided multiple bands in MDCK cells (e.g. Fig. 12). However, Pgp antibody from Signet provided two sharp bands with expected molecular weight (e.g. Fig. 22). It seems, that in MDCK cells anti-Pgp antibody provided two signals with approximately molecular weight 140 kDa and 160 kDa. However, in many studies investigating Pgp induction in other cell lines, researchers showed only one band with approximately molecular weight of 160 kDa (Maitra et al., 2001). Nonetheless, there are also articles in which both bands were described in MDCK cells (Loo and Clarke, 1999b; Tang et al., 2002). In addition to that, Jetté et al. (Jetté et al, 1997) have found that glycosylated monomeric subunit of Pgp in brain capillaries had molecular weight of 162 kDa, whereas in renal brush border membranes had 140 kDa. That study is in line with our findings in immortalized rat brain GPNT and kidney MDCK cells.

All those problems were reason to modify and change the Western blotting protocol. The established protocol (for details see Table 2) was used for next experiments.

14.1.2 Pgp induction in MDCK cells after exposure to antiepileptic drugs

After many modifications in Western blotting method and trying C219 Pgp antibody from various companies (Table 2), specific Pgp antibody has still recognized two bands in MDCK cells. One band appeared with molecular weight approximately of 140 kDa and another with approximately 160 kDa. Those both bands were characteristic for MDCK cells and presented different glycosylation forms of Pgp (Loo and Clarke, 1999b). However, the occurrence of double bands could be a problem in experiments in which MDCK cells are treated with drugs and changes in expression have to be calculated. In such situation, the questionable fact can be, on which band drugs influence and which band should be taken into calculation. In order to answer those questions, two bands were counted separately (Fig. 19). Both calculated bands provided similar results, but only quantification of upper band led to significant data (Fig. 19A). It seemed like both forms of Pgp proteins were influenced by drugs, but upper band (app. 160 kDa) might have been more sensitive towards treatment.

In regard to glycosylation process in Pgp, it is known that core-glycosylated Pgp protein (app. 140 kDa) are retained in the endoplasmic reticulum (ER) in association with chaperone proteins like

calnexin and Hsc70 (heat shock cognate 71 kDa protein). When it is required, core-glycosylated form can be converted into the mature (app. 160 kDa) protein and be trafficked to the cell surface. Such situation can happen in presence of multidrug transporters substrates (Loo and Clarke, 1995).

Furthermore, fully glycosylated Pgp protein is a form of functional active protein (Loo and Clarke, 1999a) and this form was under investigation in presented thesis.

To affirm the position of mature Pgp protein (app. 160 kDa) in MDCK cells, experiments in other transfected cells were performed. Comparison between MDCK wild type cells or transfected with human MDR1 gene and other cell lines (GPNT, LLC) is shown in Fig. 20, 21 and 22. Cells transfected with human Pgp gene, such as: porcine kidney cells (LLC-MDR1) and MDCK-MDR1 and rat brain endothelial cells (GPNT) used in those experiments, functioned as controls to confirmed positions of mature Pgp protein.

In all investigated cell lines, Pgp bands appeared with molecular weight around 160 kDa (Fig. 21). Additionally, in MDCK cells second band with molecular weight around 140 kDa was present and constituted as core-glycosylated form of Pgp.

The evidence for existence of two different glycosylated forms of P-glycoprotein in MDCK cells came from the experiment, in which MDCK-WT cells were treated with PNGase enzyme (Fig. 23).

PNGase enzyme removed glycosylated residues from Pgp protein resulted in detection of only one band (app. 140 kDa) by Pgp antibody. In contrast to MDCK cells untreated with PNGase enzyme both bands (app. 140 kDa & 160 kDa) appeared on immunoblot (Fig. 23).

In Part II of Results chapter, modified protocol for Western blotting was checked in MDCK cells that were treated with two AEDs: phenobarbital and carbamazepine. Protein expressions of Pgp and mRNA level for Mrd1 and MRPs did not show any indication of Pgp upregulation after AEDs (Fig. 16, 17 & 18). However, significant induction was observed in MDCK cells treated with phenobarbital in Fig. 19. This significant result was obtained with statistical test unpaired t-test, not like in others experiment after ANOVA analysis.

14.1.3 Experiments in GPNT cells

As another model to investigate possible influence of AEDs on Pgp expression and function, immortalized rat brain endothelial cells (GPNT) were used (Roux and Couraud, 2005). Cells were examined in Western blotting method according to the optimized protocol (protocol no. 2) (see Table

2) and in uptake assays. Since there were some differences in medium compositions (Table 4), GPNT cell line was cultured in two media compositions and Pgp expression (Fig. 24) and functionality (Fig. 37 & 38) were tested. Cells cultured for uptake assay were treated with two Pgp substrates vinblastine and digoxin (Fig. 37 & 38). As a reference, Pgp inducer dexamethasone was used.

Significant Pgp upregulation after dexamethasone was observed in both medium compositions and for both Pgp substrates Pgp expression had similar trend. Thus, for purpose of this work, GPNT cells were cultured in medium 2 (Table 4). Digoxin was chosen as a Pgp substrate for uptake assays at concentration digoxin [1 μM]+^{3}H-digoxin [10 kBq/ml].

Several AEDs were tested in GPNT cells like phenobarbital, carbamazepine and phenytoin. As positive internal controls, cells were treated with known Pgp inducers puromycin (Demeuse et al., 2004), doxorubicin (Fardel et al., 1997) and dexamethasone (Régina et al., 1999).

Taking together all data, there was no significant increase in Pgp expression and functionality after treatment with AEDs. Overall, dexamethasone used in uptake assay experiments led to significantly decreased digoxin uptake and significantly increased digoxin uptake after cell exposure to Pgp inhibitor tariquidar (Fig. 47).

However, there was some variability in individual experiments. Cells exposed to inducers gave contradictory results, e.g. treatment with doxorubicin resulted in significant increase in Pgp expression (Fig. 26D), but in another experiment under the same conditions doxorubicin significantly decreased Pgp expression (Fig. 31A). Similar contradictory data were also observed in other experiments with dexamethasone (Fig. 29A *vs* Fig. 32A), which was previously shown to induce Pgp activity (Régina et al., 1999). One of the explanations for such variable data could be that Pgp inducers, e.g doxorubicin, influences Pgp expression only in cell lines different from rat brain endothelial cells (GPNT), e. g. rat liver (Fardel et al., 1997). In single experiment, enhanced Pgp expression after phenobarbital (Fig. 32) and altered functionality after phenytoin (Fig. 34) were observed. Variabilities in data could be also due to differences in Pgp expression in particular passages of cells. It was shown by Roux's group (Demeuse et al., 2004) and in presented thesis (Fig. 22) that Pgp level could depend on passage numbers. Cells with higher passage numbers expressed more Pgp and such variability in Pgp expression could influence on results. Another intriguing issue was decreased Pgp expression after tariquidar treatment (Fig. 31 & 32) that may be due to problems with C219 antibody binding. Tariquidar structure might be too large and could cover the place which was recognized by Pgp antibody.

14.2 General discussion

Epilepsy, as mentioned previously, is a common neurological disease and it is characterized by unprovoked seizures. Epileptic features have a great impact on the life quality of epileptic patients. Thus, it is important to find out which mechanisms lead to epilepsy. Due to several miscellaneous conditions like seizures, disturbed sensations, mental disturbance, epilepsy is considered to be caused by multiple factors. The main problem in therapy is the effectiveness of the treatment. Around 30% of patients do not respond to the AEDs. This pharmacoresistance is defined as a persistence of the symptoms despite the treatment with at least two different AEDs in certain time.

There are two main hypotheses explaining the mechanisms that underlie the pharmacoresistance. According to first, multidrug transporter hypothesis, enhanced expression of multidrug transporters in BBB leads to unsufficient drug concentration in the brain by ATP-dependent drug efflux to the blood stream. According to the second, drug target hypothesis, alteration in neurotransmitter receptors or voltage-gated ion channels on which AEDs act, might cause those targets less responsive to AEDs.

Presented thesis was concentrated on the multidrug transporter hypothesis and was devoted to answer the question whether AEDs can cause overexpression of Pgp.

Starting point for multidrug hypothesis was study by Tishler et al. (Tishler et al., 1995), who showed overexpression of Pgp in capillary endothelium in samples from epileptic patients both in mRNA and protein levels. Furthermore, overexpression of Pgp in patients with intractable epilepsy was also shown by Dombrowski et al. (Dombrowski et al., 2001). However, what caused the upregulation of Pgp is still under the question. The idea of presented thesis was to investigate whether changes in Pgp expression (one of the mechanism for pharmacoresistance) could be due to applied AEDs.

Studies on cancer cells have shown increased in Pgp expression after vinblastine exposure (Sulová et al., 2009). Thus, interesting is also whether commonly used AEDs, (most of them are substrates for Pgp) contribute to changes in Pgp expression.

14.2.1 Pgp expression and functionality in different cell lines

As a model to investigate Pgp expression several cell lines like MDCKII, LLC, GPNT, and RBE4 were tested.

MDCK type II, Madin-Darby canine kidney cells are epithelial cells derived by S. H. Madin and N. B. Darby from the kidney tissue of an adult female cocker spaniel (Madin and Darby, 1958). MDCK cells are used as a model of BBB to study multidrug resistance phenomenon (Mahar Doan et al., 2002). Expression of tight junctions (TJ) plays a role in specialized functions of apical and basolateral membranes of MDCK cells and makes MDCK cells an useful tool in drugs transport experiments (Baltes et al., 2007b). MDCK cells express Pgp protein but can be also easily transfected with human Pgp (MDR1 gene) (Pastan et al., 1988). In presented thesis, MDCK cells were used in context of Pgp role in pharmacoresistance in epilepsy. Thus, cells were exposed to different AEDs for certain time and Pgp expression was analyzed by Western blotting.

The Western blotting in MDCK cells resulted in multiple patterns of bands that were recognized by specific Pgp antibody (clone C219). In first experiments Pgp antibody has identified many signals, which resembled rather as dots then bands. Problem with shape of bands and unexpected positions could be due to method condition and was solved after protocol for Western blotting was established. After the Western blotting protocol was established, Pgp antibody still recognized double bands, but the signal appeared with reported molecular weights (app. 140 kDa and 160 kDa).

Presence of two bands could be a hurdle in experiments that are meant to quantify expression of Pgp. Multiple bands can lead to question, which band should be calculated, that is which band represents changes in Pgp expression or maybe both are altered by the treatment with drugs. The goal of presented thesis was to investigate the influence of AEDs on mature protein.

In order to check, which band represented the functional form of Pgp, MDCK cells were treated with AEDs (Fig. 19) and then, bands representing Pgp protein were quantified separately. It came out that quantification of both bands gave the same trend after treatment, but only quantification of upper band (app. 160 kDa) gave significant results. That was a hint to calculate upper band, as a representative signal for changes in Pgp expression. However, it would be interesting to investigate also changes in core-glycosylated form of Pgp.

MDCK and LLC cells transfected with human Pgp (MDCK-MDR1, LLC-MDR1) were used as internal controls (Fig. 20 & 21).

It has been shown that Pgp exists in two forms, which differ in glycosylation (Loo and Clarke, 1999b). The core-glycosylated form is reported to have molecular weight around 140 kDa, and mature fully glycosylated Pgp has molecular weight of approximately 160 kDa. To improve the position of mature protein on the blot, special enzyme endoglycosidase PNGase F was tested in MDCK cells. PNGase F removed the internal glycosylated residues from Pgp and resulted in only one band (app. 140 kDa) recognized by Pgp antibody (Fig. 23). This experiment was an evidence for the soundness of upper (app. 160 kDa) band quantification, which might be a mature form of Pgp.

Lack of the effect after AEDs treatment might be also explained by the fact, that MDCK cells express lot of different multidrug transporters. Thus, it might happen that used AEDs are substrates for other transporters than Pgp and resulted in their enhanced expression.

Apart from MDCK cells, immortalized rat brain endothelial cell line, GPNT cell line was used. GPNT cells were obtained after transfection of GP8.3 cells, with plasmid containing puromycin resistance gene. The immortalized brain endothelial cells GP8.3 were characterized by Greenwood et al. (Greenwood et al., 1996) and preserve phenotype reminiscent BBB endothelium (Demeuse et al., 2004).

GPNT cells express higher level of Pgp than parental cells GP8.3 or brain endothelial cells in primary culture (Régina et al., 1999). Pgp expression and functionality can be enhanced by routine treatment with puromycin (Demeuse et al., 2004).

High level of endogenous Pgp in GPNT has been shown by Demeuse (Demeuse et al., 2004). Increased Pgp expression might be a disadvantage in presented study. Possibly, lack in alteration of Pgp expression and function, in presented individual experiments, could be due to already high Pgp level in GPNT. In such situation, AEDs were not able to increase more Pgp expression.

However, the data from individual trials showed also significant increase in Pgp expression after dexamethasone (as expected) (Fig. 24 & 29), therefore upregulation in Pgp expression could be possible. Such variable data might be due to distinct Pgp expression from passage to passage (Demeuse et al., 2004). Enhancement in Pgp expression has been shown also in presented thesis (Fig. 22). Cells with higher passage number, without puromycin treatment, expressed more Pgp, in comparison to cells with lower passage number. Time dependent and puromycin dependent increase in Pgp expression in GPNT cells might be a disadvantage in experiments devoted to investigate changes in Pgp expression. It could happen that highly expressed Pgp masks the effect of examined drugs.

The advantage of using GPNT cells was presence of single, sharp band recognized by Pgp antibody (Fig. 24), easily to quantify for Western blotting results.

Overall, no significant upregulations in Pgp expression by AEDs were found. However, dexamethasone and tariquidar treatment resulted in significant alterations in Pgp functionality (Fig. 47). Those observations came from experiments using digoxin and vinblastine, a Pgp substrate. However, no Pgp induction after the treatment with antiepileptic drugs could be due to specific properties of digoxin. Digoxin is recommended by FDA for transport experiments. Hovewer, it might be not ideal substrate for uptake assays because of highly permeable features.

Improper time of the beginning or short period of the treatment (from 3 to 6 days) with AEDs could be also a reason for no effect of AEDs on Pgp expression. It has been shown that four AEDs (carbamazepine, phenobarbital, phenytoin and valproic acid) induced Pgp expression in astocytes cultured from postnatal rats. Observed Pgp induction was time and dose dependent and was significantly affected after 30 days (Yang et al., 2004). In another study by Yang et al. (Yang et al., 2008), Pgp was upregulated by AEDs (carbamazepine, phenobarbital, phenytoin and valproic acid) and also by rifampicin on both protein and functionality level in primary cultured rat brain microvascular endothelial cells. Protein level and functionality of Pgp was investigated 60 days after treatment has begun. This long exposure to drugs seems astonishing, because of used sensitive primary culture. However, the viability of the cells was proved by MTT assay. Apart the experiment in cell culture, another study on AEDs induction was performed by Wen et al., 2008 (Wen et al., 2008). Three AEDs (phenobarbital, phenytion and carbamazepine), orally given to rats twice a day for 21 days, altered Pgp expression and functionality, however, no positive control was applied.

Period of the treatment might have influence on Pgp induction and could be a reason for prolonged treatment in MDCK and GPNT cells. However, cell exposure to potentially toxic compounds such as AEDs, should demonstrate fast protective response, which would be at least enhancement in Pgp functionality. Long time induction to AEDs might be due to increase in gene expression and synthesis of new proteins. This possibility is in line with experiment done by Yang et al., (Yang et al., 2008), in which Pgp functionality was increased in parallel to Pgp expression. Whereas, in presented thesis, experiments in GPNT cells showed increased functionality only after dexamethasone treatment without robust changes on Pgp expression. Lack of the effect after AEDs treatment might be also due to not right time point for the beginning of treatment. Data from confocal microscopy might suggest time dependent Pgp expression in culture (Fig. 25). Possibly, it is more effective to start the treatment when cells express more Pgp (after 6 days of confluence or even later, Fig. 25C) and when Pgp

became fully specialized multidrug transporter protein localized in cell membrane. However, in several studies investigating Pgp induction in GPNT, cells were exposured to the drugs from the day of confluence (Demeuse et al., 2004; Régina et al., 1999). Study by Lombardo et al. (Lombardo et al., 2008) have shown upregulation in Pgp expression by AEDs (topiramate, tiagabine, levetiracetam, phenobarbital, carbamazepine and phenytoin) in GPNT cells using Western blotting and immunocytochemistry methods. Drug treatment lasted for 3 days and began on the day of confluence. These experimental results differed from those presented in this thesis, where no Pgp upregulation in GPNT cells have been seen. In single experiment induction after PB was detected. However, it was no robust effect.

In Lombardo's paper, expression of last three mentioned AEDs was correlated with increased activation of PXR and CAR receptors. Lombardo et al. (Lombardo et al., 2008) were able to detect Pgp upregulation after AEDs. However, no information could be found about Pgp activity, and none of known Pgp inducers was tried. The positive data from Lombardo might be due to distinct culturing conditions of GPNT cells that could influence Pgp expression. Moreover, induction of Pgp by AEDs might depend on cell types (species differences), similar to nuclear receptor family members (PXR, CAR), in which species differences were investigated and proven (Kliewer and Willson, 2002). As an example, rifampicin activated the human and rabbit PXR but had no activity on the mouse or rat receptors (Kocarek et al., 1995). This would explain lack of robust effect in Pgp expression after doxorubicin exposure in GPNT cells, but significant alteration in Pgp in rat liver cells (Fardel et al., 1997).

Described thesis was meant to investigate possible Pgp upregulation after AEDs treatment, as one of the mechanism of pharmacoresistance. However, studies on two different cell lines (MDCK, GPNT) did not reveal significant changes in Pgp expression after AEDs. Functionality of Pgp was not altered by AEDs, but significant decrease in accumulation of Pgp substrates within cells was observed after the treatment with dexamethasone. Pgp inhibitor tariquidar significantly increased uptake of digoxin, which was an evidence for functionality of Pgp in investigated cells.

It seems that tested AEDs do not affect Pgp expression and function. However, many factors like culturing conditions (adding puromicin), time point of the treatment (day of confluence, after confluence) or period of the treatment (3 days or longer) might influence such results.

15 Summary

Methods of investigating drug-induced increase in the expression of the drug efflux transporter P-glycoprotein in different cell types

Kamila Ambroziak

Epilepsy is one of the most common neurological disorders, which affects humans and animals, like dogs and cats. Drug treatment is still the most often used therapy. However, about one third of epileptic patients do not respond to distinct AEDs. The mechanisms of pharmacoresistance are poorly understood. Nonetheless, there are two major hypothesis explaining pharmacoresistance, multidrug hypothesis and drug target hypothesis (Schmidt and Löscher, 2009).

According to the first hypothesis, overexpression of multidrug transporters in blood-brain barrier decreases drug uptake in the brain, which lead to insufficient drug concentration needed for effective treatment. An evidence for correlation between overexpression of multidrug transporter and pharmacoresistance came from work by Tishler et al. (Tishler et al., 1995). Group showed upregulation, one of the most examined multidrug transporters, Pgp in epileptic brain tissue.

Drug target hypothesis, suggests that alterations in the structure and/or functionality of AED targets in epileptogenic brain regions lead to reduced drug effects (Löscher and Potschka, 2005a). For instance, experimental studies have shown that changes in the structure and function of inhibitory GABA(A) receptors may contribute to drug resistance in epilepsy (Bethmann et al., 2008).

Presented thesis was concentrated on multidrug transporter hypothesis, especially on Pgp, as a main principal for pharmacoresistance in epilepsy.

In order to overcome the pharmacoresistance in epilepsy, it is relevant to explore the mechanisms that lead to refractory epilepsy. Thus, presented thesis was meant to investigate whether commonly used AEDs might cause overexpression of multidrug transporters resulting in decrease drugs concentration in the brain.

For purpose of thesis two cell lines were tested: MDCK epithelial kidney cells and GPNT rat brain endothelial cells. The reason for using those cells was mostly that they are recognized as a model of blood-brain barrier. Further, MDCK cells were used in study on drug transport, GPNT express several endothelial markers and for both cell lines cultering conditions were established.

Study in cell lines began from establishing of protocols for Western blotting and uptake assay methods, which are used to investigate expression and functionality of proteins. Cells were treated with antiepileptic drugs for certain period of the time, and then protein expression and functionality were analyzed. Pgp protein in MDCK cells was recognized by anti-Pgp antibody as double bands with molecular weights of approximately 140 kDa and 160 kDa. Both bands seemed to be Pgp proteins that differed in glycosylation. As it was shown, Pgp with molecular weight around 140 kDa is core-glycosylated form of Pgp, the signal around 160 kDa in turn seems to be fully glycosylated, mature form of Pgp. In presented thesis, mature form of Pgp was investigated. Thus, for quantification of the Pgp expression, bands around 160 kDa were taken as signals representing mature proteins. In contrast to MDCK, in GPNT cells anti-Pgp antibody has recognized only single band with molecular weight around 160 kDa.

Individual experiments in MDCK and GPNT cells differed in results. The observed variance could be due to particular characteristics of the used cell lines, for instance changes in Pgp expression during the culturing that could depend on passage of the cells. Furthermore, period and beginning of the treatment (differentiation of the cells) might be relevant.

In general, treatment with AEDs did not influence Pgp expression in MDCK cells. In GPNT cells, Pgp expression and functionality were not affected as well. However, Pgp was functionally active in GPNT cells, which was improved by significant increase in uptake of Pgp substrates after exposure to Pgp inhibitor tariquidar. No effect on Pgp fuctionality after exposure to AEDs was observed in uptake assays with both Pgp substrates: digoxin and vinblastine.

Thus, the results of presented thesis did not indicate any robust effect on Pgp expression and functionality after AEDs treatment. However, GPNT and MDCK cells might be not ideal models for studying drug-induced alterations in Pgp expression, because of high level of endogenous Pgp and other multidrug transporters.

More research must be done to verify multidrug transporter hypothesis and find suitable models for Pgp expression investigation.

16 Zusammenfassung

Methoden zur Untersuchung der Arzneimittel-induzierten Erhöhung der Expression von P-glycoprotein, einem Medikamenten-Efflux-Transporterprotein, in verschiedenen Zelltypen

Kamila Ambroziak

Epilepsie ist eine der häufigsten neurologischen Erkrankungen und betrifft Menschen und Tiere, wie zum Beispiel Hunde und Katzen. Die medikamentöse Behandlung ist die am häufigsten gewählte Therapieform. Allerdings reagieren ungefähr ein Drittel der Patienten nicht in ausreichendem Maße auf die verschiedenen, eingesetzten Antiepileptika. Die Mechanismen für diese Pharmakoresistenz sind weit gehend unbekannt. Es gibt aber zwei Hypothesen, welche die Gründe für Pharmakoresistenz zu erklären versuchen: die so genannte Transporter-Hypothese und die Target-Hypothese (Schmidt und Löscher, 2009).

Entsprechend der ersten verringern Multidrug-Transporterproteine in der Blut-Hirn-Schranke die Aufnahme von Substanzen in das Gehirngewebe und führen so zu einer geringen Substanzkonzentration, die keine ausreichende Wirkung ermöglicht. Tishler et al. (Tishler et al., 1995) erbrachten Hinweise für eine Verbindung zwischen der Überexpression von Multidrug-Transportern und Pharmakoresistenz, indem sie im epileptischen Hirngewebe eine Hochregulation von P-Glycoprotein (Pgp) zeigten, eines der am besten untersuchten Multidrug-Transporterproteine.

Die Target-Hypothese handelt von strukturellen und/oder funktionellen Veränderungen an den Zielstrukturen der Antiepileptika im epileptischen Gewebe. Diese veränderten Zielstrukturen bewirken dann einen verminderten Effekt der Substanzen (Löscher und Potschka, 2005a). Beispielsweise haben Studien gezeigt, dass strukturelle und funktionelle Veränderungen der inhibitorischen GABA(A) Rezeptoren an der Resistenz gegenüber Substanzen zur Epilepsiebehandlung beteiligt sein können (Bethmann et al., 2008).

Die hier beschriebene Arbeit konzentrierte sich auf die Transporter-Hypothese, und besonders auf Pgp, als ein möglicher Hauptgrund für Pharmakoresistenz bei Epilepsie.

Um die Pharmakoresistenz zu überwinden, ist es notwendig die Mechanismen aufzuklären, die ihr zugrunde liegen. Daher wurde in dieser Arbeit untersucht, ob häufig eingesetzte Antiepileptika eine

Überexpression von Multidrug-Transportern hervorrufen und so eine verringerte Substanzkonzentration im Gehirn bewirken.

Zu diesem Zweck wurden zwei Zelllinien verwendet: MDCK, eine epitheliale Nierenzelllinie, und GPNT, eine Endothelzelllinie aus dem Gehirn der Ratte. Diese Zellen wurden vor allem deswegen benutzt, weil sie als Modell für die Blut-Hirn-Schranke gelten. MDCK Zellen haben den Vorteil, dass sie bereits in Untersuchungen zum Substanztransport eingesetzt wurden, GPNT Zellen dagegen exprimieren einige endotheliale Markerproteine. Für beide Zelllinien sind die Kulturbedingungen bereits bekannt.

Zunächst mussten die Protokolle für die verwendeten Methoden zur Untersuchung von Proteinexpression und –funktion, Western Blot und Uptake Assay, etabliert werden. Die Zellen wurden mit Antiepileptika über bestimmte Zeit behandelt und anschließend wurden Expression und Funktionalität untersucht. In MDCK Zellen wurde Pgp durch den spezifischen Antikörper als doppelte Banden nachgewiesen mit Signalen bei Molekulargewichten von 140 und 160 kDa. Es lässt sich vermuten, dass beide Signale Pgp darstellen, allerdings in unterschiedlich glykosylierter Form. Es konnte gezeigt werden, dass die Bande bei 140 kDa das kern-glykosylierte Pgp darstellt, während die bei 160 kDa das voll-glykosylierte, reife Protein repräsentiert. Da in der vorliegenden Arbeit die funktionstüchtige Form von Pgp untersucht werden sollte, wurde für die Auswertungen die Bande bei 160 kDa herangezogen. Im Gegensatz zu MDCK konnte bei GPNT Zellen nur eine Bande bei 160 kDa nachgewiesen werden.

Die einzelnen Experimente mit MDCK und GPNT Zellen variierten in den Ergebnissen. Diese Beobachtung könnte in den Besonderheiten der beiden Zelllinien begründet sein. Zum Beispiel kommt es zu Veränderungen in der Pgp-Expression während der Kultivierung. Die Expression scheint dabei von der Anzahl der Passagen der jeweiligen Zellen abzuhängen. Darüber hinaus dürften Dauer und Beginn der Behandlung mit Antiepileptika im Hinblick auf die Differenzierung der Zellen von Relevanz sein.

Im Allgemeinen ergab sich durch Behandlung von MDCK Zellen mit Antiepileptika keine Beeinflussung der Pgp-Expression. Auch in GPNT Zellen wurden Expression und Funktion unter Behandlung nicht verändert. Hier konnte allerdings funktionelles Pgp nachgewiesen werden. So zeigte sich im Uptake Assay nach Zugabe des Pgp-Inhibitors Tariquidar eine signifikant erhöhte Aufnahme von Pgp-Substraten in die GPNT Zellen. Nach Behandlung mit Antiepileptika dagegen wurde keine veränderte Pgp-Funktion im Transport von zwei Pgp-Substraten, Digoxin und Vinblastin, beobachtet.

Insgesamt konnten die Ergebnisse der vorliegenden Arbeit keine robusten Effekte auf Pgp-Expression und -Funktion nach Behandlung mit Antiepileptika zeigen. Allerdings könnte es sein, dass sich die hier gewählten Zelllinien, GPNT und MDCK, nicht als Modelle für diese Untersuchungen eignen, entweder aufgrund eines hohen endogenen Pgp-Spiegels oder wegen hoher Expression anderer Multidrug-Transporter.

Daher wären weitere Experimente mit anderen Zelllinien notwendig, um die Transporter-Hypothese noch besser zu untersuchen und möglicherweise geeignetere Modelle für Pgp-Expressionstudien zu finden.

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18 Appendix

18.1 Experiments in MDCK cells (Table 6)

18.2 Experiments in GPNT cells (Table 7 & 8)

18.3 Uptake assay experiments in different cell lines (Table 9 & 10)

Table 6 Experimental conditions to investigate Pgp expression in MDCK wild type cell line									
MDCK cells									
date of experiment/beginning of the treatment	**AEDs**	**period of the treatment**	**sample size**	**lysis buffer**	**Pgp antibody (name & dilution)**	**loading protein (name of antibody and dilution)**	**Protein amount loaded on the gel**	**results**	**Fig.no.**
24.10.06- day of the confluence	30, 100, 300 µM PB; 30, 100 µM PHT;10, 30, 100 µM TPM; 30, 100 µM CBZ **(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS **(**)**	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	100 µg	no significant differences between samples after merging	data not shown
30.10.06- day of the confluence	**(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS **(**)**	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	100 µg		
13.11.06- day of the confluence	**(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS **(**)**	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	100 µg		
18.01.07-day of the confluence	**(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS (**)	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	100 µg		

During experiments problems with unstable signals from loading control (tubulin) and Pgp antibodies, multiply bands from Pgp antibody have occurred. Drugs solutions were kept in -20^{o}C and thawed every day before treatment. Next step was to check Pgp expression in differentiated cells and start treatment 3 days after confluence.								no significant differences between samples after merging the data
19.11.06- 3d after confluence	**(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS **(**)**	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	100 µg	
4.12.06- 3d after confluence	**(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS (**)	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	100 µg	
10.12.06- 3d after confluence	**(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS (**)	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	100 µg	
During experiments problems to obtain stable signals from tubulin and Pgp antibodies. Drugs solutions were kept in -20^{o}C and thawed every day before treatment. Because of problems with Pgp protein detection Pgp antibody from another company was tried in next experiments. In order to have specific interaction and to eliminate multiply bands from Pgp antibody less protein amount was loaded on the gel.								
24.01.07-day of the confluence	**(*)**	3 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Calbiochem, 1:500 in 5% milk in PBS **(**)**	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	50 µg	
Since there was not effect of AED's on Pgp expression in next experiments beginning of the treatment was prolonged and cells were treated with AED's and Pgp inducer 5 days after confluence.								

05.02.07-day of the confluence	300 µM PB, 25 µM RIF	5 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Calbiochem, 1:500 in 5% milk in PBS **(**)**	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	50 µg	no significant differences between samples after merging	9
19.02.07- day of the confluence	300 µM PB, 25 µM RIF	5 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Calbiochem, 1:500 in 5% milk in PBS (**)	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	50 µg		10
25.02.07-day of the confluence	300 µM PB, 25 µM RIF	5 days	2	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Alexis, 1:250 in 5% milk in PBS (**)	alpha-tubulin (Sigma), 1:5000 in 5% milk in PBS	50 µg		12
The treatment was prolonged up to 5 days, starting from the day of confluence. There was no Pgp upregulation after PB and RIF.									
26.03.07- day of confluence	25 µM RIF, 300 µM PB	2 days	1	20 mM Tris pH=7.5, 10% SDS (+ protease inhibitor)	C219 Calbiochem, 1:250 in 5% milk in PBS **(**)**	Villin (BD Transduction Laboratory), 1:200 in 5 % milk in PBS	50 µg	a tendency to increase Pgp expression after PB and RIF	13
To optimize condition, different Pgp antibody dilutions and time of treatment were tried. Because of ongoing problems with loading control, tubulin was replaced by villin. No robust effect of AED's on Pgp expression could be due to not optimized condition for cell treatment (beginning of the treatment, period of the treatment) and protein preparation. Important is also preparation of drug solutions, it is better to avoid DMSO as a solvent (even at low concentration, 0.1%), because its can influence on the results.									

Experiments based on new protocol (protocol no. 2), modified by Dr. Konstantin Kuteykin-Teplyakov									
22.08.07- different time of treatment and cells collection- look attached graph of treatment	300 µM PB	3 and 6 days	1	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3)	actin (Sigma), 1:5000 in 2% milk in TBS-T	50 µg	no differences in Pgp expression after PB after merging	16
16.11.07- different time of treatment and cells collection- look attached graph of treatment	300 µM PB	3 and 6 days	1	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3)	actin (Sigma), 1:5000 in 2% milk in TBS-T	50 µg		16
Treatment with PB at different cell confluence and for various periods. To optimalise condition a new lysis buffer is applied. New antibodies are used for loading control and Pgp detection. Drugs were dissolved in medium and kept +4^{o}C.									
11.10.07- different time of treatment and cells collection- look attached graph of treatment	70 µM CBZ	3 and 6 days	1	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3)	actin (Sigma), 1:5000 in 2% milk in TBS-T	50 µg	no differences in Pgp expression after PB after merging	18

12.11.07-different time of treatment and cells collection-look attached graph of treatment	70 µM CBZ	3 and 6 days	1	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3)	actin (Sigma), 1:5000 in 2% milk in TBS-T	50 µg	no differences in Pgp expression after PB after merging	18
Treatment with CBZ at different cell confluence and for various periods. Drugs were dissolved in MeOH and kept +4^{o}C during experiment.									
30.01.08- 3 days after confluence	100 µM PB, 30 µM PHT, 100 µM PHT,	3 days	3	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3)	actin (Sigma), 1:5000 in 2% milk in TBS-T	50 µg	significant Pgp upregulation after 100 µM PB	19
Treatment with AEDs in different concentrations for 3 days. No Pgp induction after the treatment could be due to inadequate storing condition of the drugs (e.g. often freezing and thawing) and lost effectiveness. Again the effect of AED's and Pgp inducer is not robust and permanent, even if there is an effect after PB in one experiment the results cannot be repeated in the next one. Meaning that either conditions for methods which are used are not proper or cell line is not suitable for such experiments. That way the next step is to perform similar work with another cell line, an immortalized rat brain endothelial cell line GPNT.									

Table 7 Experimental conditions to investigate Pgp expression in GPNT cell line									
GPNT cells									
date of experiment/beginning of the treatment	**AEDs**	**period of the treatment**	**sample size**	**lysis buffer**	**Pgp antibody (name & dilution)**	**loading protein (name of antibody and its dilution)**	**µg protein loaded on the gel**	**results**	**Fig.no.**
30.04.07- day of the confluence	5 ug/ml Pur; 50 µM PB;50 µM PB/+Pur; 150 µM PB; 150 µM PB/+Pur	3 and 6 days		25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Calbiochem, 1:250 in 5% milk in PBS **(**)**	alpha-tubulin (Sigma), 1:1000 in 5% milk in PBS	25-50 µg	not calculated because of unstable signal from Pgp and tubulin	data not shown
12.12.07 - on the day of confluence	5 ug/ml Pur; 50 µM PB;50 µM PB/+Pur; 150 µM PB; 150 µM PB/+Pur	3 days	3	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Calbiochem, 1:250 in 5% milk in PBS (**)	actin (Sigma), 1:5000 in 5% milk in PBS	25 µg	no significant differences between samples	26A
15.01.08- on the day of confluence	50 µM PB, 150 µM PB, 0.92 µM DOX	3 days	3	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3) **(**)**	actin (Sigma), 1:5000 in 2% milk in PBS-T	25 µg	no significant differences between control and PB treated cells; significant difference after DOX	26B

31.01.08- 3 days after confluence	30 µM PB, 100 µM PB, 30 µM PHT, 100 µM PHT, 0.92 µM DOX	3 days	from 1 to 3	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3) **(**)**	actin (Sigma), 1:5000 in 2% milk in PBS-T	25 µg	no significant difference between samples	28
All drugs were dissolved in medium and stored at +4^{o}C or in -20^{o}C, however those storing condition could influence on drugs properties. 25-50 µg protein is enough to detect strong sharp, single band of Pgp.									

Table 8 Experimental conditions to investigate Pgp expression in GPNT cell line, experiment were done in parallel to uptake assays

GPNT cells										
date of experiment/beginning of the treatment	**AEDs**	**Pgp inducer**	**sample size**	**period of the treatment**	**lysis buffer**	**Pgp antibody (name & dilution)**	**loading protein (name of antibody and dilution)**	**µg protein loaded on the gel**	**results**	**Fig.no.**
17.02.08- 7 days after confluence	100 µM PB	1 µM DEX, 0.92 µM DOX	3	3 days	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3) **(**)**	actin (Sigma), 1:5000 in 2% milk in PBS-T	25 µg	significant Pgp up-regulation after DEX	29A

7.03.08- day of the confluence	300 µM PB	1 µM DEX, 0.92 µM DOX	3	3 days	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3) **(**)**	actin (Sigma), 1:5000 in 2% milk in PBS-T	25 µg	significant Pgp down-regulation after DOX nad TQ	31
19.03.08- 6 days after confluence	300 µM PB	1 µM DEX, 0.92 µM DOX	3	3 days	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05% NaN_3) **(**)**	actin (Sigma), 1:5000 in 2% milk in PBS-T	25 µg	significant Pgp overexpression after PB	32A
19.11.08-day of the confluence	100 µM PB, 50 µM PHT, 30 µM CBZ	1 µM DEX	3	3 days	25 mM Tris HCl, 50 mM NaCl, 0.5% Na - Desoxycholate, 0.5% Triton X-100 (+ protease inhibitor)	C219 Signet, 1:200 in 2% milk in TBS-T (+0.05 % NaN_3) **(**)**	actin (Sigma), 1:5000 in 2% milk in PBS-T	25 µg	no significant differences between samples	33A
DEX, DOX were dissolved in medium, drug solutions were prepared every day or store as aliquotes at +4^{o}C. There was no problem with detection loading control and Pgp signals. Antibody for Pgp recognized one specific band in around 140 kDa.										

Footnotes:

(*) 30 µM, 100 µM, 300 µM PB; 30 µM, 100 µM PHT; 10 µM, 30 µM, 100 µM TPM; 30 µM, 100 µM CBZ -drugs were dissolved in DMSO (DMSO final concentration 0.1%)

(**) secondary antibody: Dako antibody for Pgp (anti-mouse) 1:1000; for actin (anti-rabbit) 1:5000 in 2% milk in PBS-T

Table 9 Establishing of the conditions for uptake assays in different cell lines										
date of experiment/beginning of the treatment	**cell line and passage**	**AEDs**	**Pgp inducer**	**period of the treatment**	**sample size**	**time of the assay**	**Pgp substrate**	**results**	**changes in digoxin uptake (after drug stimulation)**	**Fig. no.**
11.12.07	GPNT P30	no AEDs	0.5 μM TQ	-	3	4th day of confluence	Digoxin [100 nM] + ^{3}H-Digoxin [1,85 kBq/ml]	no significant changes in digoxin uptake	↔TQ*	34A
13.12.07	GPNT P30	no AEDs	0.5 μM TQ	-	3	4th day of confluence	Digoxin [100 nM] + ^{3}H-Digoxin [1,85 kBq/ml]	no significant changes in digoxin uptake	↔TQ	34B
20.10.07	MDCK-WT P14	no AEDs	0.5 μM TQ	-	3	day of the confluence	Digoxin [100 nM] + ^{3}H-Digoxin [1,85 kBq/ml]	significantly increase in digoxin uptake after TQ stimulation	↑ TQ	34C
20.10.07	RBE 4 P60	no AEDs	0.5 μM TQ	-	3	day of the confluence	Digoxin [100 nM] + ^{3}H-Digoxin [1,85 kBq/ml]	not significantly changes in digoxin uptake	↔TQ	34D
10.04.08/day of the confluence	GPNT P33	no AEDs	1 μM DEX, 0.5 μM TQ	3 days	from 1 to 3	4th day of confluence	I) Digoxin [10 nM] + ^{3}H-Digoxin [1,85 kBq/ml], II) Digoxin [10 nM] + 3H-Digoxin [10 kBq/ml], III) Rhodamine 123 [3 μM]	not significantly changes in digoxin uptake	↔DEX; ↔TQ**	I) 34E; II) 34F; III) 35A

30.05.08	GPNT P28	no AEDs	0.5 μM TQ	-	3	4th day of confluence	Rhodamine 123 [10 μM]	significantly rhodamine accumulation in the cells after TQ	↑ TQ	35B
The work was meant to formulate proper condition of the experiments. Functionality of Pgp was investigated by uptake of Pgp substrates: digoxin and rhodamine in different cell lines. In next experiments digoxin was chosen as a more reliable and specific substrate for Pgp. In order to obtain robust effect concentration of hot and cold digoxins was increased.										
02.05.08	GPNT P38	no AEDs	0.5 μM TQ	-	3	4th day of confluence	Digoxin [1 μM] + ^{3}H-Digoxin [10 kBq/ml]	significantly increase in digoxin uptake after TQ	↑ TQ (measurment based on DPM; protein was not measured)	36A
12.06.08	GPNT P32	no AEDs	0.5 μM TQ	-	3	4th day of confluence	Digoxin [1μM] + ^{3}H-Digoxin [10 kBq/ml]	significantly increase in digoxin uptake after TQ	↑ TQ	36B
Due to differences in medium composition for uptake assays in literature, next experiments were performed in two kinds of often used media for accumulation test. The aim was to check whether the medium type could influence on results in uptake assay.										
21.10.08/day of the confluence	GPNT P30	no AEDs	1 μM DEX, 0.5 μM TQ*	-	from 1 to 3	4th day of confluence	Digoxin [1 μM] + ^{3}H-Digoxin [10 kBq/ml]	no significantly differences between control and DEX treated cells in medium 1 & 2**	↔DEX; ↔TQ**	37
Because of different DPM values after TQ treatment another more unspecific Pgp substrate was used.										

30.10.08/day of the confluence	GPNT P32	no AEDs	1 μM DEX, 0.5 μM TQ*	3 days	from 1 to 3	4th day of confluence	Vinblastine [1 μM] + ^{3}H-Vinblastine [10 kBq/ml]	significantly decrease in vinblastine uptake after DEX treatment in medium no. 1 & 2	↓DEX; ↔TQ**	38
The aim of above named experiments was to find out the proper condition for uptake assays (medium, concentration of digoxin, time point) in various cell lines. Cells were not treated with AED's but just stimulated with known Pgp inducer DEX and Pgp inhibitor TQ. To optimize condition distinct Pgp substrate were used. Next step was to apply tested experimental condition in relation to the antiepileptic drugs. For the next experiments digoxin was chosen as a substrate, because is recommended by FDA in transport experiments and it is a specific for Pgp protein.										

Table 10 Results of uptake assays after the treatment with different antiepileptic drugs

date of experiment/beginning of the treatment	cell line and passage	AEDs	Pgp inducer	period of the treatment	sample size	time of the assay	Pgp substrate	results	changes in digoxin uptake (after drug stimulation)	Fig. no.
7.03.08/day of the confluence	GPNT P35	300 μM PB	1 μM DEX, 0.92 μM DOX, 0.5 μM TQ*	3 days	3	4th day of confluence	Digoxin [100 nM] + ^{3}H-Digoxin [1,85 kBq/ml]	significantly digoxin uptake only after TQ	↑ TQ; ↔PB; ↔DEX; ↔DOX	31C

17.02.08/6 days after confluence	GPNT P30	100 µM PB	1 µM DEX, 0.92 µM DOX	3 days	3	9th day of confluence	Digoxin [100 nM] + ^{3}H-Digoxin [1,85 kBq/ml]	significantly decrease in digoxin uptake after DEX and significantly increase in digoxin uptake after TQ	↓ DEX; ↔DOX; ↔PB; ↑ TQ	29D
19.03.08/6 days after confluence	GPNT P35	300 µM PB	1 µM DEX, 0.92 µM DOX, 0.5 µM TQ*	3 days	3	9th day of confluence	Digoxin [100 nM] + ^{3}H-Digoxin [1,85 kBq/ml]	significantly digoxin uptake only after TQ	↑ TQ; ↔PB; ↔DEX; ↔DOX	32C
First experiments in GPNT cells were done with lower concentration of digoxin. Cells were treated with PB at different time of differentation. In next step another AEDs were added and concentration of digoxin was increased.										
20.06.08/day of the confluence	GPNT P35	300 µM PB	1 µM DEX, 0.5 µM TQ*	3 days	3	4th day of confluence	Digoxin [1 µM] + ^{3}H-Digoxin [10 kBq/ml]	Significant.y digoxin uptake only after TQ	↑ TQ; ↔PB; ↔DEX	39
10.07.08/day of the confluence	GPNT P30	30 µM CBZ	1 µM DEX, 0.5 µM TQ*	3 days	from 1 to 3	4th day of confluence	Digoxin [1 µM] + ^{3}H-Digoxin [10 kBq/ml]	not significantly diffrences between samples in digoxin uptake	↔CBZ; ↔DEX; ↔TQ**	42A
02.08.08/day of the confluence	GPNT P32	30 µM CBZ	1 µM DEX, 0.5 µM TQ*	3 days	from 1 to 5	4th day of confluence	Digoxin [1 µM] + ^{3}H-Digoxin [10 kBq/ml]	not significantly diffrences between samples in digoxin uptake	↔CBZ; ↔DEX; ↔TQ**	42B
22.08.08/day of the confluence	GPNT P31	50 µM PHT	1 µM DEX, 0.5 µM TQ*	3 days	from 1 to 5	4th day of confluence	Digoxin [1 µM] + ^{3}H-Digoxin [10 kBq/ml]	not significantly diffrences between samples in digoxin uptake	↔PHT; ↔DEX; ↔TQ**	41

15.09.08/day of the confluence	GPNT P31	30 µM CBZ	50 µM DEX, 0.92 µM DOX, 0.5 µM TQ*	3 days	from 1 to 3	4th day of confluence	Digoxin [1 µM] + ^{3}H-Digoxin [10 kBq/ml]	not significantly diffrences between samples in digoxin uptake	↔CBZ; ↔DEX; ↔DOX; ↔TQ**	42C
05.12.08/6 days after confluence (assay was performed by Carlos Luna Tórtos)	GPNT P39	100 µM PB	1 µM DEX, 0.92 µM DOX, 0.5 µM TQ*	3 days	3	4th day of confluence	Digoxin [1 µM] + ^{3}H-Digoxin [10 kBq/ml]	significantly decrease in digoxin uptake after DEX and increase after TQ treatment; no changes in uptake after AEDs treatment	↓DEX; ↔PB; ↔DOX; ↑TQ	29D
04.11.08/day of the confluence	GPNT P32	100 µM PB	1 µM DEX, 0.92 µM DOX, 0.5 µM TQ*	7 days	from 1 to 3	8th day of confluence	Digoxin [1 µM] + ^{3}H-Digoxin [10 kBq/ml]	not significantly diffrences between samples	↔PB; ↔DEX; ↔DOX; ↔TQ**	40
In experiments described above GPNT cells were treated with various antiepileptic drugs, starting at the day of confluence for different time periods. As a control known Pgp inducers DOX and DEX were used. The purpose on next experiments was to investigate the uptake of vinblastine, which could be better Pgp substrate in such experiments.										
19.11.08/day of the confluence (assay was performed by Carlos Luna Tórtos)	GPNT P36	100 µM PB, 50 µM PHT, 30 µM CBZ	1 µM DEX, 0.5 µM TQ*	3 days	3	4th day of confluence	Vinblastin [1 µM] + ^{3}H vinblastin [4.2 kBq/ml]	significantly decrease in vinblastine uptake after DEX treatment; and significantly increase after TQ; no changes in uptake after AEDs treatments	↓DEX; ↔PB; ↔PHT; ↔CBZ; ↑TQ	43
16.12.08/6 days after confluence (assay was performed by Carlos Luna Tórtos)	GPNT P40	100 µM PB, 50 µM PHT, 30 µM CBZ	1 µM DEX, 0.5 µM TQ*	3 days	3	9th day of confluence	Vinblastine [1 µM] + ^{3}H-Vinblastine [4.2 kBq/ml]	significantly decrease in vinblastine uptake after DEX treatment; significantly increase in vinblastine uptake after PHT treatment and TQ treatment	↓DEX; ↔PB; ↑PHT; ↔CBZ; ↑TQ	44

27.01.09/day of the confluence (assay was performed by Carlos Luna Tórtos)	MDCK-WT P9	100 μM PB, 50 μM PHT, 30 μM CBZ	1 μM DEX, 0.5 μM TQ*, 50 μM MK571	3 days	3	4th day of confluence	Vinblastine [1 μM] + ^{3}H-Vinblastine [4.2 kBq/ml]	significantly increase in vinblastine uptake after TQ and MK571; no changes in vinblastine uptake after AEDs treatment	↑ TQ; ↑MK571; ↔PB; ↔PHT; ↔CBZ; ↔DEX	45
20.02.09/day of confluence (assay was performed by Carlos Luna Tórtos)	MDCK-WT P16	100 μM PB, 50 μM PHT, 30 μM CBZ	1 μM DEX, 0.5 μM TQ*, 50 μM MK571	3 days	3	4th day of confluence	Digoxin [1 μM] + ^{3}H-Digoxin [10 kBq/ml]	significantly increase in digoxin uptake after TQ; no changes in digoxin uptake after AEDs treatment	↑ TQ; ↔PB; ↔PHT; ↔CBZ; ↔DEX	46
Uptake assays were performed with various cell lines treated with AEDs in different time. Three Pgp substrates at different concentrations were used to find the most adequate conditions of experiments. In general there was no significant uptake of Pgp substrate after AEDs treatment, whereas the uptake of Pgp substrate was decreased after Pgp inducer DEX. The functionality of Pgp was proved by using specific inhibitor-TQ which inhibits accumulation of Pgp substrates.										

Footnotes:

* ↔ no changes in uptake after drug treatment; ↑increase or ↓ decrease in uptake after drug treatment

** For those experiments nTQ=1

*** For media compositions see Table no. 4, if not mentioned, assays were performed in medium no. 2.

18.4 Western blotting protocol

Protein preparation

1) Remove old medium from the plates where the cells are seeded
2) Wash cells monolayer 3x 10 ml of PBS
3) Add 10 ml of PBS and scrub the cells with the cells scrubber and transfer them to the 15 ml plastic tube (additionally wash the flask with 5 ml of PBS)
4) Centrifuge at 2000 rpm / 10 min
5) Remove the supernatant, leave the pellet on ice
6) Add 200 µl of Lysisbuffer* to each pellet very quickly
7) Leave cells on ice for 30 min
8) Centrifuge cells lysed at 13-15000 g at +4^{o}C for 15 min
9) Replace supernatant (cell lysates) to the new tubes and measure proteat concentration

Lysisbuffer

25 mM Tris HCl
50 mM NaCl
0.5% Na-Desoxycholate
0.5% Triton X-100

Adjust pH to 8.0. Prior to use add protease inhibitors (1x Protease Inhibitor Cocktail Complete® from Roche, or 0.4 mM PMSF) to required amount of buffer.

Detremination of protein concentration by BCA-Kit (Pierce Protein Research Products, ThermoScientific)

1. Take the 96 wells plate and load 10 µl of the standard solutions (bovine serum albumin) and 10 µl of investigated protein lysed
2. Incubate the samples with BSA-kit solutions (solution A: B in ratio 50:1)
3. Incubate 30 min at 37°C
4. Measure optical density using the calorymeter

Preparation of the gels for electrophoresis

7% Separating gel (described for 10 ml):

2.3 ml acrylamid
2.5 ml Tris-HCl pH=8.8
5 ml H_2O
100 µl 10% SDS
100 µl 10% APS
10 µl TEMED

4% Stacking gel:

100 ml Rotiophorese gel 30%
250 ml 0.5 M Tris-HCl pH=6.8
10 ml 10% (w/v) SDS in water
650 ml water
Degas and store at +4^{o}C in dark place.

For gel preparation (10 ml for 2 gels) take:
10 ml Stacking gel
100 µl 10% APS
10 µl TEMED

Western blotting

1. Wash adherent cells twice in the dish or flask with ice-cold PBS and drain off PBS.
2. Collect the cells with scraper and PBS, centrifuge at 800-1000 g at +4°C for 5 minutes to pellet the cells (if necessary, take aliquot for RNA isolation and freeze without PBS at -20°C).
3. Resuspend cells in ice-cold lysis buffer (app. 40-80 µL for 10^6 cells), incubate on ice for 30 min (with agitation)
4. Centrifuge at 12.000-16.000 g (max speed) at +4°C for 15 minutes. Collect supernatant (protein lysate) to new tube and measure concentration with BCA assay (undiluted and dilution 1:3 and 1:10) or Bradford method. Discard the pellet (cell debris). Store supernatant (cell lysate) at -20°C (make aliquots to avoid repeated thaw/freeze!).
5. Take 4 volumes of cell lysate (30-100 µg of total protein) and add 1 volume of Laemmli loading buffer 5x, add 2M DTT solution up to 100 mM. Spin down to collect all the drops and mix well (vortex or pipette).
6. Incubate protein samples at +37°C for 30-40 min, then spin to collect drops.
7. Load samples on the gel and run SDS-PAGE (max. voltage U=70 V; for overnight run use 20-25 V).
8. When electrophoresis will be completed, put the gel into transfer buffer for 15-30 min with gentle agitation.
9. Cut 10-12 pieces of filter paper and 1 piece of PVDF membrane (size equal to the gel). Label the membrane by writing on dry membrane with soft pencil
10. Soak PVDF membrane in few ml of methanol for re-hydration, and then rinse with Transfer buffer for 1-2 min with agitation.
11. Place 5-6 pieces of filter paper on the anode with red cable (wet each filter paper piece with transfer buffer), then membrane, then gel followed by 5-6 pieces of filter paper, then cathode with black cable.
12. Carry out the transfer at current I=2 mA/cm² for 2 hours.
13. After transfer, rinse the membrane in PBS-T buffer for 1 min and put the gel in Coomassie solution for staining.
14. Stain the membrane with Ponceau for 1-2 min and de-stain in water (or 1% solution of acetic acid, if membrane will be scanned).
15. Block the membrane with 5% non-fat milk in PBS-T (or 4% BSA in PBS-T, if antibodies are not compatible with milk) for 1-2 hours at room temperature (overnight at +4°C is also possible).
16. Rinse membrane in PBS-T and pack in PE (polyethylene) film with Primary antibody solution, incubate 1-2 h at room temperature or overnight at +4°C.
17. After incubation, collect the antibody solution and immediately freeze at -20°C (it's possible to re-use antibody 2-5 times).
18. Wash membrane 3 times for 5 min each with PBS-T.

19. Pack the membrane in PE film with Secondary antibody solution; incubate 1-2 h at room temperature or overnight at +4°C.
20. Wash membrane 2 times for 5 min each with PBS-T.
21. Wash membrane for 5 min with PBS 1x (without Tween-20).
22. Prepare ECL working solution (mix two reagents 1:1); app. 10 µl/cm². Place membrane in this solution and incubate 5 min, then put on special film and expose with X ray film
23. Wash membrane with PBS-T and store at +4°C.

Laemmli Loading buffer

5x Laemmli sample buffer (5 mL)	1x Concentration
0.5 g SDS	2% (w/v)
2.5 mL 0.5 M Tris, pH 6.8	50 mM
5 mg bromphenol blue	0.2 mg/mL
0.385 g DTT	0.1 M
2.5 mL glycerol	10% (v/v)

Transfer buffer

25 mM Tris (base)
192 mM glycine
10% methanol

PBS-T

137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.47 mM KH_2PO_4
0.1% Tween-20

Solution to dilute the antibody

2% milk
0.05% NaN_3 in PBS-T

18.5 Uptake assay protocol

Cells are cultured on 78 cm^2 plates until are confluence. At least 3 plates per series are necessary for the test.

1) Prepare assay-medium with inhibitor (Tariquidar; TQ):
- Dissolve 10,49 mg Tariquidar in 2,5 ml DMSO (stock solution)
- Medium with inhibitor: 15 ml for 3 wells
- Add Tariquidar stock solution for a final concentration of 0.5 µM:
  1,6 µl TQ stock sol. for 16 ml medium

2) Check the monolayers under microscope. Identify the plates that will be tested with inhibitor.

3) Remove medium, and replace it with Optimem® +/- inhibitor. Use 5 ml medium for each well.

4) Incubate the cells for 1 h (37°C, 95% humidity, 5% CO_2), to allow them equilibrating with the new medium, on shaker

5) Prepare assay-medium with substrate (digoxin), with/without inhibitor:

Preparation of assay-medium with two different concentration of digoxin:

- **^{3}H-Digoxin [1,85kBq/ml]**

40 ml Optimem® + 4 µl cold digoxin stock **sol.#2** + 2 µl ^{3}H-digoxin
From it:
6 ml Optimem® with digoxin (radioactive and cold digoxin mixed)
+ 0,6 µl Tariquidar stock sol.

- **^{3}H-Digoxin [10kBq/ml]**

40 ml Optimem® + 4 µl digoxin stock **sol.#2** + 10,8 µl ^{3}H-digoxin
From it:
6 ml Optimem® with digoxin (radioactive and cold digoxin mixed)
+ 0,6 µl Tariquidar stock sol.

- **Rhodamine 123 [3 µM]**

Prepare 40 ml Optimem® + 3,1µl Rh 123 stock sol.
From it:
6 ml Optimem ® + 0,6 µl Tariquidar stock sol.

6) Remove the medium from flasks, and replace it with transport-medium +/- inhibitor

7) Incubate the plate (37°C, 95% humidity, 5% CO_2) on a shaker (50 cpm).
Take samples at **120 min**.

8) Prepare the tubes to collect the samples

9) Prepare centrifuge at 2°C
Sampling method for measurement of radioactivity:

1) Take the plates from the incubator, take respective samples of medium and remove the remaining medium as fast as possible. Keep the plates on ice

2) Wash the cells 3 times with ice cold PBS, keep the plate on ice (remove completely the PBS from all plates after last washing – all plates in the same homogenous conditions)

3) Scrape the cells and collect them in Eppendorf tubes. Keep the plate inclined and add 400 µl cold PBS, while flushing the rest of cells with it. Recover the whole content

4) Centrifuge the cells at 1250 rpm for 5 min.

5) Remove supernatant, and add 200 µl lyses buffer, resuspend the pellet avoiding foam

7) If samples are not measured immediately, keep them in the freezer (room for radioactive substances, toxicology)

8) Perform the measurements in β-Counter. Program: 8 (for H-3 isotopes). Analyse the results with GraphPrism® software.

Sampling method for measurement the fluorescent:

- Procedure like above until lysation step

5) Remove supernatant, and add 200 µl lyses buffer, resuspend the pellet avoiding foam

6) Take 100 µl of cells suspension and transfer to the black 96-wells plate

7) Cover the plate with another one (avoid the light)

8) Place the plate to the Fluoroscan II and measure

18.6 Devices and materials used in this thesis

Table 11 Materials and its suppliers used in Western blotting method		
materials	**suppliers**	**product information**
Acetic acid	Applichem	A0369,2500
Ammonium Persulfate (APS)	USB Corporation	76322
Basic fibroblast growth factor (bFGF)	Invitrogen	PHG0024
Bovine Serum Albumin (BSA)	Linaris	GSL0344UC
Bromphenol blue	Carl Roth GmbH, Karlsruhe	T116.1
Chamber for electrophoresis	Biometra	011 - 200
CL-Xposure™ film, Clear Blue X-Ray film (5x7 inches)	Thermo Scientific	34090
Collagen type I-coated plates	Roche	14474700
Coomassie	Applichem	C.I.42660
Deoxycholate, Sodium Salt (DOC)	Carl Roth GmbH, Karlsruhe	3484,1
Dithiothreitol (DTT)	Carl Roth GmbH, Karlsruhe	6908,3
Doxorubicin . hydrochloride	Alexis	ALX-380-042-M010
Electrophoresis power supply	Whatman Biometra	PS 304
Fetal calf serum	Linaris	SBF3111YK
Geneticin	Gibco	10131019
Glutamine	Sigma-Aldrich	G6392
Glycerol	Applichem	A0567,1000
Glycine	Carl Roth GmbH, Karlsruhe	3790,1
Ham's F-10 + Glutamax Nutrien Mix cell culture medium	Sigma-Aldrich	41550
Insulin	Sigma-Aldrich	I6634
Methanol	Carl Roth GmbH, Karlsruhe	X948.1
Mini rocker shaker	PEQLAB Biotechnologie GmbH, Erlangern Germany	MR-1
Mowiol 4-88	Calbiochem	475904

N,N,N',N'-Tetramethyl ethylenediamine	Merck	1107320100
N-Glycosidase F	BioLabs	P0704S
Nitrocellulose membrane	Carl Roth GmbH, Karlsruhe	10401197
PageBlue™ Protein Staining Solution	Ferentas Life Science	R0571
PageRuler™Plus Prestained Protein Ladder	Ferentas Life Science	SM1811
PageRuler™Plus Prestained Protein Ladder	Ferentas Life Science	SM0671
Penicillin-Streptomycin	Gibco	15140
PerfectBlue™, Semi-Dry-Electroblotter	PEQLAB Biotechnologie GmbH, Models SEDEC M	52-1010
Ponceau S	Carl Roth GmbH, Karlsruhe	A2756.10000
Potassium chloride (KCl)	Applichem	A2939,5000
Precision Plus Protein Standards, Dual Color	Bio-rad	161-0374
Primary anti-human Villin (mouse)	BD Transduction Laboratories™	610358
Primary monoclonal antibody to P-glycoprotein (C219) (mouse)	Alexis	ALX-801-002-C100
Primary monoclonal antibody to P-glycoprotein (C219) (mouse)	Calbiochem	517310
Primary monoclonal antibody to P-glycoprotein (C219) (mouse)	Signet	SIG-38710-1000
Primary monoclonal ß-actin (rabbit)	Sigma-Aldrich	A2066
Primary monocolonal α-tubulin DM1A (mouse)	Sigma-Aldrich	T 2690
Protease Inhibitor Coctail Tablets	Carl Roth GmbH, Karlsruhe	04 693 124 001
Puromycin	Sigma-Aldrich	P9620
Rothiophorese gel 30%	Carl Roth GmbH, Karlsruhe	3037,1

Roti®-PVDF membrane	Carl Roth GmbH, Karlsruhe	T830.1
Saponin	Prolabo	27534.187 (0403516)
Secondary goat-anti-rabbit	Dako	P0449
Secondary rabbit-anti mouse-HRP	Dako	P0260
Selenium standard for atomic absorption spectroscopy	Fluka	84896
Skim milk in puder (0.9% fatt)	Sucofin	Fa. TSI GmbH & Co KG
Sodium azide	Sigma-Aldrich	S8032
Sodium chloride	Applichem	A4661,1000
Sodium Dodecyl Sulfate	Carl Roth GmbH, Karlsruhe	CN30.1
Sodium phosphate	Sigma-Aldrich	S5011
Spectra ™ Multicolor Broad Range Protein Ladder	Ferentas Life Science	SM1841
Transferrin	Gibco	11107018
Tris	Applichem	A2756.10000
Trypsin 2.5%	Gibco	15090
Tween 20	Carl Roth GmbH, Karlsruhe	9127,1
α-Minimal Essential Medium + Glutamax cell culture medium	Gibco	32561

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